A Role of CFTR Cl^- Channel in the Protection from Ischemic Injury in Neonatal Rat Ventricular Myocytes.

Hiromi Uramoto

DOCTOR OF PHILOSOPHY

Department of Physiological Sciences
School of Life Science
The Graduate University for Advanced Studies

2003

Summary

The physiological or pathophysiological function of cardiac CFTR (cCFTR) is not well known. In this study, I examined whether CFTR is expressed and functioning as a Cl^- channel in ischemic cardiomyocytes and whether cCFTR plays a role in protection from ischemic injury in neonatal rat ventricular cardiomyocytes in primary culture. After the cells were subjected to ischemia, time-dependent changes in CFTR expression were examined in the membrane fraction by western blot. A peak expression of mature CFTR protein was found at 3 h after ischemia (O_2 and glucose deprivation), and thereafter the signal disappeared gradually. Relative optical densities of these bands at 3 h and 8 h after ischemia were 166% ($P<0.01$) and 44% ($P<0.01$) of the control (0 h), respectively. In contrast, the results of RT-PCR indicated a same expression of CFTR mRNA between 0 h and 4 h after ischemia. Immunohistochemical examination showed that the expression of CFTR proteins on the plasma membrane was most prominent at 3 h after ischemia. However, the plasmalemmal CFTR signal was markedly reduced at 8 h after ischemia. The time-dependent downregulation was partially prevented by co-application of activators for PKA and PKC. Whole-cell recordings showed that cultured neonatal cardiomyocytes responded to cAMP with activation of time-independent, I-V linear currents which contained an anion-selective component sensitive to a CFTR Cl^- channel blocker but not to a stilbene-derivative conventional Cl^- channel blocker. This cAMP-activated Cl^- channel current was found to be enhanced by 3 to 4 h application of ischemic stress. Only blockers effective for the CFTR channel, among various Cl^- channel blockers tested, aggravated cell injury observed 4 days after 8-h ischemic treatment. In contrast, activation of CFTR by co-application of PKA and

PKC activators was found to enhance cell viability after ischemic stress. The ameliorating effect of PKA and PKC activators was completely abolished by CFTR channel blockers, when applied simultaneously. Neither a CFTR inhibitor nor CFTR activators affected ischemic injury, when applied prior to or after, but not during, ischemic treatment. Rhythmic contractility of cultured monolayer cardiomyocytes was impaired during ischemic insult. Four days after reoxygenation contractility recovered partially. The post-ischemic recovery of contractility became perfect by CFTR activators applied during ischemic treatment, whereas this was abolished by a CFTR channel blocker. Taken together, it is concluded that expression of CFTR Cl^- channel on plasma membrane is transiently enhanced under glucose-free hypoxic conditions, and also that activation of CFTR Cl^- channels under ischemic conditions may play a crucial role in protection against injury in cardiomyocytes subjected to ischemic stress.

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR), a member of the ATP-binding cassette (ABC) membrane transporter superfamily, is a cAMP- and protein kinase A (PKA)-regulated Cl^- channel and a regulator of other ion channels (Kunzelmann, 2001; Schwiebert et al., 1999). Abnormality of this gene is responsible for cystic fibrosis (CF), a channelopathy (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989). In normal, non-CF epithelial cells and in cells transfected with recombinant CFTR, patch-clamp single-channel recording showed that low-conductance Cl^- channel events are activated in response to an increase in intracellular cAMP (Berger et al., 1991; Cliff et al., 1992). In epithelial cells derived from CF patients, low-conductance CFTR Cl^- channel events were never observed (Kunzelmann, 2001). CF is the most common fatal genetic disease in humans (Cheng et al., 1990). The most prominent symptoms associated with cystic fibrosis are attributable to reduced transepithelial transports of chloride ions (Welsh & Fick, 1987). CFTR is expressed in luminal membranes of both secretory and absorptive epithelia, and plays a predominant role in secretion of electrolytes. Apart from its secretory function, CFTR also regulates, absorption of electrolytes by controlling the activity of epithelial Na^+ channel (ENaC) in absorptive epithelial cells in colon, airways, and sweat ducts (Kunzelmann, 2001). A gene associated with CF, epithelial CFTR, was isolated in 1989 (Rommens et al., 1989; Riordan et al., 1989).

On the other hand, in cardiac myocytes, it was demonstrated that a time- and voltage-independent anion leak conductance is tightly linked to regulation by the adenylyl cyclase-cAMP-protein kinase A (PKA) pathway in 1989 (Bahinski et al., 1989;

Harvey & Hume, 1989). This finding provided new impetus for further studies of Cl^- channels in the heart. In 1993, deletion of exon 5 in the cardiac form of CFTR was confirmed using Southern analysis (Horowitz et al., 1993). And the cDNA encoding the complete cardiac CFTR isoform was subsequently cloned and sequenced from rabbit heart (Hart et al., 1996). Comparison of the amino acid sequence of human epithelial CFTR with the deduced sequence from rabbit heart indicated deletion of a 30-amino acid segment in the first cytoplasmic loop of CFTR suggesting that the CFTR is an alternatively spliced (exon 5 -) isoform in heart (Horowitz et al., 1993; Warth et al., 1996). Then this gene was demonstrated to encode a cardiac cAMP-dependent chloride channel (Hart et al., 1996). Putative PKA and PKC phosphorylation sites are highly conserved in the two isoforms. Since PKC stimulation potentiates the rate and magnitude of PKA stimulation, it is clear that synergistic interactions between PKA and PKC phosphorylation play an important role in the regulation of both epithelial CFTR (eCFTR) (Jia et al., 1997; Liedtke & Cole, 1998; Hume et al., 2000) and cardiac CFTR (cCFTR) (Middleton & Harvey, 1998; Duan et al., 1999; Hume et al., 2000).

Electrophysiological studies have indicated a significant species and tissue variability in the cardiac expression of PKA-activated Cl^- current (James et al., 1996; Hume et al., 2000). In general, it is most often found in adult ventricular, but not in atrial or sinoatrial nodal, cells in guinea pig, rabbit and cat (James et al., 1996; Hume et al., 2000). In contrast, no evidence for the conductance has yet been found in adult rat or mouse hearts (Hume et al., 2000), although the conductance has been reported in rat and mouse neonatal myocytes (Hume et al., 2000, Tilly et al., 1996). In human, evidence for functional expression of CFTR is controversial (Hume et al., 2000), the studies have failed to detect cAMP-dependent chloride conductance in atrium (Oz & Sorota, 1995;

Yajima et al., 1997; Lader et al., 2000) whereas mRNA of CFTR channels has been detected in human atrium and ventricle by nested PCR (Oz & Sorota, 1995) and in human ventricle by RT-PCR (Hart et al., 1994). Furthermore, immunohistochemical studies in non-diseased human ventricle showed slight immunoreactivity (Yajima et al., 1997).

A first clue of a possible physiological role of cardiac CFTR was provided by the reports that the action potential duration became shorten by forskolin or by stimulation of β-adrenoceptors in heart (Tominaga et al., 1995; Leblais et al., 1999). Stimulation of β_3-adrenoceptors was actually observed to reduce the action potential duration in human myocytes obtained from non-CF patients, but not in myocytes from ΔF508 CF patients (Leblais et al., 1999). However, it is not clear as yet whether or not significant electrocardiogram abnormalities may occur, especially during strong sympathetic stress, such as ischemia, in CF patients (Hume et al., 2000). Since myocardial necrosis and fibrosis is a rare complication of CF causing sudden and unexpected death in infancy due to cardiac arrest (Wiebicke et al., 1993; Zebrak et al., 2000), there is a possibility that eCFTR plays some pathophysiological role. Cardiac cell swelling was reported to be induced by accumulation of anaerobic metabolites during ischemia (Tranum-Jensen et al., 1981; Steenbergen et al., 1985; Jennings et al., 1986). Wang et al. (1997) reported evidence that cCFTR involve in the regulatory volume decrease (RVD) under stimulation of β-adrenergic receptor. Also, activation of volume-regulatory chloride channel and enhanced cell volume regulation were suggested to play a protective role against ischemic injury in cardiomyocytes (Diaz et al., 1999; 2003).

In light of these facts, there arises a possibility that CFTR exerts a protective action in cardiomyocytes subjected to ischemic stress. However, severe hypoxia (<0.05% O_2) led

to downregulation of protein synthesis and RNA synthesis in a muscle cell line C_2C_{12} and cardiomyocytes (Arthur et al., 2000; Casey et al., 2002). Casey et al. (2002) estimated that at least 88% of the resting respiration rate of isolated neonatal non-contracting cardiomyocytes under normoxic conditions is used to fuel the major ATP-consuming (27% protein synthesis, 20% RNA synthesis, and 22% Na^+/K^+-ATPase activity) and ATP-wasting (19% proton leak) processes. Cellular protein synthesis and RNA synthesis were major energy-consuming pathways. Therefore, they are downregulated during severe hypoxia, when oxygen concentrations were low enough to induce energetic stress, quantitatively defined as being any situation in which phosphocreatine concentrations had fallen by more than 40% (Casey et al., 2002). A previous investigation for effects of ATP depletion induced by adding respiratory and glycolytic inhibitors on the CFTR expression in the human airway epithelium showed that the expression of the mature CFTR was down-regulated, whereas the steady-state level of CFTR mRNA semiquantified by RT-PCR kinetics remained constant throughout ATP depletion (Brezillon et al., 1997).

There has been no study on the time-dependent change in the expression of CFTR under ischemic conditions and a role of the functional expression of CFTR during ischemia in heart. Therefore, the present study was performed to address following two questions: 1) Whether CFTR is expressed and functioning as a Cl^- channel in ischemic cardiomyocytes? 2) Whether CFTR exerts a protective action in cardiomyocytes subjected to ischemic stress?

Materials and Methods

Neonatal rat ventricular myocyte preparation and ischemic treatment

Primary cultures of neonatal rat cardiac myocytes were prepared by a modification of a protocol reported previously by Simpson & Savion (1982). In brief, the hearts were removed from neonatal rats (day 4), and the ventricles were minced in 0.05% trypsin-EDTA (Gibco, NY). These tissue fragments were digested by stepwise trypsin-induced dissociation at 37°C in a CO_2 incubator. To reduce the number of contaminating non-myocardial cells, the dissociated cells were pre-plated for 1 h. The non-adherent myocytes were then plated onto the collagen-coated substrate at a density of $7.2\times10^5/cm^2$ for a dish and 1.2×10^5/well for a 96-well plate. Cells were cultured in plating medium consisting of M199 supplemented with 10% fetal bovine serum. The final myocyte cultures were estimated to contain >90% cardiac myocytes by immunostaining with α-sarcomeric actin antibody (Sigma, St. Louis, MO). The cells were maintained at 37°C in the presence of 5% CO_2 in a humidified incubator. Bromodeoxyuridine (BrdU 0.1 mmol/L) was included in the medium after plating to inhibit fibroblast growth. The culture medium was changed on day 1. BrdU was present continuously except the experiment period. Cells were provided for experiments 4 days after culture. Cells were divided into 2 groups, the half was used as a non-ischemic control group, and the remaining group was used as the ischemic group. Cardiomyocytes of ischemic group were, under hypoxic conditions, incubated for a given time period in glucose-free solution, which contained (mM) 108 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, 2.5 Tris (pH adjusted to 7.4 with $NaHCO_3$; osmolarity adjusted with mannitol to 300 mosmol/kg-H_2O) and bubbled with CO_2 for over 1 h

before application. Hypoxic conditions were produced by placing the dishes with cultured cardiomyocytes in an air-tight incubator where normal air was replaced with a gas mixture of 95% Ar-5% CO_2. The pO_2 in the media reached down to a level of <10 mmHg. In the non-ischemic group, solution was replaced with new M199, and then left in a CO_2 incubator. In some experiments, ischemic treatment was performed in the presence of dbcAMP (1 mM), forskolin (10 μM), 3-isobuthyl-1-methylxanetine (IBMX, 0.5 μM), isoproterenol (ISO, 1 mM), and/or phorbol 12-myristate 13-acetate (PMA, 100 nM) with or without adding a Cl^- channel blocker.

RNA isolation and RT-PCR of CFTR mRNA

Poly $(A)^+$ RNA was extracted from rat ventricular myocytes cultured for 4 days using direct mRNA Purification Kit with the magnetic porous glass (MGP) (VPG Inc., NJ). Briefly, the cells were homogenized in extraction-hybridization buffer, which contained 100 mM Tris-HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulphate (LiDS) and 5 mM dithiothreitol (DTT). Poly $(A)^+$ RNA was magnetically isolated from the homogenate by binding with MPG-bound oligo$(dT)_{25}$. The poly $(A)^+$ RNA of 200 ng was used for single-strand cDNA synthesis using SuperScript Preamplification System (Life technologies, Rockville, MD). In brief, RNA samples were reverse-transcribed at 4°C for 50 min by incubation with 20 μl of an RT mixture containing the following constituents: 500 ng of oligo$(dT)_{12\text{-}18}$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dNTPs, 10 mM DTT and 200 units of SuperScript Ⅱ RT. The reverse transcriptase was then inactivated by heating at 70°C for 15 min. The resultant first-strand cDNA was used for the PCR procedure. PCR was performed in a total volume of 50 μl of a buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM

NTPs, and 250 units of Ampli Taq Gold (Perkin Elmer, NJ), using the following set of primers (200 nM each), which were based on the sequence of the rat and mouse CFTR. Two pairs of sense and antisence primers (A QIAGEN Co./SAWADY TECHNOLOGY, Tokyo, Japan) were 5'-ATAGCACACTGAACATCACCG-3' and 5'-TGAAGACGCTCAGAGCAAGC-3', and 5'-TTGTCTC GGCTTTACAACC-3' and 5'-AAGACTCTGCTCACAGATCGC-3', respectively. Both sets of primers are predicted to yield 354-bp and 472-bp products. The optimum temperature cycling protocol was determined to be 94°C for 1 min, 55°C for 2 min and 72°C for 3 min for 1 round of 35 cycles using a programmable thermal cycler (GeneAmp PCR System 9600: Perkin Elmer). Two sets of negative control-experiment were performed by including primers but no cDNA or RNA that was not reverse-transcribed. G3PDH, a house-keeping protein with abundant expression, was used as a positive control. PCR products were purified by pGEM-T & pGEM-T Easy Vector System (Promega, Madison, WI) and then subjected to sequencing using an ABI PRISM 310 automatic sequencer (Perkin-Elmer).

Western blot analysis

Subconfluent cardiomyocytes, pretreated with or without ischemia, were scraped into buffer (10 mM Tris・HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, with protease inhibitors (complete, Mini, EDTA-free)) and sonicated. Postnuclear supernatants were obtained by centrifugation (15,000 rpm for 30 min). The membrane fraction was obtained by centrifugation of the postnuclear supernatants at 100,000×g for 60 min. The pellet was dissolved in lysis buffer (50 mM Tris・HCl, 1% Nonidet P-40. 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, (pH 7.4)) with protease inhibitors

at 4°C overnight and sonicated after suspension, then the membrane proteins were obtained by centrifugation at 15,000 rpm for 10 min, at 4°C. After measuring the protein concentration, the membrane lysate samples diluted with 3 x SDS-PAGE sample buffer. The membrane lysates containing an equal amount of protein were subjected to SDS-PAGE using 7.5% polyacrylamide gels and transferred to PVDF membranes. The blocking and reaction with antibody were performed, using ECL Advance (Amersham Bioscience, Buckinghamshire, England) according to the manufacture's instructions. The blots were incubated with anti-CFTR antibodies (clone M3A7: Upstate Biotechnology, Lake Placid, NY) at room temperature for 1 h. For antibody detection, horseradish peroxidase-conjugated secondary antibodies (Amersham Bioscience) and detection solution were used. Enhanced chemiluminescences were exposed to Hyperfilm^{TM} ECL^{TM} (Amersham Bioscience) for an appropriate duration. As positive control, HEK293T cells transfected with WT CFTR were used, as described previously (Ando-Akatsuka et al., 2002). Relative integrated optical densities of the bands were quantified using Scion image. Since actin, which is generally used as the control protein, was markedly decreased by ischemic stress (data not shown), data were normalized by the whole protein, but not actin, in the sample.

Immunofluorescence of CFTR

To analyze localization of CFTR on the plasma membrane, a cell surface labeling technique with the lectin, wheat germ agglutinin (WGA), was used as reported previously (Stegemann et al., 1990). The cells cultured on coverslips were fixed with cooled methanol for 10 min at –20°C. The cells were then washed with PBS, incubated with FITC-conjugated lectin (100 μg/ml purified WGA; EY Laboratories, INC, San

Mateo, CA) before permeabilization, and washed three times with PBS. The cells were then permeabilized with PBS containing 0.2% TritonX-100 for 10 min, washed three times with PBS, and soaked in a blocking solution (PBS containing 4% normal donkey serum) for 1 h. Next, they were incubated with polyclonal anti-CFTR (a peptide mapping near the amino terminus, N-20) goat antibodies (1:100 dilution; Santa Cruz Biotechnology, INC, Santa Cruz, CA) as a primary antibody for 1 h and washed three times with PBS. The cells were then incubated with rhodamine red-X-conjugated donkey anti-goat IgG (1:50 dilution; Jackson Immunoresearch, West Grove, PA) as the secondary antibody for 30 min, followed by washing with PBS. The cells were then embedded in 90% glycerol-PBS containing 0.1% *p*-phenylendiamine and 1% *n*-propyl gallate. The confocal images of stained samples were visualized using a Zeiss Axiophot fluorescence microscopy (Carl Zeiss Jena, Jena, Germany) and LaserSharp2000 (MRC1024; Bio Rad, Hercules, CA). As a negative control, 1% albumin-PBS was applied instead of anti-CFTR.

Electrophysiology

Whole-cell recordings were performed, as described previously (Zhou et al., 1998), using wide-tipped electrodes at room temperature. The patch electrodes were fabricated from borosilicate glass capillaries using a micropipette puller (Sutter Instrument, Novato, CA). Electrodes, filled with pipette solution, had a resistance of about 2 MΩ. Series resistance (usually < 5 MΩ) was compensated (to 60-75%) to minimize voltage errors. Currents were recorded using Axopatch 200A amplifier (Axon Instruments, Foster city, CA), filtered at 1 kHz using a four-pore Bessel filter and digitized at 4 kHz. The PCLAMP software (version 6.0.2, Axon Instruments) was used

for command pulse control, data acquisition and analysis. The time course of current activation and recovery was monitored by repetitively applying (every 15 s) ramp pulses (1.5 s duration) to ±80 mV for cAMP-activated Cl^- currents from a holding potential of 0 mV. The time- and voltage-independent properties of cAMP-activated Cl^- currents were examined by applying step pulses (1 s duration) from –80 to +80 mV in 20 mV increments. The amplitude of instantaneous current was measured 2.5 ms after the step pulse onset. The patch pipette contained (in mM) 85 CsCl, 10 EGTA, 20 TEA-Cl, 5 MgATP, 3 phosphocreatine-Na_2, 0.5 $MgCl_2$, 5 Na_2ATP, and 10 HEPES (pH adjusted to 7.34 with CsOH; osmolarity to 290 mosmol/kg-H_2O). The bathing solution contained (in mM) 103 NaCl, 47 Na-gluconate, 3 $MgCl_2$, 1 $CdCl_2$, 5.5 glucose, and 5 HEPES (pH adjusted to 7.4 with NaOH; osmolarity to 315 mosmol/kg-H_2O). Whole-cell currents were measured in symmetrical Cl^- conditions or extracellular low Cl^- conditions where NaCl in bath solution was replaced with Na-gluconate. CFTR Cl^- currents were activated by applying a cAMP-stimulating cocktail containing 1 mM dbcAMP, 0.5 μM IBMX and 10 μM FSK.

Cell viability assay

Viability of cells cultured in the 96-well culture plates was assessed by mitochondrial dehydragenase activity using the colorimetric MTT assay, based on the fact that viable cells (but not dead cells) can reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mossman, 1983), using the Cell counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Observation of contractility of cardiomyocytes by microvideoscopy

The cells cultured on coverslips were used for contractility observations. The experiment was performed under light microscopy using the video system composed of a CCD camera, a video set, an image capture adapter and a personal computer. Cell images were recorded for 5 s at the rate of 300/s for every cell.

DNA fragmentation assay

Internucleosomal DNA fragmentation was detected by modified TUNEL method, using Cell Death Detection ELISA kit ^{PLUS} (Roche, Mannheim, Germany) according to the manufacturer's instructions. It was measured in each group, 0 day and 4 days after ischemic stress.

Chemicals

All the reagents except for EGTA, HEPES (Dojindo, Kumamoto, Japan), protease inhibitor cocktail (Roche Molecular Biotech UK, Buckinghamshire, England) and FITC-conjugated WGA (EY Laboratories, INC, San Mateo, CA) were obtained from Sigma (St. Louis, MO). PMA was dissolved in ethanol as the stock solution and diluted 1,000 times in the experimental solution. 5-nitro-2-2 (3-phenylpropylamino)-benzoate (NPPB), 4-acetamido-4'-iso-thiocyanostilbene (SITS), indanyloxyacetic acid (IAA-94), 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), furosemide, IBMX and FSK were dissolved in dimethyl sulfoxide (DMSO) as the stock solution and diluted 1,000 times in the experimental solution. Glibenclamide and gemfibrozil were dissolved in DMSO as stock solution and diluted 500 times in experimental solution. Anthracene-9-carboxylate (9AC) was dissolved in DMSO as stock solution and diluted 100 times in experimental solution. The vehicle (ethanol or DMSO) alone at the

employed concentration (0.1% to 1%) did not affect the MTT assay. isoproterenol (ISO) was dissolved in H_2O as stock solution and diluted 1000 times in experimental solution.

Data analysis

Data analysis was performed using SPSS (11.0J; SPSS Japan Inc.) software packages. All data are summarized and expressed as mean $\pm$ SEM. For all data except electrophysiological data, ANOVA analysis followed by Scheffe's post hoc test was performed to determine significant differences ($P<0.05$) among groups and between two groups, respectively. Statistical differences of the electrophysiological data were evaluated by Student's t-test and considered significant at $P<0.05$.

Results

Part 1: Ischemic Stress-induced Enhancement of CFTR Expression on the Plasma Membrane in Neonatal Rat Ventricular Myocytes.

Time-dependent changes in CFTR expression by ischemia

Expression of the CFTR protein was previously confirmed in neonatal rat ventricular myocytes (Tilly et al., 1996), but the effect of ischemic stress on the expression was not examined in cardiomyocytes. Therefore, next experiments were carried out according to the protocol shown in Figure 1C. RT-PCR was performed on the poly $(A)^+$ RNA isolated from non-ischemic and ischemic cardimyocytes subjected to ischemic stress. Figure 2 shows the results of RT-PCR amplification. The PCR products of the expected size (354 and 472 base pairs) were, to an essentially similar extent, amplified in reverse-transcribed RNA in both non-ischemic and ischemic cardiomyocytes. However no PCR product was amplified when the reverse transcriptase was omitted from the reaction in both conditions. These results suggest that the CFTR gene expression was not affected by ischemic stress in neonatal cardiomyocytes. I then estimated the effect of ischemia on CFTR protein expression by western blotting (Fig. 3A). As shown in Figure 3A (Fig. 1A as protocol), the time course of expression of CFTR protein showed a transient enhancement. A peak expression of mature CFTR protein band was found at 3 h after ischemia and thereafter the bands became gradually faint. Relative optical densities of mature CFTR at 3 h and 8 h after ischemia resulted in 166.1±7.8 % ($P<0.01$) and 44.4±7.8 % ($P<0.01$) compared with the control (0 h), respectively (Fig. 3A, Fig. 1B as protocol). Immature CFTR also showed time-dependent change in the expression similar to mature CFTR, though transient enhancement at 3 h after ischemia was less prominent.

Taken together, it is concluded that the mature CFTR protein expresses in the membrane fraction throughout the 8 h ischemic period, and that ischemia induces a

transient of the enhanced expression of mature CFTR proteins by the posttranscriptional control in neonatal rat cardiomyocytes.

Effects of ischemia on expression of plasmalemmal CFTR

In order to detect expression of the CFTR protein on the plasma membrane before and during application of ischemic stress, immunostaining was performed using a polyclonal anti-CFTR antibody. Such as described above, mature CFTR proteins in the membrane fraction showed peak expression at 3 h after ischemia. Therefore using a protocol shown in Fig. 1B, localization of CFTR proteins on the plasma membrane was next examined using dual staining with WGA, which binds to the glycoside residue on the plasma membrane, and anti-CFTR. As shown in Figure 4, most CFTR proteins were found to exist on plasma membrane at 3 h after ischemia, but the CFTR signal was prominently reduced at 8 h. These findings were in agreement with the time course of mature CFTR protein expression in the membrane fraction (Fig. 3).

Effect of co-application of PKA and PKC activators on expression of CFTR in cardiomyocytes subjected to ischemia

Next, I examined effects of co-application of PKA and PKC activators on expression of CFTR in cardiomyocytes subjected to ischemia (Fig. 4 and 5). Downregulation of the mature CFTR protein expression in the membrane fraction at 8 h after ischemia was prevented by co-application of PKA activator (FSK+IBMX) and a PKC activator (PMA), as shown in Fig. 5. Similarly, downregulation of the plasmalemmal CFTR expression by 8 h ischemic stress could be partially prevented by co-application of FSK, IBMX and PMA, as shown in Figure 4 (bottom photos).

Effects of ischemia on the CFTR channel activity

To examine the effect of ischemic stress on the funcional expression of CFTR

channel on the plasma membrane in cardiomyocytes, patch-clamp whole-cell recordings were performed using the protocol shown in Figure 6. Stimulation with a cAMP cocktail resulted in activation of whole-cell currents (Fig. 7 and Fig. 8). The current responses to step pulses exhibited time-independent activation (Fig. 7A). The current-voltage curve exhibited a linear relationship (Fig. 7B). These characteristics are typical of the CFTR currents. The CFTR-like current became more prominent in cardiomyocytes after applying ischemic stress for 3 to 4 h (Fig. 7). As shown in Figure 8 (A and B), the cAMP-activated currents recorded from ischemic cardiomyocytes were significantly, though in part, suppressed by a CFTR channel blocker, NPPB or glibenclamide, but never affected by a conventional Cl^- channel blocker, SITS, which is known to be ineffective for CFTR Cl^- channel. Reduction of extracellular Cl^- concentration (from 111 to 8 mM) induced a rightward shift (by 6.4 $\pm$ 1.2 mV, n=3) of the reversal potential (Fig. 8C). These results indicate that the cAMP-activated time- and voltage-independent currents contained a sizable component of CFTR Cl^- channel current, and that the functional expression of plasmalemmal CFTR was enhanced by ischemic stress for 3 to 4 h in cardiomyocytes.

Conclusions

Not only molecular, but also functional expression of CFTR could be confirmed on the plasma membrane in neonatal rat cardiomyocytes in primary culture. Ischemic stress was found to augment the plasmalemmal CFTR expression at 3 to 4 h after ischemia and downregulate it at 8 h after ischemia. However, activation of PKA and PKC was found to largely prevent the downregulation induced by long-term ischemia.

Part 2: Protective Role of CFTR Cl^- Channel against Ischemic Injury in Neonatal Rat Ventricular Myocytes

Experimental protocols for optimization of ischemic conditions

Figure 9 shows experimental protocols for selection of optimal ischemic conditions. Effects of changes in periods of ischemia and post ischemia on cell viability were examined.

Figure 10 shows the results of this experiment. From these results, I selected the time period of ischemic stress of 8 h and that of post-ischemic recovery period of 4 days as the most optimal ischemic conditions for cell viability assays.

Monolayer cardiomyocytes regularly exhibited rhythmic contractions in a synchronous fashion (data not shown). During an ischemic insult, the contraction was found to cease completely (data not shown). However, cardiomyocyte contractility became recovered, though not perfectly, after reoxygenation. After 8-h ischemia and 4-day reoxygenation, irregular contraction was found in most cardiomyocytes (data not shown).

Effects of various Cl^- and K^+ channel blockers on post-ischemic recovery

Employing the protocol summarized in Figure 11 effects of ion channel blockers on post-ischemic recovery of ischemic cell injury were examined. Among a variety of K^+ and Cl^- channel blockers tested, CFTR Cl^- channel blockers (Hume et al., 2000), NPPB (50 μM), glibenclamide (500 μM), 9AC (2 mM), gemfibrozil (2 mM) (Walsh &Wang, 1996), were found to inhibit markedly post-ischemic recovery of the cell viability, as shown in Figure 12. Glibenclamide was less effective at 100 μM (data not shown, n=3). This fact is consistent with the previous report that this drug could block cardiac CFTR current only partially (Tominaga et al., 1995) and may exclude a possible action of this drug on K_{ATP} channel which is known to be completely inhibited by 100 μM glibenclamide (Light & French, 1994; Wellman et al., 1999). In contrast, such aggravating effects were never observed by blockers for other types of Cl^- channel (SITS, DIDS and IAA-94), a blocker for Na^+-K^+-$2Cl^-$ or Na^+-Cl^- cotransporter (furosemide) and a K^+ channel blocker (TEA), as also shown in Figure 12. These results

suggest that the endogenous activity of CFTR Cl^- channel exerts a protective action to ischemic injury in cardiomyocytes.

Post-ischemic recovery of cardiomyocyte contractility was abolished by NPPB (50 μM) applied during, but not before or after, the ischemic insult (data not shown).

Effects of treatment with dbcAMP, PMA, forskolin and isoproterenol on post-ischemic recovery

Since cardiac CFTR, like epithelial CFTR, is known to be activated by PKA (Hume et al., 2000), and this PKA-induced activation is to be potentiated by PKC (Middleton & Harvey, 1998), I next examined effects of PKA activators (dbcAMP, FSK, IBMX and ISO) and a PKC activator (PMA) on post-ischemic recovery of neonatal cardiomyocytes. The results demonstrate that recovery of cell viability observed 4 days after ischemic stress was significantly ameliorated by co-application of PKA and PKC activators (Fig. 13).

Post-ischemic recovery of cell contractility also became enhanced by this maneuver. Cardiomyocytes exhibited synchronously rhythmic contractions, just like control conditions, when dbcAMP and PMA were applied during the ischemic treatment (data not shown).

Effects of CFTR channel blockers on enhancement of post-ischemic recovery by dbcAMP and PMA

Next I examined whether the enhancement of cell viability by co-application of PKA and PKC activators is in fact mediated by activation of CFTR Cl^- channel. As shown in Figure 14, enhanced cell viability by PKA and PKC activators was completely abolished by either CFTR channel blocker. The degree of inhibition was comparable with that by a CFTR blocker alone (Fig. 12). Thus, it is confirmed that the enhancement of cell viability by co-application of PKA and PKC activators is mediated by activation

of CFTR Cl^- channel. Post-ischemic recovery of cardiomyocyte contractility in the presence or absence of these reagents paralleled that of cell viability (data not shown).

Comparison of effects of treatment, pre-treatment and post-treatment with CFTR activators and blocker

To examine the relationship between the timing of CFTR action and that of ischemia, I then observed the effects of pre-treatment and post-treatment with a CFTR blocker or CFTR activators, using the protocols summarized in Figure 15, and compared these effects with the effects of these reagents applied simultaneously during the ischemic treatment (simultaneous treatment: Fig. 15). As shown in Figure 16B, a significant inhibitory effect of NPPB was observed only in the case of simultaneous treatment, but neither of pre-treatment nor of post-treatment. Also, significant enhancing effects of dbcAMP and PMA were observed only when applied simultaneously with the ischemic treatment (Fig. 16C). Thus, it is concluded that the endogenous activity of CFTR Cl^- channel exerts a protective action just for the ischemic period.

Lack of effects of CFTR activators and blocker on apoptosis in ischemic cardiomyocytes

Two types of cell death, apoptosis and necrosis, have been simultaneously observed in ischemic hearts (Kurrelmeyer et al., 2000; Depre & Taegtmeyer, 2000) and neonatal rat cardiomyocytes (Schafferr et al., 2000). Apoptotic cells were found to be increased by ischemia or ischemia/reperfusion in the heart (Gottlieb et al., 1994; Fliss & Gattlieb at al., 1996; Bialik et al., 1997; Maulik et al., 1999; Kurrelmeyer et al., 2000; Borutaite et al., 2001), and in cultured neonatal rat cardiomyocytes (Tanaka et al., 1994; Long et al, 1997; Mackay & Mochly-Rosen, 1999; Schaffer et al., 2000; Adachi et al., 2001). Thus, it is possible that CFTR-sensitive cell death in ischemic cardiomyocytes was due to apoptosis. As shown in Figure 17, however, apoptotic internucleosomal DNA

fragmentation, which was slightly increased 4 days after ischemia, was not affected either by NPPB or by dbcAMP plus PMA. These results suggest that CFTR-sensitive cell death in ischemic cardiomyocytes is due to non-apoptotic (necrotic) cell death.

Conclusions

Ischemia-induced (non-apoptotic) cell injury was aggravated by application of CFTR Cl^- channel blocker, but not conventional Cl^- channel blocker, during (but neither before nor after) ischemic treatment in neonatal rat cardiomyocytes in primary culture. On the other hand, recovery from ischemia-induced cell injury was greatly enhanced when PKA and PKC activators were applied during (but neither before nor after) ischemic treatment in cardiomyocytes.

Discussion

It is suggested from the results obtained in neonatal rat cardiomyocytes in this study that the function of cardiac CFTR (cCFTR) is closely associated with endogenous protective mechanisms against cell injury under glucose-free hypoxic conditions, which are an *in vitro* model of ischemia. Since physiological and pathophysiological functions of cCFTR has not been clear, the obtained findings will provide new evidence for a pathophysiological function of cCFTR.

There is enough reliable evidence for expression of cardiac CFTR (cCFTR) (Horowitz et al., 1993, Warth et al., 1996, Hart et al., 1996) and detection of cAMP-activated Cl^- current in the mammalian heart (Hume et al., 2000). However, functional expression of CFTR Cl^- channel varies with animal is variability in species and tissues (Hume et al., 2000). In murine heart, neonatal cardiac myocytes express a cAMP-dependent Cl^- channel identified as the CFTR protein (Tilly et al., 1996; Lader et al., 2000), but adult cardiac myocytes do not (Hume et al., 2000). In human heart a cAMP-dependent Cl^- current has not been detected (Oz & Sorota, 1995, Yajima et al., 1997), whereas the CFTR mRNA is detected (Yajima et al., 1997). A recent study has shown that stimulation of β_3-adrenoceptors reduces the action potential duration in human cardiomyocytes obtained from non-CF patients, but not in cardiomyocytes from ΔF508 CF patients (Leblais et al., 1999). Therefore, I was interested in the changes in expression of cCFTR under ischemic conditions where cardiomyocytes must be subjected to strong sympathetic stress.

The present study demonstrated, using the *in vitro* model of stimulated ischemia by O_2 and glucose deprivation in neonatal rat cardiomyocytes, that a transient enhancement

of expression of endogenous CFTR proteins is induced by ischemic stress. Both western blot and immunohistochemical studies revealed localization of mature CFTR protein on the plasma membrane, and whole-cell recoding indicated that the CFTR is functional as a cAMP-activated Cl^- channel. Furthermore, short-term (3-4 h) application of ischemic stress was found to enhance the plasmalemmal expression of CFTR and to augment the CFTR Cl^- channel activity. The enhanced expression of CFTR protein could be attributed to the posttranscriptional control. However, long-term (8 h) application of ischemic stress caused downregulation of CFTR protein expression. This finding is in agreement with an observation in epithelial cells by Casey et al. (2002).

Downregulation of the CFTR protein may be due to inhibition of protein synthesis by ATP depletion caused by severe hypoxia. However, the mechanism for transient upregulation of plasmalemmal expression of the CFTR protein is unknown. Intracellular cAMP was reported to increase expression of the CFTR mRNA in colon carcinoma epithelial HT-29 cells (Bargon et al., 1998). However, CFTR mRNA levels were kept at 4 h after ischemia in this study. Since downregulation of gene expression of proteasome β3 was found to contribute to a transient enhancement of expression of the CFTR protein (Jiang et al., 2002) , it is possible that this mechanism is involved in transient CFTR upregulation after short-term ischemia in cardiomyocytes. To verify or test this possibility, however, further studies are needed.

The present findings clearly indicate that plasmalemmal expression of CFTR Cl^- channel is enhanced under glucose-free hypoxic conditions especially in the presence of PKA and PKC activators. Therefore, I next explored a role of CFTR Cl^- channel in ischemic cardiomyocytes.

Although it has been controversial that some Cl^- channel is capable of protective

action to the ischemic injury in cardiomyocytes (Diaz et al., 1999), recent data showed that volume regulation by volume-regulatory Cl^- channel is responsible for the protective mechanism against ischemic injury in cardiomyocytes (Diaz et al., 2003). The β-adrenergic signaling modulates cell survival in heart (Lochner et al., 1999; Zhu et al., 2001), and β_2-adrenergic, but not β_1-adrenergic, receptors are responsible for the β-adrenergic signaling (Zhu et al., 2001). Stimulation of β_1-adrenergic receptors increases apoptosis, whereas stimulation of β_2-adrenergic receptors inhibits apoptosis via a Gi-coupled pathway in cardiomyocytes (Communal et al., 1999). The β_2-adrenergic receptor constitutes a macromolecular complex together with CFTR and the Na^+/H^+ exchanger regulatory factor (NHERF), and the complex is regulated by PKA-dependent phosphorylation through the regulatory domain of CFTR in airway epithelial cells (Naren et al., 2003). Also, Wang et al. (1997) reported that CFTR Cl^- channels are activated under adrenergic stimulation and involved in volume regulation after osmotic swelling in cardiomyocytes. Furthermore, it was reported that myocardial necrosis or fibrosis is a rare complication of cystic fibrosis (CF) (Wiebicke et al., 1993; Zebrak et al., 2000). These findings support our data that only effective blockers for CFTR channel, but not other Cl^- channel blockers, showed injurious effects in ischemic cardiomyocytes, and imply that the protective effect may be, at least in part, induced by volume regulation during ischemia.

Epithelial CFTR (eCFTR) is regulated by PKC as well as cAMP activated-PKA pathway (Jia et al., 1997; Liedtke & Cole, 1998; Liedtke et al., 2002). cCFTR is also regulated by cAMP activated-PKA pathway, and PKC potentiates PKA-dependent responses, although PKC alone does not significantly regulate cCFTR Cl^- channel activity (Middleton & Harvey, 1998). In this experiment, CFTR activation by

co-application of PKA and PKC activators, but not only PKA or PKC activator alone, was found enhanced cell survival of ischemic cardiomyocytes, and CFTR blockers abolished completely the enhanced cell survival by both activators. These results together with the observation of recovery of contractility by these activators provide strong evidence that CFTR has a protective function in ischemic cardiomyocytes. It seems possible that the protective effect involves PKCε translocation and activation of PKCε, because activated PKCε was reported to protect hearts from post-ischemic contractile dysfunction (Cross et al., 2002), and the activity of PKCε is necessary for cAMP-dependent CFTR function through its binding to a receptor for activated C kinase (RACK1) and NHERF1 (Liedtke et al., 2002).

As protective system in ischemic heart, preconditioning is well known. Stimulation of adenosine receptor (AR) or activation of PKC can mimic preconditioning in heart (Ladilov et al., 1999; Cohen et al., 2000; Cross et al., 2002). In the heart, it is well known that adenosine nucleotides are released to the interstitial space during hypoxia (Paddk & Burnstak, 1974; Forrester & Williams, 1977; Clemens & Forrester, 1981; Vial et al., 1987; Borst & Schrader, 1991) and ischemia or ischemic preconditioning (Borst & Schrader, 1991; Kuzmin et al., 1998; 2000; Ninomiya et al., 2002). Released ATP was shown to be degraded by ectoATPase and ectonucleotidase to adenosine, thereby stimulating adenosine receptors (AR) in rat heart (Ninomiya et al., 2002). Adenosine and its nucleotides are capable of activating eCFTR-dependent halide permeability through stimulation of A_{2B} AR (Clancy et al., 1999). In cardiac preconditioning PKCε plays an important role among various PKC isoforms (Gray et al., 1997; Cohen et al., 2000; Liu et al., 2001; Cross et al., 2002). A recent report indicated that PKCε regulates eCFTR through binding to a receptor for activated C kinase (RACK1) which binds to

NHERF1 (Liedtke et al., 2002). And preconditioning selectively activates PKCε in the particulate fraction (Liu et al., 2002). This finding suggests that the target of PKCε is closely associated with the plasma membrane. It is still unknown which PKC phosphorylation sites on CFTR are involved in activation of CFTR. Although there are two phosphorylation sites on the R-domain, the functional effects of phosphorylation of these residues on PKC-induced regulation of eCFTR channels have not been assessed (Hume et al., 2000). Furthermore, Lochner et al. (1999) showed that an increase in cAMP induced by preconditioning is involved in myocardial protection against ischemia. Taken together, it is suggested that cCFTR is one of key players in the preconditioning effect.

In the heart, PKA is known to activate a variety of ion channels and transporters, including CFTR Cl^- channel, Na^+ pump and L-type Ca^{2+} channel. The effect of β-adrenergic receptor on the L-type Ca^{2+} current is well documented (McDonald et al., 1994). Activation of β-adrenergic receptor results in an increase in peak inward current and slowdown of inactivation via phosphorylation of the channel protein by PKA. Hypoxia inhibits the basal activity of L-type Ca^{2+} channel and increases its sensitivity to β-adrenergic receptor (Hool et al., 2000; 2001). By contrast, much less is known about the modulation of the cardiac Na^+-K^+ pump via the cAMP-PKA pathway (Gao et al., 1994; Kockskamper et al., 2000, 2001, Erlenkamp et al., 2002). Kockskamper et al. (2001) confirmed differences in the PKA-dependent regulation of CFTR Cl^- channel and Na^+ pump in cardiomyocytes. Thus, they showed that there is a tight functional coupling between PKA and CFTR Cl^- channels. On the other hand, Na^+ pumps, though also regulated by PKA, appear to be loosely coupled to the kinases (Kockskamper et al., 2001). The activity of Na^+ pump is reduced during anoxia (Inoue et al., 1999), because

the pump needs a large amount of ATP for the activity. However, rapid resumption of Na^+ pump activity upon reperfusion may be beneficial by allowing efflux of Na^+ without concomitant influx of Ca^{2+}. There is a report that CFTR may contribute to the Na^+ pump activity by regulating the rate of Na^+ entry in Calu-3 cells (Ito et al., 2000). Thus, I think it is useful to maintain or upregulate expression of the CFTR proteins in ischemic cardiomyocytes subjected to prolonged ischemia.

Because CFTR is an integral membrane protein, it will be, of necessity, trafficked through vesicular membrane compartments. Therefore, one should expect, at some level, to detect CFTR within intracellular compartments such as the endoplasmic reticulum, Golgi stacks, and vesicular membranes of the endosomal/lysosomal pathways. Analysis of proteins coprecipitated with CFTR revealed the association with α-adaptin, a coat protein component of endocytic clathrin-coated vesicles (Bradbury, 1999). Furthermore, it was reported that the endocytic recycling of CFTR is regulated by an interaction with the PDZ domain (Swiatecka-Urban et al., 2002).

The possible explanations for the mechanism of protective effect of CFTR in cardiomyocytes subjected to ischemic stress are as follows:

1) inducing volume regulation of swollen cardiomyocytes during ischemia by cCFTR (Wang et al., 1997) thereby preventing the necrotic volume increase (NVI) which is an early step of necrosis (Okada et al., 2001);

2) controlling the intracellular pH, because CFTR may function also as a chloride channel within intracellular organelles of the endosomal and biosynthetic pathways, thereby potentially modifying their acidification as well as regulating the trafficking properties of intracellular organelles (Bradbury, 1999); and

3) suppressing NHE-activation at just after reoxygenation, because inhibition of the

sarcolemmal NHE affords significant protection to myocardium subjected to ischemia and reperfusion predominantly through reduced intracellular accumulation of Na^+ (Xiao & Allen, 1999) and consequently Ca^{2+} (An et al., 2001; Avkiran et al., 2002). Since there have been many reports the protein-protein interaction between CFTR and NHERF via the PDZ1 domain (Kunzelmann, 2001), the interaction of CFTR with NHE3 may be mediated by NHERF. In fact, NHERF was reported to mediate NHE3 regulation by β_2-receptor (Hall et al., 1998), cAMP (Zizak et al., 1999) and CFTR (Bagorda et al., 2002). Although, the cardiac isoform is NHE1 (Fliegel & Wang, 1997; Frohlich & Karmazyn, 1997) which is upregulated the expression of mRNA by ischemia (Gan et al., 1999), it is possible that NHERF and CFTR also mediate cAMP-induced suppression of NHE1, because cardiac NHE1 was found to be essential for the detrimental cardiac effectors of β_1-receptor stimulation in the heart (Engelhardt et al., 2002).

Here, I demonstrated that cCFTR plays a crucial role, under ischemic conditions, in protection of neonatal rat cardiomyocytes. However, for the precise mechanism remains to be clarified in future studies.

Acknowledgments

I gratefully thank Prof. Y. Okada, S. Morishima and R.Z. Sabirov for their guidance and support throughout my study. I would like to thank gratefully Y. Ando-Akatsuka and A.K. Dutta for support with immunostaining and whole-cell recording, respectively, and E. Maeno, S. Mori, N. Takahashi and T. Shimizu for helpful discussion throughout this research. I would like to thank gratefully M. Ohara for technical assistance, and K. Tsuchiya, K. Shigemoto and T. Kondo for support throughout my study. I am grateful to all other members of our laboratory for their generous supports.

References

Adachi, S, Ito, H., Tamamori-Adachi, M., Ono, Y., Nozato, T., Abe, S., Ikeda, M., Marumo, F. & Hiroe, M. (2001). Cyclin A/cdk2 activation is involved in hypoxia-induced apoptosis in cardiomyocytes. Circ. Res. 88, 408-414.

An, J., Varadarajan, S. G., Camara, A., Chen, Q., Novalija, E., Gross, G. J. & Stowe, D. F. (2001). Blocking Na^+/H^+ exchange reduces $[Na^+]_i$ and $[Ca^{2+}]_i$ load after ischemia and improves function in intact hearts. Am. J. Physiol. 281, H2398-H2409.

Ando-Akatsuka, Y., Abdullaev, I. F., Lee, E. L., Okada, Y. & Sabirov, R. Z. (2002). Down-regulation of volume-sensitive Cl^- channels by CFTR is mediated by the second nucleotide-binding domain. Pflugers Arch. Eur. J. Physiol. 445, 177-186.

Arthur, P. G., Giles, J. J and Wakeford, C. M. (2000). Protein synthesis during oxygen conformance and severe hypoxia in the mouse muscle cell line C_2C_{12}. Biochim. Biophys. Acta 1475, 83-89

Avkiran, M., Marber, M. S. & Faha, F. (2002). Na^+/H^+ exchange inhibitors for cardioprotective therapy: progress, problems and prospects. J. Am. Col. Cardiol. 39, 747-753.

Bagorda, A., Guerra, L., Sole, F. D., Hemle-Kolb, C., Cardone, R. A., Fanelli, T., Reshkn, S. J., Gisler, S. M., Murer, H. & Casavola, V. (2002). Reciprocal protein kinase

A regulatory interactions between cystic fibrosis transmembrane conductance regulator and Na^+/H^+ exchanger isoform 3 in a renal polarized epithelial cell model. J. Biol. Chem. 277, 21480-21488.

Bahinski, A., Nairn, A. C., Greengard, P. & Gadsby, D. C. (1989). Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. Nature 340, 718-721.

Bargon, J., Loitsch, S., Dauletbaev, N., Mallinckrodt, C. V. & Buhl, R. (1998). Modulation of cystic fibrosis transmembrane conductance regulator gene-expression by elevation of intracellular cyclic AMP. Eur. J. Med. Res. 3, 256-262.

Berger, H. A., Anderson M. P., Gregory, R. J., Thompson, S., Howard, P. W., Maurer, R. A., Mulligan, R., Smith, A. E. & Welsh, M. J. (1991). Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel. J. Clin. Invest. 88, 1422-1431.

Bialik, S., Geenen, D. L., Sasson, I. E., Cheng, R., Horner, J. W., Evans, S. M., Lord, E. M., Koch, C. J & Kitsis, R. N. (1997). Myocyte apoptosis during acute myocardial infarction in the mouse localizes to hypoxic regions but occurs independently of P53. J. Clin. Invest. 100, 1363-1372.

Borst, M. M. & Schrader, J. (1991). Adenosine nucleotide release from isolated perfused guinea pig hearts and extracellular formation of adenosine. Circ. Res. 68,

797-806.

Borutaite, V., Budriunaite, A., Morkuniene, R & Brown, G. C. (2001). Release of mitochondrial cytochrome c and activation of cytosolic caspases induce by myocardial ischaemia. Biochim. Biophys. Acta 1537, 101-109.

Bradbury, N. A. (1999). Intracellular CFTR; localization and function. Physiol. Rev. 79, S175-S191.

Brezillon, S., Zahm, J.-M., Pierrot, D., Gaillard, D., Hinnrasky, J., Millart, H., Klossek, J.-M., Tummler, B. & Puchelle, E. (1997). ATP depletion induces a loss of respiratory epithelium functional integrity and down-regulates CFTR expression. J. Biol. Chem. 272, 27830-27838

Casey, T. M., Pakay, J. L., Guppy, M. & Arthur P. G. (2002). Hypoxia causes downregulation of protein and RNA synthesis in noncontracting mammalian cardiomyocytes. Circ. Res. 90, 777-783.

Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O'Riordan, C. R. & Smith, A. E. (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 63, 827-834.

Clancy, J. P., Ruiz, F. E. & Sorscher, E. J. (1999). Adenosine and its nucleotides activate wild-type and R117H CFTR through an A_{2B} receptor-coupled pathway. Am. J. Physiol.

276, C361-C369.

Clemens, M. G. & Forrester, T. (1981). Appearance of adenosine triphosphate in the coronary sinus effluent from isolated working rat heart in response to hypoxia. J. Physiol. (Lond) 312, 143-158.

Cliff, W. H., Schoumacher, R. A. & Frizzell, R. A. (1992). cAMP-activated Cl channels in CFTR-transfected cystic fibrosis pancreatic epithelial cells. Am. J. Physiol. 262, C1154-1160.

Cohen, M. V., Baines, C. P. & Downey, J. M. (2000). Ischemic preconditioning: From adenosine receptor to K_{ATP} channel. Annu. Rev. Physiol. 62, 79-109.

Communal, C., Singh, K., Sawyer, D. B. & Colucci, W. S. (1999). Opposing effects of β1- and β2-adrenergic receptors on cardiac myocyte apoptosis; Role of a pertussis toxin-sensitive G protein. Circulation 100, 2210-2212.

Cross, H. R., Murphy, E., Bolli, R., Ping, P. & Steenbergen, C. (2002). Expression of activated PKC epsilon (PKCε) protects the ischemic heart, without attenuating ischemic H^+ production. J. Mol. Cell. Cardiol. 34, 361-367.

Depre, C. & Taegtmeyer, H. (2000). Metabolic aspects of programmed cell survival and cell death in the heart. Cardiovascular Res. 45, 538-548.

Diaz, R. J., Losito, V. A., Mao, G. D., Ford, M. K., Backx, P. H. & Wilson G.. J. (1999). Chloride channel inhibition blocks the protection of ischemic preconditioning and hypo-osmotic stress in rabbit ventricular myocardium. Circ. Res. 84, 763-775.

Diaz, R. J., Armstrong, S. C., Batthish, M., Backx, P. H., Ganote, C. E. & Wilson, G.. J. (2003). Enhanced cell volume regulation: a key protective mechanism of ischemic preconditioning in rabbit ventricular myocytes. J. Mol. Cell. Cardiol. 35, 45-58.

Duan, D., Ye, L., Britton, F., Miller, L. J., Yamazaki, J., Horowitz, B. & Hume, J. R. (1999). Purinoceptor-coupled Cl^- channels in mouse heart: a novel, alternative pathway for CFTR regulation. J. Physiol. 521, 43-56.

Engelhardt, S., Hein, L., Keller, U., Klambt, K. & Lohse, M. J. (2002). Inhibition of Na^+-H^+ exchange prevents hypertrophy, fibrosis, and heart failure in β_1-adrenergic receptor transgenic mice. Circ. Res. 90, 814-819.

Erlenkamp, S., Glitsch, H. G. & Kockskamper, J. (2002). Dual regulation of cardiac Na^+-K^+ pumps and CFTR Cl^- channels by protein kinases A and C. Pflugers. Arch. Eur. J. Physiol. 444, 251-262.

Fliegel, L. & Wang, H. (1997). The regulation of the Na^+/H^+ exchanger in the mammalian myocardium. J. Mol. Cell. Cardiol. 29, 1991-1999.

Fliss, H. & Gattinger, D. (1996). Apoptosis in ischemic and reperfused rat myocardium.

Circ. Res. 79, 949-956.

Forrester, T. & Williams, C. A. (1977). Release of adenosine triphosphate from isolated adult heart cells in response to hypoxia. J. Physiol. (Lond) 268, 371-390.

Frohlich, O. & Karmazyn, M. (1997). The Na-H exchanger revisited. An update on Na-H exchange regulation and the role of the exchanger in hypertension and cardiac function in health and disease. Cardiovas. Res. 26, 138-148.

Gottlieb, R. A., Burleson, K. O., Kloner, R. A., Babior, B. M. & Engler, R. L. (1994). Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J. Clin. Invest. 94, 1621-1628.

Gan, X. T., Chakrabarti, S. & Karmazyn, M. (1999). Modulation of Na^+/H^+ exchange isoform 1 mRNA expression in isolated rat hearts. Am. J. Physiol. 277, H993-H998.

Gao, J., Cohen, I. S., Mathias, R. T. & Baldo, G. J. (1994). Regulation of the β-stimulation of the Na^+-K^+ pump current in guinea-pig ventricular myocytes by a cAMP-dependent PKA pathway. J. Physiol. 477, 373-380.

Gray,, M. O., Karliner, J. S. & Mochly-Rosen, D. (1997). A selective ε-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. J. Biol. Chem. 272, 30945-30951.

Hall, R. A., Premont, R. T., Chow, C., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S. Weinman, E. J., Grinstein, S. & Lefkowitz, R. J. (1998). The β_2-adrenergic receptor interacts with the Na^+/H^+-exchanger regulatory factor to control Na^+/H^+ exchange. Nature 392, 626-630.

Hart, P., Geary, Y., Warth, J., Collier, M. L., Chapman, T., Hume, J. R. & Horowitz, B. (1994). Molecular and electrophysiological characterization of $CFTR_{cardiac}$ in normal and CF human hearts. Biophys. J. 66, A141, abstract.

Hart, P., Warth, J. D., Levesque, P. C., Collier, M., Geary, Y., Horowitz, B. & Hume, J. R. (1996). Cystic fibrosis gene encodes a cAMP-dependent chloride channel in heart. Proc. Natl. Acad. Sci. USA 93, 6343-6348.

Harvey, R. D. & Hume, J. R. (1989). Autonomic regulation of a chloride current in heart. Science 244, 983-985.

Hool, L. C. (2000). Hypoxia increases the sensitivity of the L-type Ca^{2+} current to β-adrenergic receptor stimulation via a C2 region-containing protein kinase C isoform. Circ. Res. 87, 1164-1171.

Hool, L. C. (2001). Hypoxia alters the sensitivity of the L-type Ca^{2+} channel to α-adrenergic receptor stimulation in the presence of β-adrenergic receptor stimulation. Circ. Res. 74, 365-507.

Horowitz, B., Tsung, S. S., Hart, P., Levesque, P. C. & Hume, J. R. (1993). Alternative splicing of CFTR Cl^- channels in heart. Am. J. Physiol. 264, H2214-H2220.

Hume, J. R., Duan, D., Collier, M. L., Yamazaki, J. & Horowitz, B. (2000). Anion transport in heart. Physiol. Rev. 80, 31-81.

Inoue M, Fujishiro, N. & Imanaga, I. (1999). Na^+ pump inhibition and non-selective cation channel activation by cyanide and anocia in guinea-pig chromaffin cells. J. Physiol. 519, 385-396.

Ito, Y., Mizuno, Y., Aoyama, M., Kume, H. & Yamaki, K. (2000). CFTR-mediated anion conductance regulates Na^+-K^+-pump activity in calu-3 human airway cells. Biochem. Biophys. Res. Commun. 274, 230-235.

James, A. F., Tominaga, T., Okada, Y. & Tominaga, M. (1996). Distribution of cAMP-activated chloride current and CFTR mRNA in the guinea pig heart. Circ. Res. 79, 201-207.

Jennings, R. B., Reimer, K. A. & Steenbergen, C. (1986). Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. J. Mol. Cell. Cardiol. 18, 769-780.

Jia, Y., Mathews, C. J. & Hanrahan, J. W. (1997). Phosphorylation by protein kinase C is required for acute activation of cystic fibrosis transmembrane conductance regulator

by protein kinase A. (1997). J. Biol. Chem. 272, 4978-4984.

Jiang, C., Lu, H., Vincent, K. A., Shankara, S., Belanger, A. J., Cheng, S. H., Akita, G. Y., Kelly, R. A., Goldberg, M. A. & Gregory, R. J. (2002). Gene expression profiles in human cardiac cells subjected to hypoxia or expressing a hybrid form of HIF-1α. Physiol. Genomics 8, 23-32.

Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M. & Tsui, L. (1989). Identification of the cystic fibrosis gene: genetic analysis. Science 245, 1073-1080.

Kockskamper, J., Erlenkamp, S. & Glitsch, H. G. (2000). Activation of the cAMP-protein kinase A pathway facilitates Na^+ translocation by the Na^+-K^+pump in guinea-pig ventricular myocytes. J. Physiol. 523, 561-574.

Kockskamper, J., Sendhoff, K., Erlenkamp, S., Bordusa, F., Cerovsky, V. & Glitsch, H. G. (2001). Differences in the protein-kinase-A-dependent regulation of CFTR Cl^- channels and Na^+-K^+ pumps in guinea-pig ventricular myocytes. Pflugers Arch. Eur. J. Physiol. 441, 807-815.

Kunzelmann, K. (2001). CFTR: Interacting with everything? News Physiol. Sci. 16, 167-170.

Kurrelmeyer, K. M., Michael, L. H., Baumgarten, G., Taffet, G. E., Peschon, J. J.,

Sivasubramanian, N., Entman, M. L. & Mann, D. L. (2000). Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. Proc. Natl. Acad. Sci. USA 97, 5456-5461.

Kuzimin, A. I., Gourine, A. V., Molosh, A. I., Lakomkin, V. L. & Vassort, G. (1998). Interstitial ATP level and degradation in control and postmyocardial infracted rats. Am. J. Physiol. 275, C766-C-771.

Kuzimin A. I., Kapelko, V. I., Lakomkin, V. L. & Vassort, G. (2000). Efects of preconditioning on myocardial interstitial levels of ATP and its catabolites during regional ischemia and reperfusion in the rat. Basic. Res. Cardiol. 95, 127-136.

Lader, A.S., Wang, Y, Jackson, G. R., Borkan, S. C. & Cantiello, H. F. (2000). cAMP-activated anion conductance is associated with expression of CFTR in neonatal mouse cardiac myocytes. Am. J. Physiol. 278, C436-C450.

Ladilov, Y. V., Balser-Schafer, C., Haffner, S., Mazeiner, H. & Piper, H. M. (1999). Pretreatment with PKC activator protects cardiomyocytes against reoxygenation-induced hypercontracture independently of Ca^{2+} overload. Cariovascular Res. 43, 408-416.

Leblais, V., Demolombe, S., Vallette, G., Langin, D., Baro, I., Escande, D. & Gauthier, C. (1999). β_3-adrenoceptor control the cystic fibrosis transmembrane conductance

regulator through a cAMP/protein kinase A-independent pathway. J. Biol. Chem. 274, 6107-6113.

Liedtke, C. M. & Cole, T. S. (1998). Antisense oligonucleotide to PKC-ε alters cAMP-dependent stimulation of CFTR in calu-3 cells. Am. J. Physiol. 275, C1357-C1364.

Liedtke, C. M., Yun, C. H. C., Kyle, N. & Wang, D. (2002). Protein kinase Cε-dependent regulation of cystic fibrosis transmembrane regulator involves binding to a receptor for activated C kinase (RACK1) and RACK1 binding to Na^+/H^+ exchange regulatory factor. J. Biol. Chem. 277, 22925-22933.

Light, P. E. & French, R. J. (1994). Glibenclamide selectively blocks ATP-sensitive K^+ channels reconstituted from skeletal muscle. Eur. J. Pharmacol. 259, 219-222.

Liu, H., McPherson, B. C. & Yao, Z. (2001). Preconditioning attenuates apoptosis and necrosis: role of protein kinase Cε and -δ isoforms. Am. J. Physiol. 281, H404-H410.

Liu, H., Zhang, H. Y., Zhu, X., Shao, Z. & Yao, Z. (2002). Preconditioning blocks cardiocyte apoptosis: role of K_{ATP} channels and PKC-ε. Am. J. Physiol. 282, H1380-H1386.

Lochner, A., Genade, S., Tromp, E., Podzuweit, T. & Moolman, J. A. (1999). Ischemic preconditioning and the β-adrenergic signal transduction pathway. Circulation 100,

958-966.

Long, X., Boluyt, M. O., Hipolito, M. L., Lundberg, M. S., Zheng, J.-S., O'Neill, L., Cirielli, C., Lakatta, E. G. & Crow, M. T. (1997). p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes. J. Clin. Invest. 99, 2635-2643.

Mackay, K. & Mochly-Rosen, D. (1999). An inhibitor of p38 mitogen-activated protein kinase protects neonatal cardiac myocytes from ischemia. J. Biol. Chem. 274, 6272-6279.

Maulik, N., Engelman, R. M., Rousou, J. A., Flack, J. E., Deaton, D. & Das, D. K. (1999). Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2. Circulation 100, SⅡ369-Ⅱ375.

McDonald, T. F., Pelzer, S., Trautwein, W. & Pelzer, D. J. (1994). Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. Physiol. Rev. 74, 365-507.

Middleton, L. M. & Harvey, R. D. (1998). PKC regulation of cardiac CFTR Cl^- channel function in guinea pig bentricular myocytes. Am. J. Physiol. 275, C293-C302.

Mossman, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immun. Meth. 65, 55-63.

Naren, A. P., Cobb, B., Li, C., Roy, K., Nelson, D., Heda, G. D., Liao, J., Kirk, K. L., Sorscher, E. J., Hanrahan, J. & Clancy, J. P. (2003). A macromolecular complex of β_2 adrenergic receptor, CFTR, and ezrin/radixin/moesin-binding phosohoprotein 50 is regulated by PKA. Proc. Natl. Acad. Sci. USA 100, 342-346.

Ninomiya, H., Otani, H., Lu, K., Uchiyama, T. & Kido, M. (2002). Complementary role of extracellular ATP and adenosine in ischemic preconditioning in the rat heart. Am. J. Physiol. 282, H1810-1820.

Okada, Y., Maeno, E., Shimizu, T., Dezaki, K., Wang, J. & Morishima, S. (2001). Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). J. Physiol. 532, 3-16.

Oz, M. C. & Sorota, S. (1995). Forskolin stimulates swelling-induced chloride current, not cardiac cystic fibrosis transmembrane-conductance regulator current, in human cardiac myocytes. Circ. Res. 76, 1063-1070.

Paddle, B. M. & Burnstock, G. (1974). Release of ATP from perfused heart during coronary vasodilatation. Blood Vessels. 11, 110-119.

Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., Drumm, M., Iannuzzi, M. C., Collins, F. S. & Tsui, L. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245, 1066-1073.

Rommens, J. M., Iannuzzi, M. C., Kerem, B., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, R., Cole, J. L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J. R., Tsui, L. & Collins, F. S. (1989). Identification of the cystic fibrosis gene: chromosome walking and junping. Science 245, 1059-1065.

Schaffer, S. W., Croft, C. B. & Solodushko, V. (2000). Cardioprotective effect of chronic hyperglycemia: effect on hypoxia-induced apoptosis and necrosis. Am. J. Physiol. 278, 1948-1954.

Schwiebert, E. M., Benos, D. J., Egan, M. E., Stutts, M. J. & Guggino, W. B. (1999). CFTR is a conductance regulator as well as a chloride channel. Physiol. Rev. 79, S145-S166.

Simpson, P. & Savion, S. (1982). Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Circ. Res. 50, 101-116.

Steenbergen, C., Hill, M. L. & Jennings R. B. (1985). Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischemic myocardium in vitro. Effects of osmotic cell swelling on plasma membrane integrity. Circ. Res. 57, 864-875.

Stegemann, M., Meyer, R., Haas, H. G. & Robert-Nicoudm, M. (1990). The cell surface of isolated cardiac myocytes – A light microscope study with use of fluorochrome-coupled lectins. J. Mol. Cell. Cardiol. 22, 787-803.

Swiatecka-Urban, A., Duhaime, M., Coutermarsh, B., Karlson, K. H., Collawn, J., Milewski, M., Cutting, G. R., Guggino, W. B., Langford, G. & Stanton, B. A. (2002). PDZ domain interaction controls the endocytic recycling of the cystic fibrosis transmembrane conductance regulator. J. Biol. Chem. 277, 40099-40105.

Tanaka, M., Ito, H., Adachi, S., Akimoto, H., Nishikawa, T., Kasajima, T, Marumo, F. & Hiroe, M. (1994). Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Circ. Res. 75, 426-433.

Tilly, B. C., Bezstarosti, K., Boomaars W. E. M., Marino, C. R., Lamers, J. M. J. & Jonge, H. R. (1996). Expression and regulation of chloride channels in neonatal rat cardiomyocytes. Mol. Cell. Biochem. 157, 129-135.

Tominaga, M., Horie, M., Sasayama, S. & Okada, Y. (1995). Glibenclamide, an ATP-sensitive K^+ channel blocker, inhibits cardiac cAMP-activated Cl^- conductance. Circ. Res. 77, 417-423.

Tranum-Jensen, J., Janse, J., Fiolet, J. ,W. T., Krieger, W. J. G., D'alnoncourt, C. N. & Durrer, D. (1981). Tissue osmolality, cell swelling, and reperfusion in acute regional myocardial ischemia in the isolated porcine heart. Circ. Res. 49, 364-381.

Vial, C., Owen, P., Opoe, L. H. & Posel, D. (1987). Significance of adenosine triphosphate and adenosine induced by hypoxia or adrenaline in perfused rat heart. J.

Mol. Cell. Cardiol. 19, 187-197.

Walsh, K. B. & Wang, C. (1996). Effect of chloride channel blockers on the cardiac CFTR chloride and L-type calcium currents. Cardiovascular Res. 32, 391-399.

Wang, Z., Mitsuiye, T., Rees, S. A. & Noma, A. (1997). regulatory volume decrease of cardiac myocytes induced by β-adrenergic activation of the Cl^- channel in guinea pig. J. Gen. Physiol. 110, 73-82.

Warth, J. D., Collier, M. L., Hart, P., Geary, Y., Gelband, C. H., Chapman, T., Horowitz, B. & Hume, J. R. (1996). CFTR chloride channels in human and simian heart. Cardiovascular Res. 31, 615-624.

Wellman, G. C., Barrett-Jolley, R., Koppel, H., Everitt, D. & Quayle, J. M. (1999). Inhibition of vascular K (ATP) channels by U-37883A: a comparison with cardiac and skeletal muscle. Br. J. Pharmacol. 128, 909-916.

Welsh, M. J. & Fick, R. B. (1987). Cystic fibrosis. J. Clin. Invest. 80, 1523-1526.

Wiebicke, W., Artlich, A. & Gerling, I. (1993). Myocardial fibrosis—a rare complication in patients with cystic fibrosis. Eur. J. Pediatr. 152, 694-696.

Xiao, X. & Allen, D. G. (1999). Role of Na^+/H^+ exchanger during ischemia and preconditioning in the isolated rat heart. Circ. Res. 85, 723-730.

Yajima, T., Nagashima, H., Tsutsumi-Sakai, R., Hagiwara, N., Hosoda, S., Querterrmouus, T., Kasanuki, H. & Kawana, M. (1997). Functional activity of the CFTR Cl^- channel in human. Heart Vessels 12, 255-261.

Zebrak, J., Skuza, B., Pogorzelski, A., Ligarska, R., Kopytko, E., Pawlik, J., Rutkewicz, E. &Witt, M. (2000). Partial CFTR genotyping and characterization of cystic fibrosis patients with myocardial fibrosis and necrosis. Clin. Genet. 57, 56-60.

Zhou, S., Takai, A., Tominaga, M. & Okada, Y. (1997). Phosphatase-mediated enhancement of cardiac cAMP-activated Cl^- conductance by a Cl^- channel blocker, anthracene-9-carboxylate. Circ. Res. 81, 219-228.

Zhu, W., Zheng, M., Koch, W. J., Lefkowitz, R. J., Kobilka, B. K. & Xiao, R. (2001). Dual modulation of cell survival and cell death by β_2-adrenergic signaling in adult mouse cardiac myocytes. Proc. Natl. Acad. Sci. USA 98, 1607-1612.

Zizak, M., Lamprecht, G., Steplock, D., Tariq, N., Shenolikar, S., Donowitz, M., Yun, C. H. C. & Weinman, E. J. (1999). cAMP-induced phosphorylation and inhibition of Na^+/H^+ exchanger 3 (NHE3) are dependent on the presence but not the phosphorylation of NHE regulatory factor. J. Biol. Chem. 274, 24753-24758.

Figure Legends

Fig. 1 Experimental protocols to examine effects of ischemia on the expression of CFTR mRNA or CFTR protein.

Three protocols were used to perform this study. Protocol A, to examine the time course of western blotting data. Protocol B, to analyze densities of the blotting bands and localization of CFTR on the plasma membrane. Protocol C, to examine expression of the mRNA. Numbers given beneath arrows indicate sampling times (in h) after ischemia.

Fig. 2 RT-PCR of CFTR mRNA in control and ischemic cardiomyocytes.

RT-PCR was performed on poly (A)+RNA isolated from cardimyocytes subjected to ischemic stress (see Fig. 1C). Positive control experiments were performed for mRNA of a house-keeping protein, G3PDH.

Fig. 3 Effect of ischemia on CFTR expression patterns.

A, Representative time course of ischemia-induced changes in expression of CFTR proteins in the membrane fraction detected by western blotting. Lane a and b were applied samples from empty vector-transfected HEK293T cells and WT CFTR vector-transfected cells, respectively. The mature CFTR bands correspond to about 180 kD protein (upper arrowhead) and the immature CFTR band to about 140 kD protein (lower arrowhead). Lanes 0 to 8 correspond to expression of CFTR proteins detected at 0 to 8 h after ischemia (Fig. 1A). Bottom numbers represent relative densities of mature

CFTR bands compared to control (0 h, taken as 100%) for these blots. B, Densitometry analysis of expressed CFTR proteins observed at 0, 1, 3, 5, 8 h after ischemia. Relative intensity values of integrated optical densities of the mature and immature bands are shown as percentages of the respective control (0 h, taken as 100%). Each column represents the average of five independent experiments, and each vertical bar represents the SE value. Asterisks; $P<0.01$, compared with the control (0 h).

Fig. 4 Effects of ischemic stress on the expression of CFTR proteins localized on the plasma membrane.

In order to detect the CFTR protein localized on the plasma membrane, the dual fluorescence staining method by immuno- and non-immuno-staining using polyclonal anti-rat CFTR antibodies and WGA, which specifically binds to residues of glycoside on the cell surface, was applied. A, B and C demonstrate expression of CFTR (red), WGA (green), co-localization visualized by image overlay (yellow), respectively on observed at 0, 1, 3 and 8 h after ischemia. The protocol shown in Fig. 1B. The bottom photos represent their expression at 8 h after ischemia with co-application of PKA activators (10 μM FSK and 0.5 μM IBMX) and a PKC activator (100 nM PMA).

Fig. 5 Effects of co-application of PKA and PKC activators on CFTR expression at 8 h after ischemia.

A, Representative expression of CFTR proteins induced by 8 h-ischemic stress under co-application of PKA activators (10 μM FSK and 0.5 μM IBMX) and a PKC activator (100 nM PMA). Expression of CFTR proteins were examined by western blotting. The mature CFTR bands correspond to a ~180 kD protein (upper arrowhead) and the

immature CFTR bands to a ~140 kD protein (lower arrowhead). B, Densitometry analysis of expressed CFTR proteins. Relative intensity values of integrated optical densities of the mature and immature bands are shown as percentages of the respective control (0 h, taken as 100%). Each column represents the average of four independent experiments, and each vertical bar represents the SE value. Asterisks; $P<0.01$, it was compared the with or without the activators.

Fig. 6 Experimental protocols to examine effects of ischemia on CFTR currents recorded under whole-cell patch-clamp.

Fig. 7 cAMP-activated, I-V linear whole-cell currents in cardiomyocytes before and after ischemic treatment.

To examine effects of ischemic stress on cAMP-activated currents, whole-cell recordings were performed in control and ischemic cardiomyocytes by using the protocols shown in Figure 6. A, Expanded traces of current responses to step pulses from –80 to +80 mV, recorded before (top panels) and after (lower panels) application of a cAMP-activating cocktail (1 mM dbcAMP + 0.5 μM IBMX + 10 μM FSK). B, I-V relationships for the mean currents before (open circles) and after (filled circles) cAMP stimulation. Each symbol represents the mean value (with the SE bar) of 3～8 observations. Asterisks; $P<0.05$, compared with the control data obtained in non-activated cells.

Fig. 8 Effects of Cl^- channel blockers and of reduction of the extracellular Cl^- concentration on cAMP-activated currents recorded

in ischemic cardiomyocytes.

The currents were recorded in cardiomyocytes subjected to ischemia stress for 3 to 4 h (the protocol shown in Fig. 6). A) Representative whole-cell currents activated by a cAMP cocktail before, during and after application of Cl^- channel blockers. B) Effects of Cl^- channel blockers on cAMP-activated whole-cell currents recorded at ±80mV. Asterisks; $P<0.05$, compared with the control data (without adding blockers). C) Effects of reduction of the extracellular Cl^- concentration on cAMP-activated whole-cell currents. Currents were recorded in symmetrical (111 mM/111 mM) or asymmetrical (111 mM/8 mM) Cl^- conditions. The asymmetrical Cl^- conditions were produced by replacing extracellular NaCl with Na-gluconate.

Fig. 9 Experimental protocols for optimization of experimental conditions.

The cells were provided for each experiment 4 days after primary culture and were divided to 6 groups, the half was used as the non-ischemic control and the remaining group was used for ischemic experiments. Ischemic stress was applied for 6, 8 or 16 h by changing the bath solution to glucose-free buffer solution and then cells were kept under hypoxic conditions. For the non-ischemic control group, bath solution was replaced by new M199 solution and then kept in a CO_2 incubator for 6, 8 or 16 h. All groups were then left in a CO_2 incubator for 4 days to attain post-ischemic recovery. Immediately after or 3 to 5 days after ischemic treatment, cell viability was assessed by the MTT assay.

Fig. 10 Effects of varying time periods of ischemia on post-ischemic

recovery of cell viability in cardiac myocytes.

Each symbol represents the mean value (with the SE bar) of 6 observations.

Fig. 11 Experimental protocol to examine effects of various reagents or drugs on the post-ischemic cell viability.

Cultured ventricular myocytes were divided to four groups; that is, iscemia and non-ischemia groups in the absence and presence of a given drug. Ischemic and post-ischemic recovery periods were 8 h and 4 days, respectively.

Fig. 12 Effects of various Cl^- and K^+ channel blockers on post-ischemic recovery: Inhibition by CFTR channel blockers.

The protocols are shown in Figure 11. To examine effects of Cl^- and K^+ channel blockers, 50 μM NPPB, 500 μM glibenclamide, 2 mM 9AC, 2 mM gemfibrozil, 300 μM SITS, 300 μM IAA-94, 300 μM DIDS, 50 μM furosemide or 20 mM TEA-Cl was added for 8 h to either M199 medium (non-ischemia with drugs) or ischemic buffer (ischemia with drugs), and then removed upon reoxygenation. Data represent the mean values (with the SE bars) of 9 observations. Asterisks; $P<0.05$, compared with the control (non-treatment) ischemia data (without drugs).

Fig. 13 Effects of treatments with dbcAMP, PMA, FSK and ISO on the post-ischemic recovery.

The protocols are shown in Figure 11. To examine effects of CFTR channel activators, 1 mM dbcAMP, 10 μM FSK, 1 μM ISO, 0.5 μM IBMX and/or 100 nM PMA were added for 8 h to either M199 medium (non-ischemia with or without drugs), or ischemic

buffer (ischemia with or without drugs) and then removed upon reoxygenation. Data represent the mean values (with the SE bars) of 9 observations. Asterisks; $P<0.05$, compared with the control (non-treatment) ischemia data (without drugs).

Fig. 14 Effects of CFTR channel blockers on enhancement of post-ischemic recovery induced by dbcAMP plus PMA.

The protocols are shown in Figure 11. To examine effects of CFTR channel blockers on the post-ischemic recovery enhanced by CFTR activators (dbcAMP plus PMA), NPPB, glibenclamide, gemfibrozil or 9AC was added to either non-ischemic M199 medium or ischemic buffer, and then removed upon reoxygenation. Data represent the mean values (with the SE bars) of 9 observations. Asterisks; $P<0.05$, compared with the control (non-treatment) ischemia data (without drugs).

Fig. 15 Experimental protocols to examine the effective timing of treatment with a CFTR blocker or its activators. NPPB or dbcAMP plus PMA were added either for 1 h prior to or for 16 h after ischemic treatment.

Fig. 16 Effects of a CFTR blocker and CFTR activators applied prior to, during or after ischemic treatment on post-ischemic recovery.

The experiments were performed in cardiomyocytes subjected to ischemic stress according to the protocols shown in Figure 15. A, Viability of the control cells (non-treatment). B, Viability of the cells treated with a CFTR blocker, NPPB (50 μM). C, Viability of the cells treated with CFTR activators, dbcAMP (1 mM) plus PMA (100 nM). Data represent the mean values (with the SE bars) of 9 observations. Asterisks;

P<0.05, compared with the control (non-treatment) ischemia data (without drugs).

Fig. 17 Effects of CFTR activators (PMA plus dbcAMP) and inhibitor (NPPB) on ischemia- and ischemia/reoxygenation-induced DNA fragmentation (apoptosis).

Intranucleosomal DNA fragmentation and cell viability were measured in cardiomyocytes, just after or 4 days after ischemia treatment in the presence or absence of CFTR activators, dbcAMP (1 mM) plus PMA (100 nM), or its blocker, NPPB (50 μM). Data represent the mean values (with the SE bars) of 6 or 9 observations. Asterisks; *P*<0.05, compared with the control (non-treatment) ischemia data (without drugs).

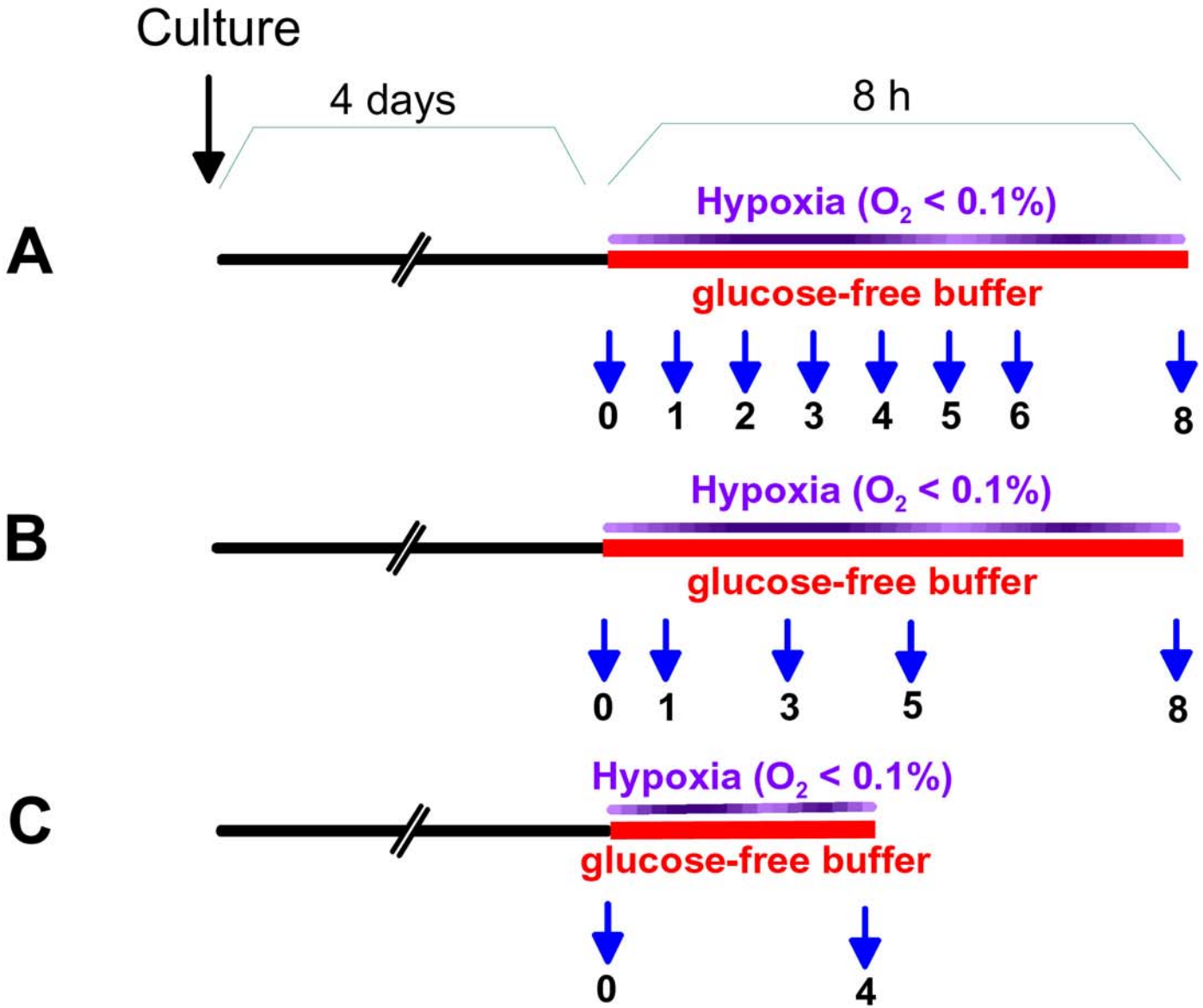

Culture
4 days
8 h
A
Hypoxia (O_2 < 0.1%)
glucose-free buffer
0 1 2 3 4 5 6 8
B
Hypoxia (O_2 < 0.1%)
glucose-free buffer
0 1 3 5 8
C
Hypoxia (O_2 < 0.1%)
glucose-free buffer
0 4

Fig. 1

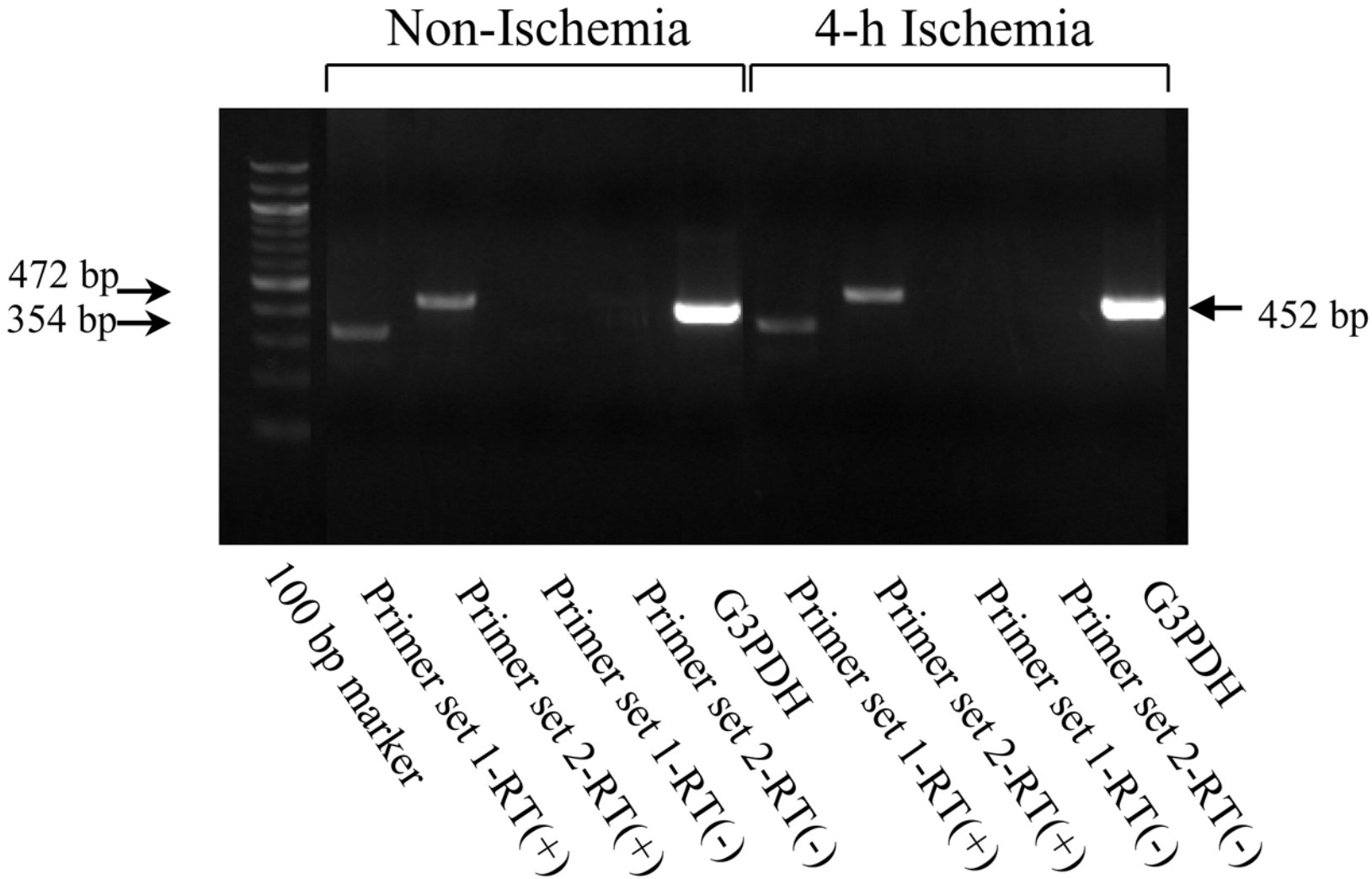

Non-Ischemia
4-h Ischemia
472 bp
354 bp
452 bp
100 bp marker
Primer set 1-RT(+)
Primer set 2-RT(+)
Primer set 1-RT(-)
Primer set 2-RT(-)
G3PDH
Primer set 1-RT(+)
Primer set 2-RT(+)
Primer set 1-RT(-)
Primer set 2-RT(-)
G3PDH

Fig. 2

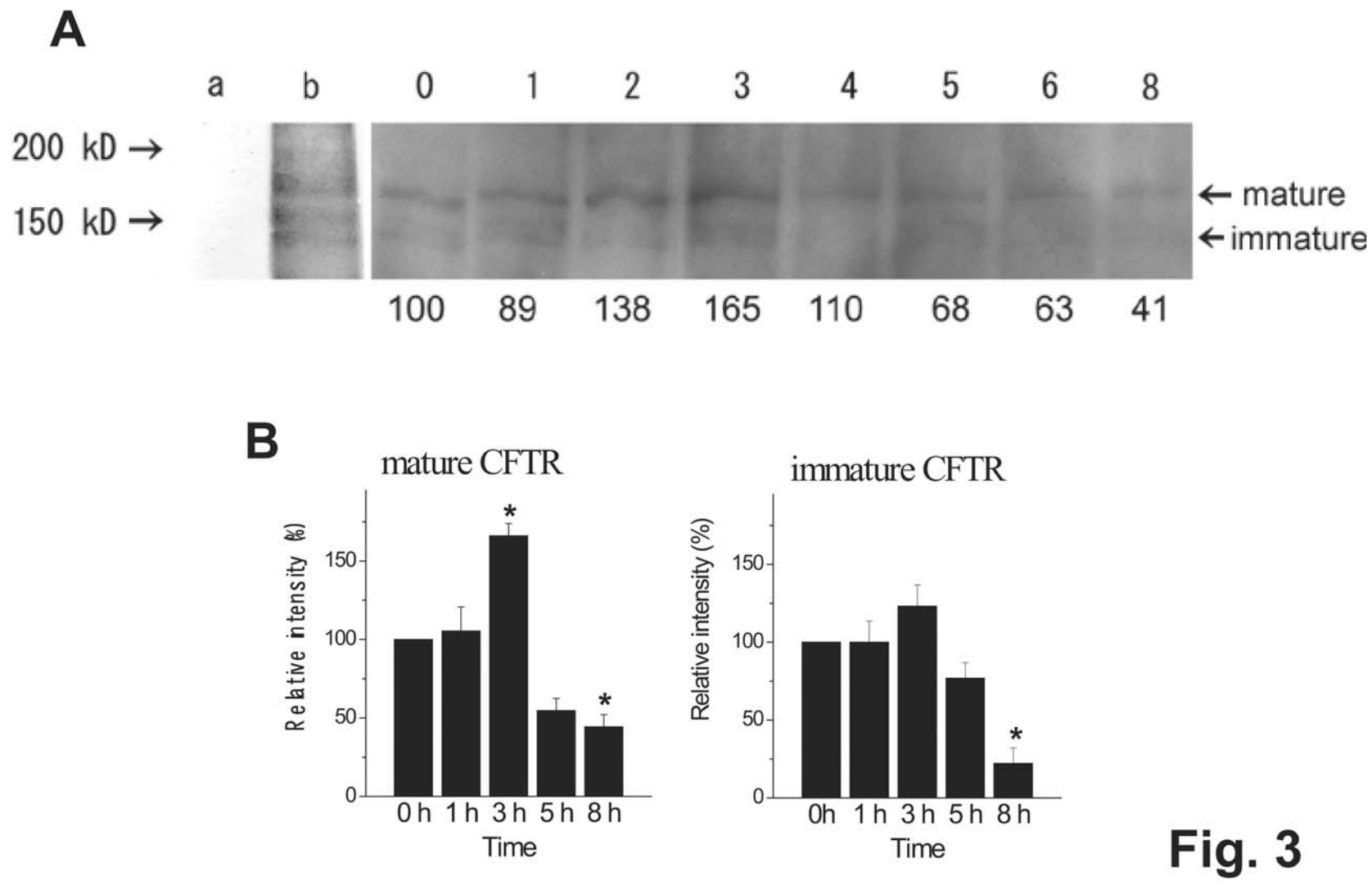
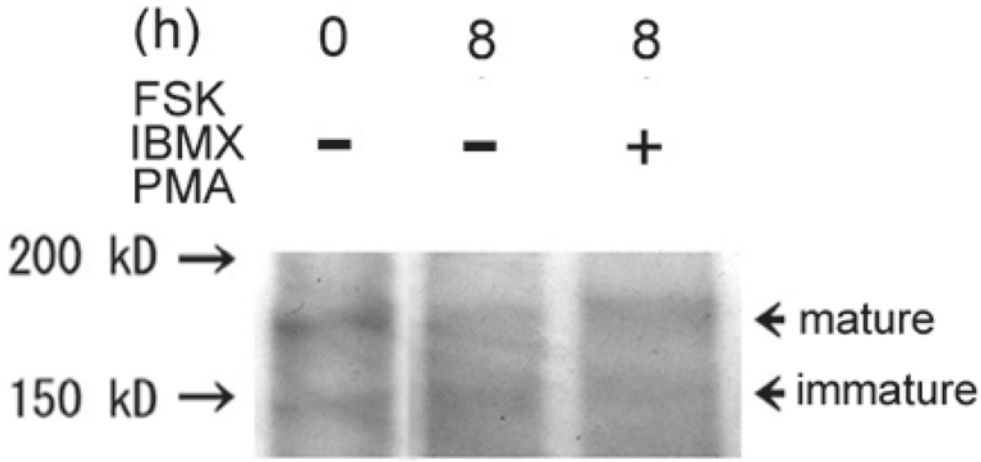

A
a
b
0
1
2
3
4
5
6
8
200 kD →
150 kD →
← mature
← immature
100
89
138
165
110
68
63
41
B
mature CFTR
immature CFTR
Relative intensity (%)
150
100
50
0
*
*
0 h
1 h
3 h
5 h
8 h
Time
Relative intensity (%)
150
100
50
0
*
0h
1 h
3 h
5 h
8 h
Time

Fig. 3


(h) 0 8 8
FSK
IBMX - - +
PMA
200 kD →
150 kD →
← mature
← immature

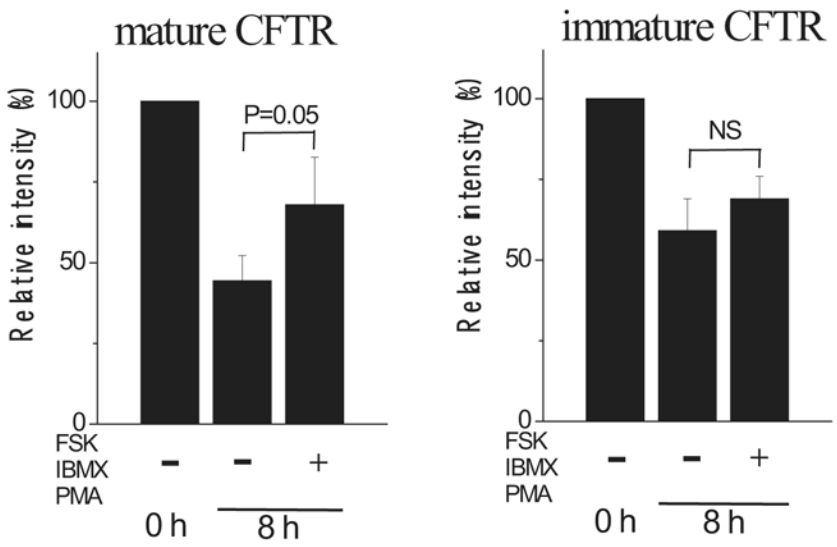

mature CFTR
Relative intensity (%)
100
50
0
P=0.05
FSK
IBMX - - +
PMA
0 h
8 h
immature CFTR
Relative intensity (%)
100
50
0
NS
FSK
IBMX - - +
PMA
0 h
8 h

Fig. 4

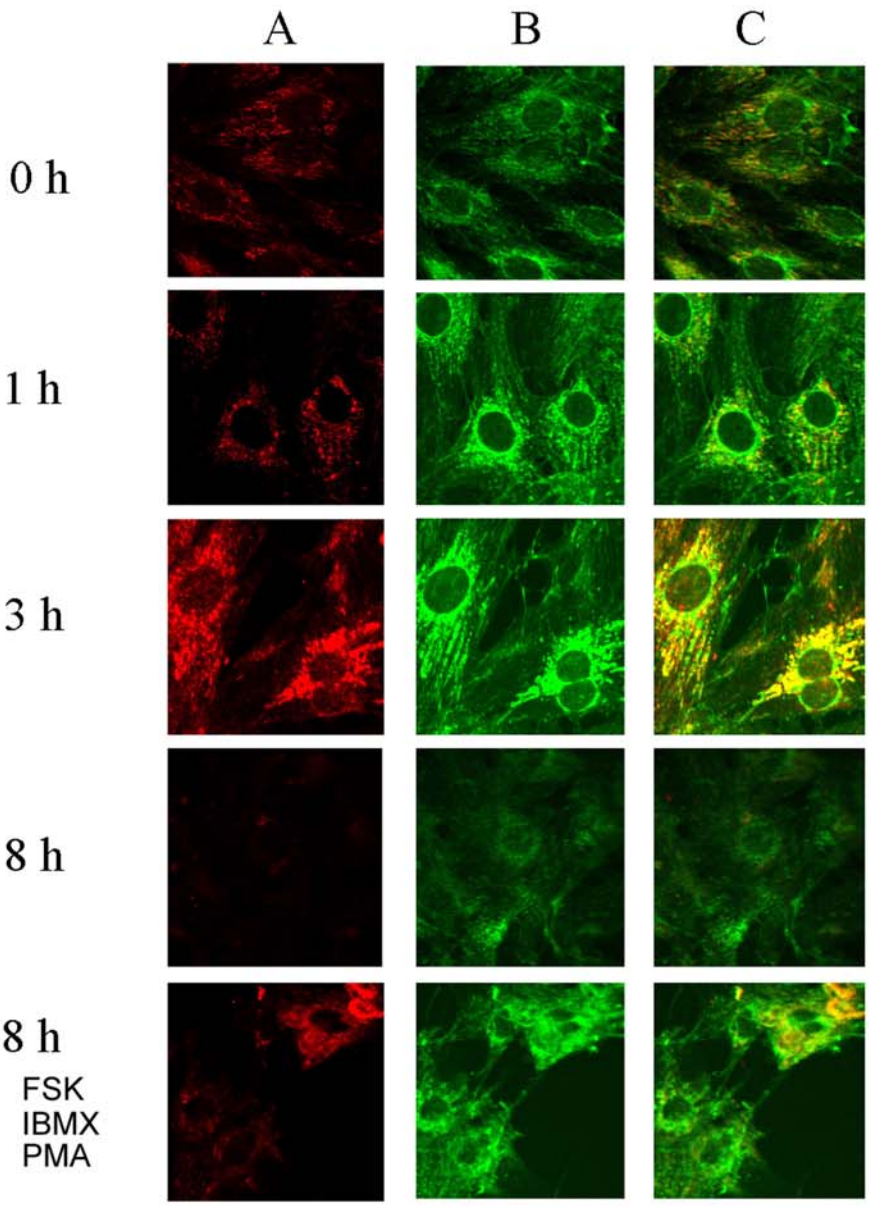


Fig. 5

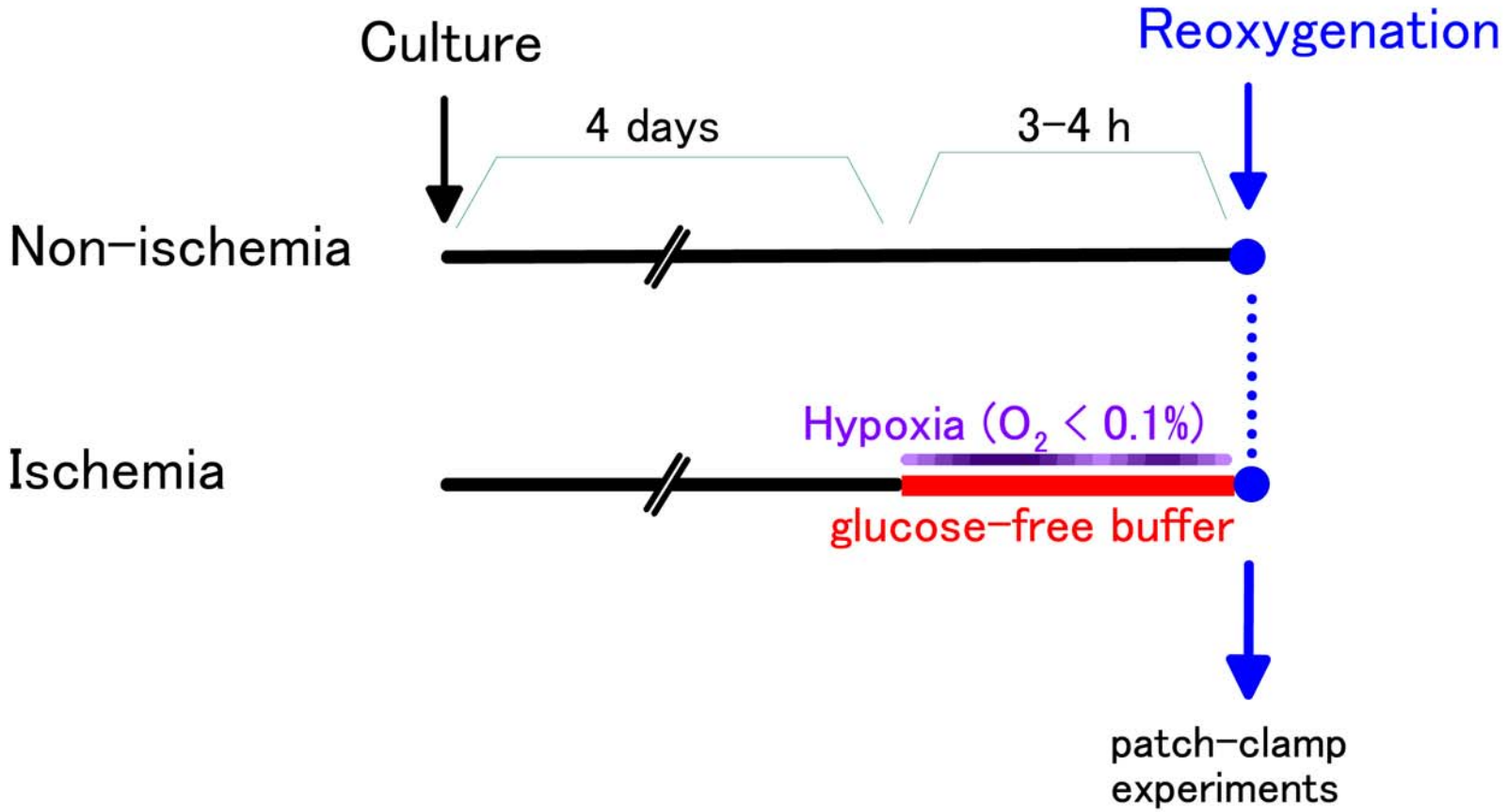

Culture
Reoxygenation
4 days
3–4 h
Non–ischemia
Ischemia
Hypoxia (O_2 < 0.1%)
glucose–free buffer
patch–clamp
experiments

Fig. 6

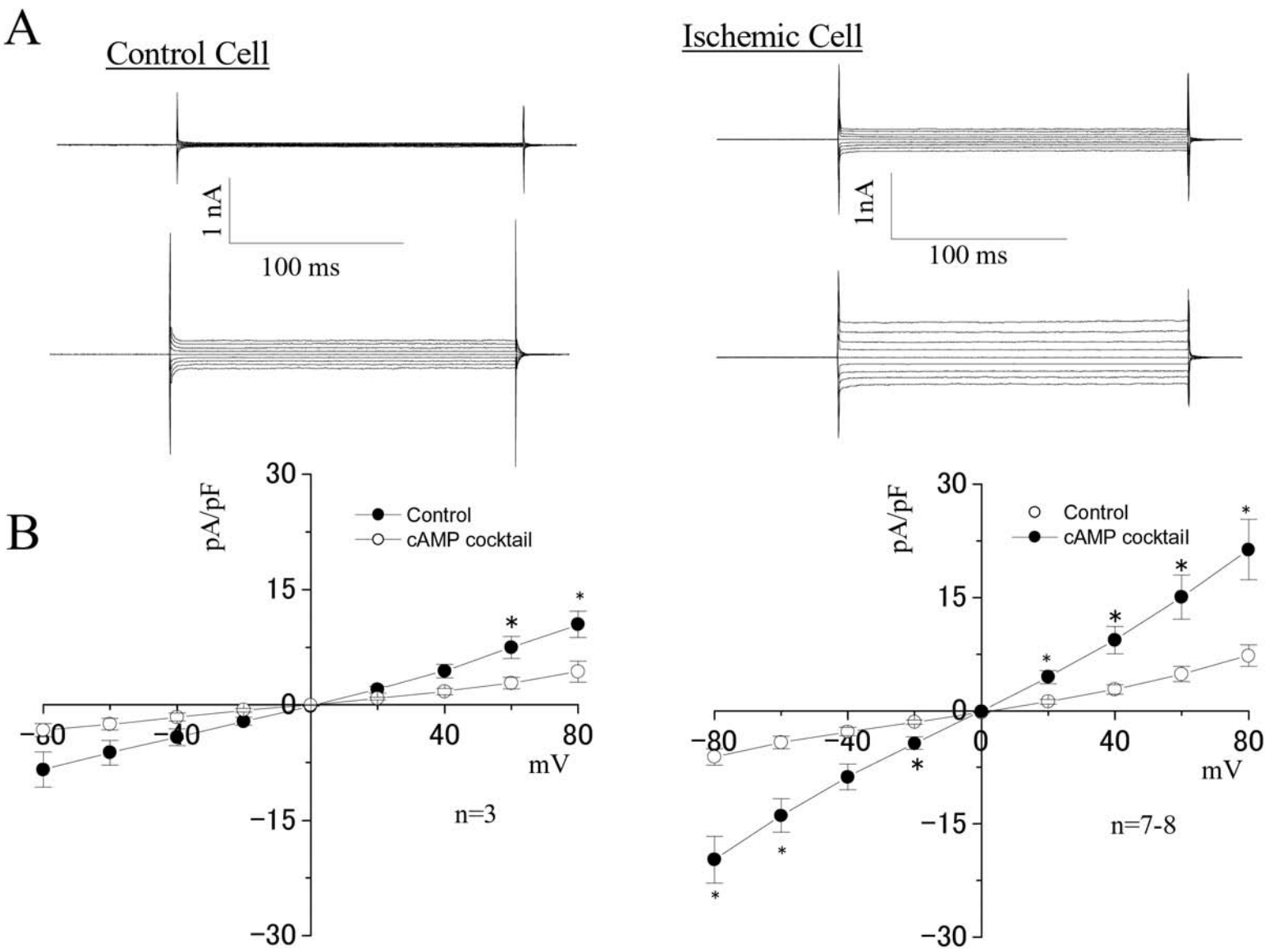

A
Control Cell
1 nA
100 ms
Ischemic Cell
1nA
100 ms
B
pA/pF
Control
cAMP cocktail
30
15
0
−15
−30
−80
−40
40
80
mV
n=3
n=7-8
*

Fig. 7

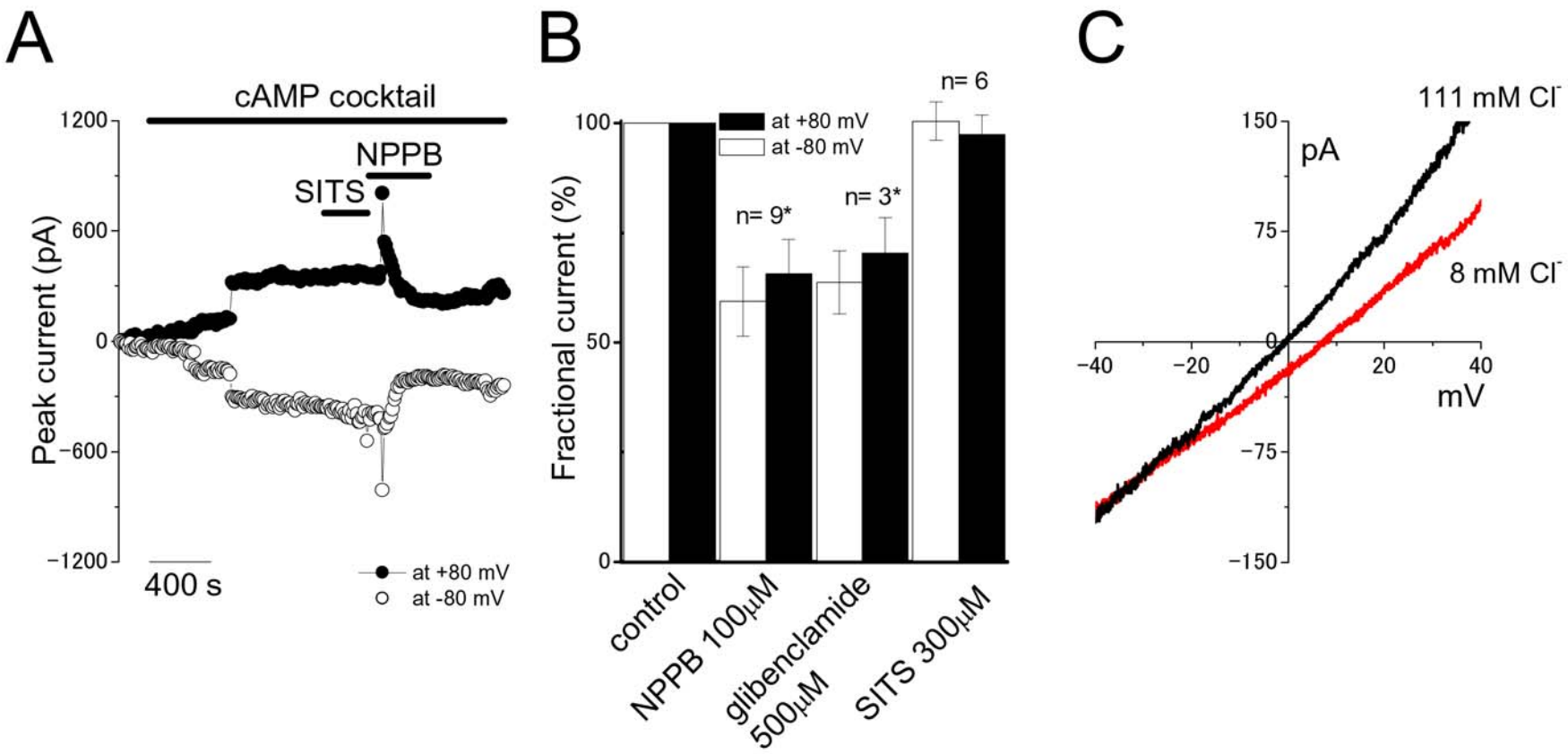

A
cAMP cocktail
NPPB
SITS
1200
600
0
−600
−1200
Peak current (pA)
400 s
at +80 mV
at -80 mV
B
100
50
0
Fractional current (%)
at +80 mV
at -80 mV
n= 9*
n= 3*
n= 6
control
NPPB 100μM
glibenclamide 500μM
SITS 300μM
C
111 mM Cl-
8 mM Cl-
150
75
0
−75
−150
pA
−40
−20
20
40
mV

Fig. 8

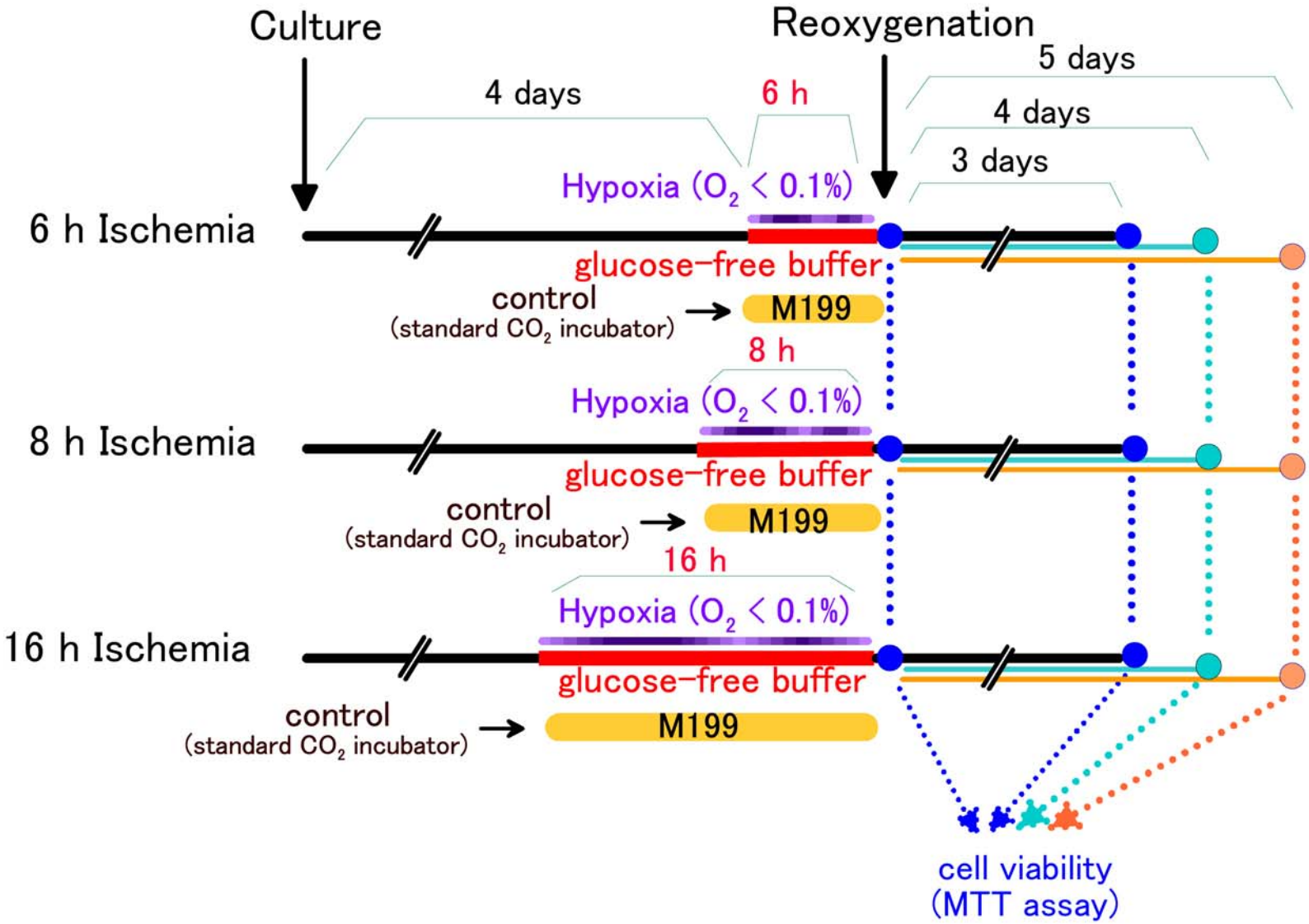

Culture
Reoxygenation
4 days
6 h
5 days
4 days
3 days
Hypoxia (O_2 < 0.1%)
6 h Ischemia
glucose-free buffer
control
(standard CO_2 incubator)
M199
8 h
Hypoxia (O_2 < 0.1%)
8 h Ischemia
glucose-free buffer
control
(standard CO_2 incubator)
M199
16 h
Hypoxia (O_2 < 0.1%)
16 h Ischemia
glucose-free buffer
control
(standard CO_2 incubator)
M199
cell viability
(MTT assay)

Fig. 9

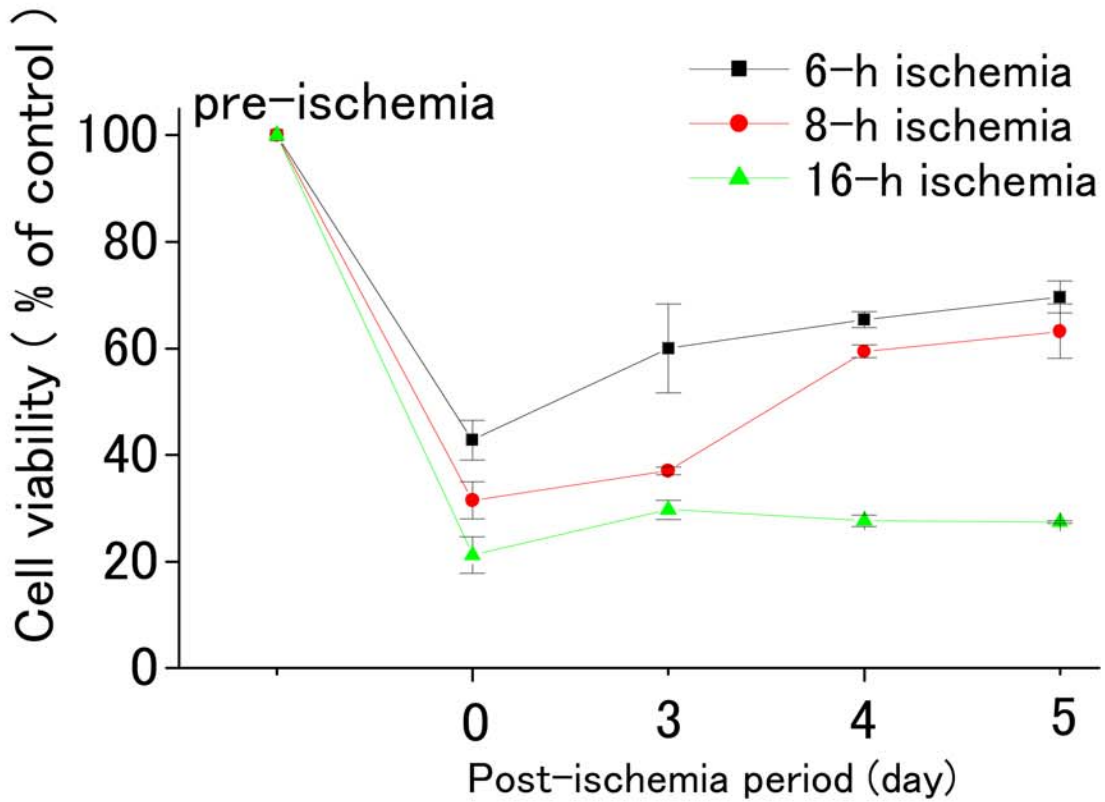

Cell viability (% of control)
pre-ischemia
6-h ischemia
8-h ischemia
16-h ischemia
100
80
60
40
20
0
0
3
4
5
Post-ischemia period (day)

Fig. 10

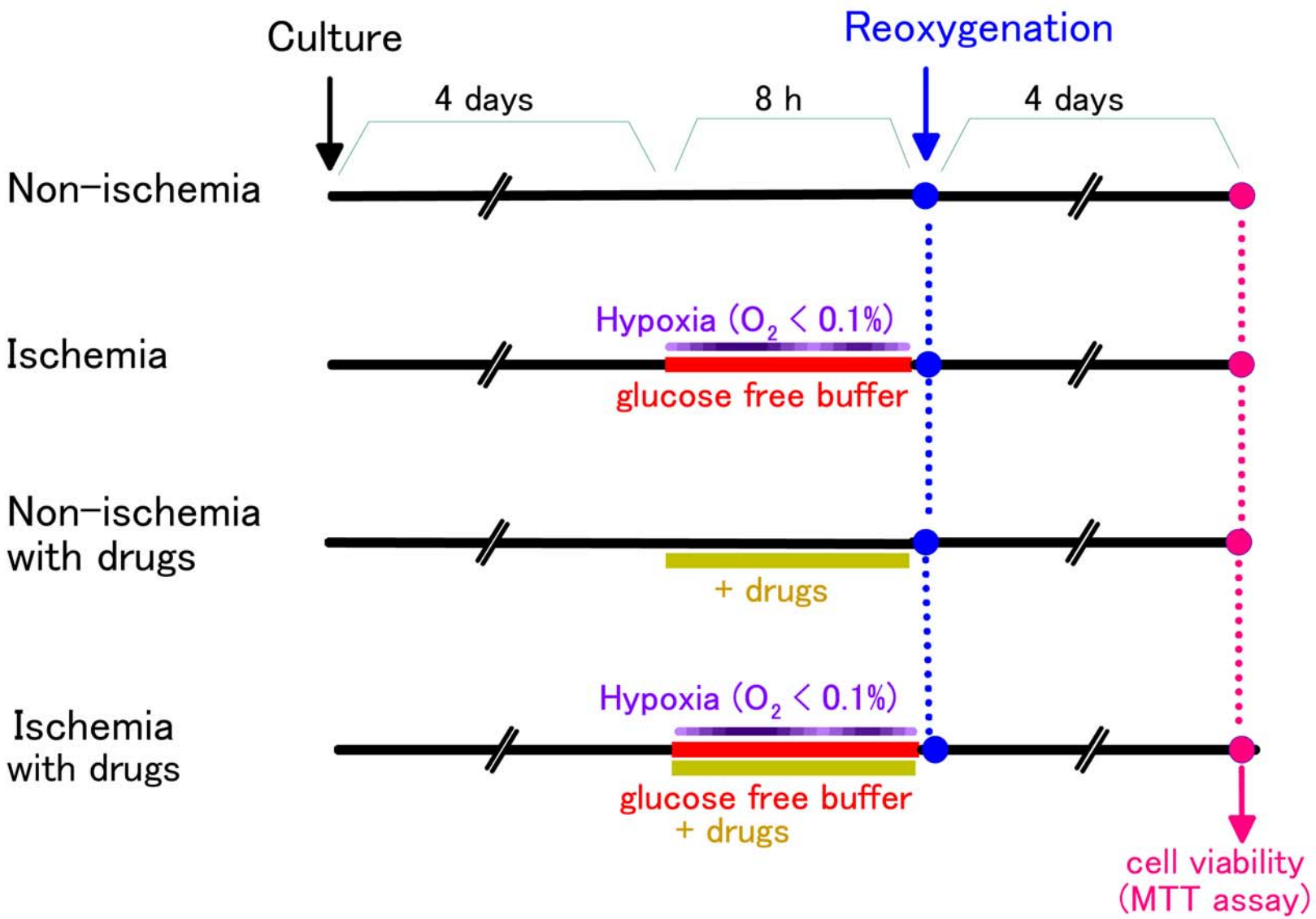

Culture
Reoxygenation
4 days
8 h
4 days
Non-ischemia
Ischemia
Hypoxia (O_2 < 0.1%)
glucose free buffer
Non-ischemia
with drugs
+ drugs
Ischemia
with drugs
Hypoxia (O_2 < 0.1%)
glucose free buffer
+ drugs
cell viability
(MTT assay)

Fig. 11

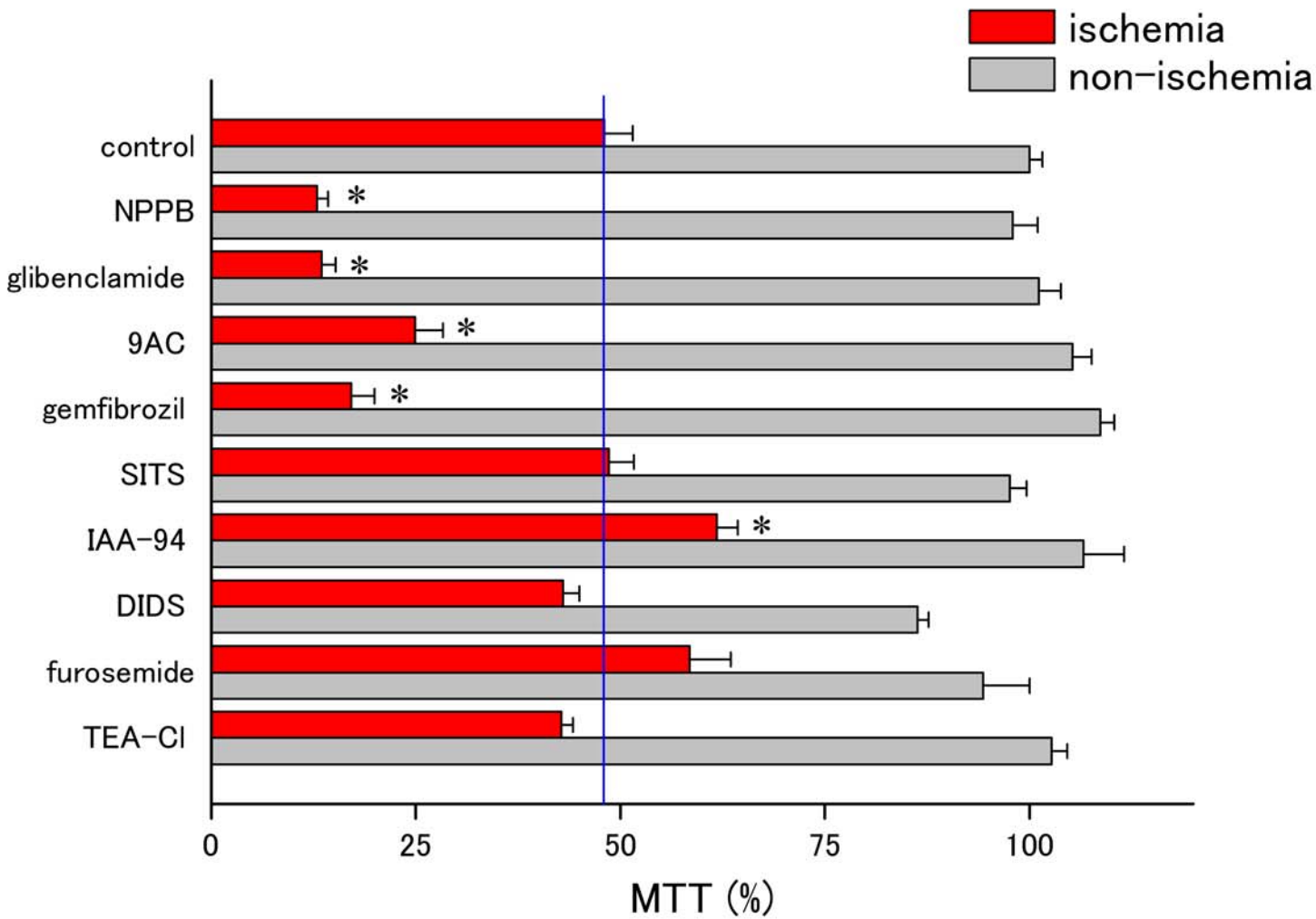

ischemia
non-ischemia
control
NPPB
glibenclamide
9AC
gemfibrozil
SITS
IAA-94
DIDS
furosemide
TEA-Cl
*
*
*
*
*
0
25
50
75
100
MTT (%)

Fig. 12

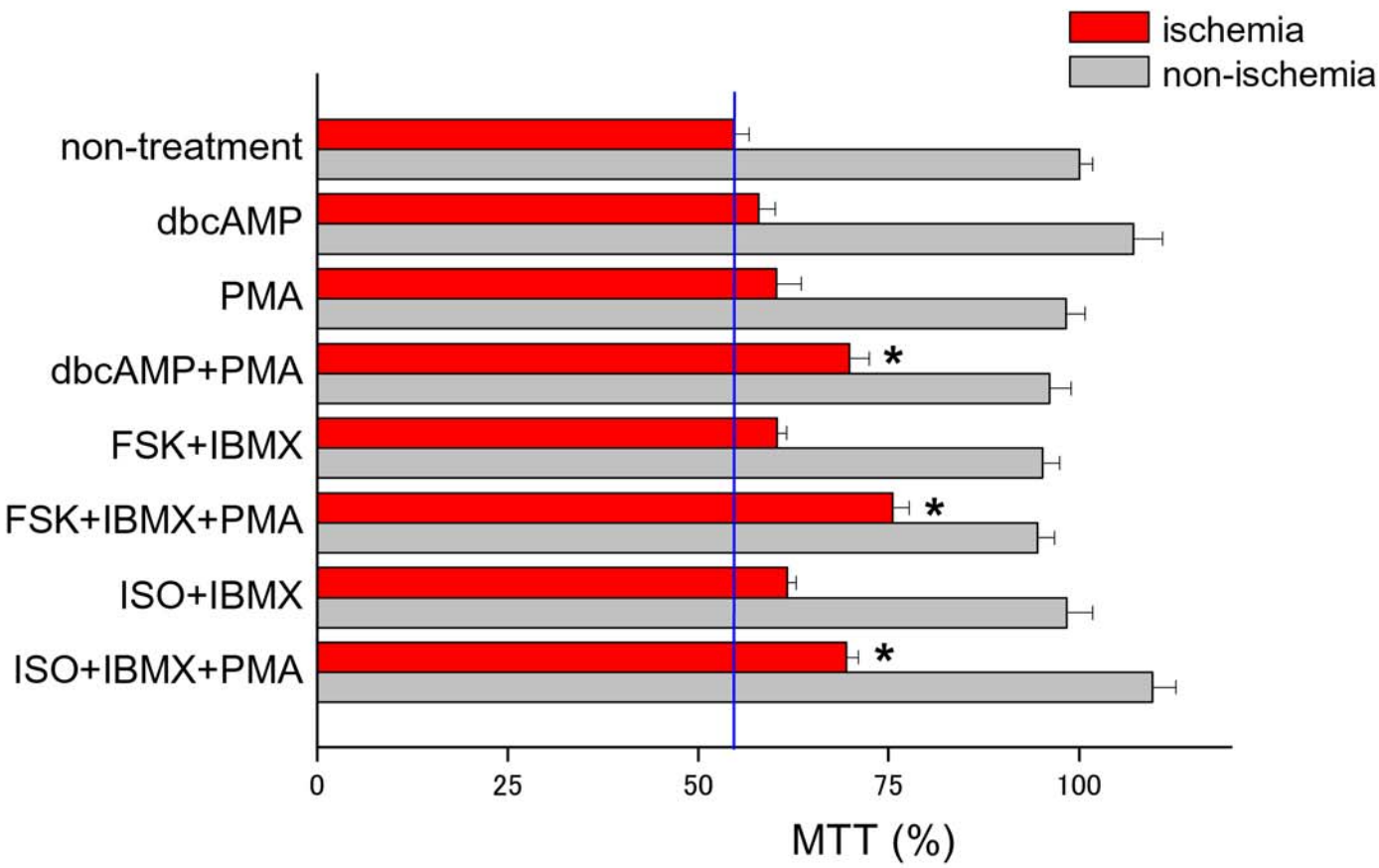

ischemia
non-ischemia
non-treatment
dbcAMP
PMA
dbcAMP+PMA
FSK+IBMX
FSK+IBMX+PMA
ISO+IBMX
ISO+IBMX+PMA
*
*
*
0
25
50
75
100
MTT (%)

Fig. 13

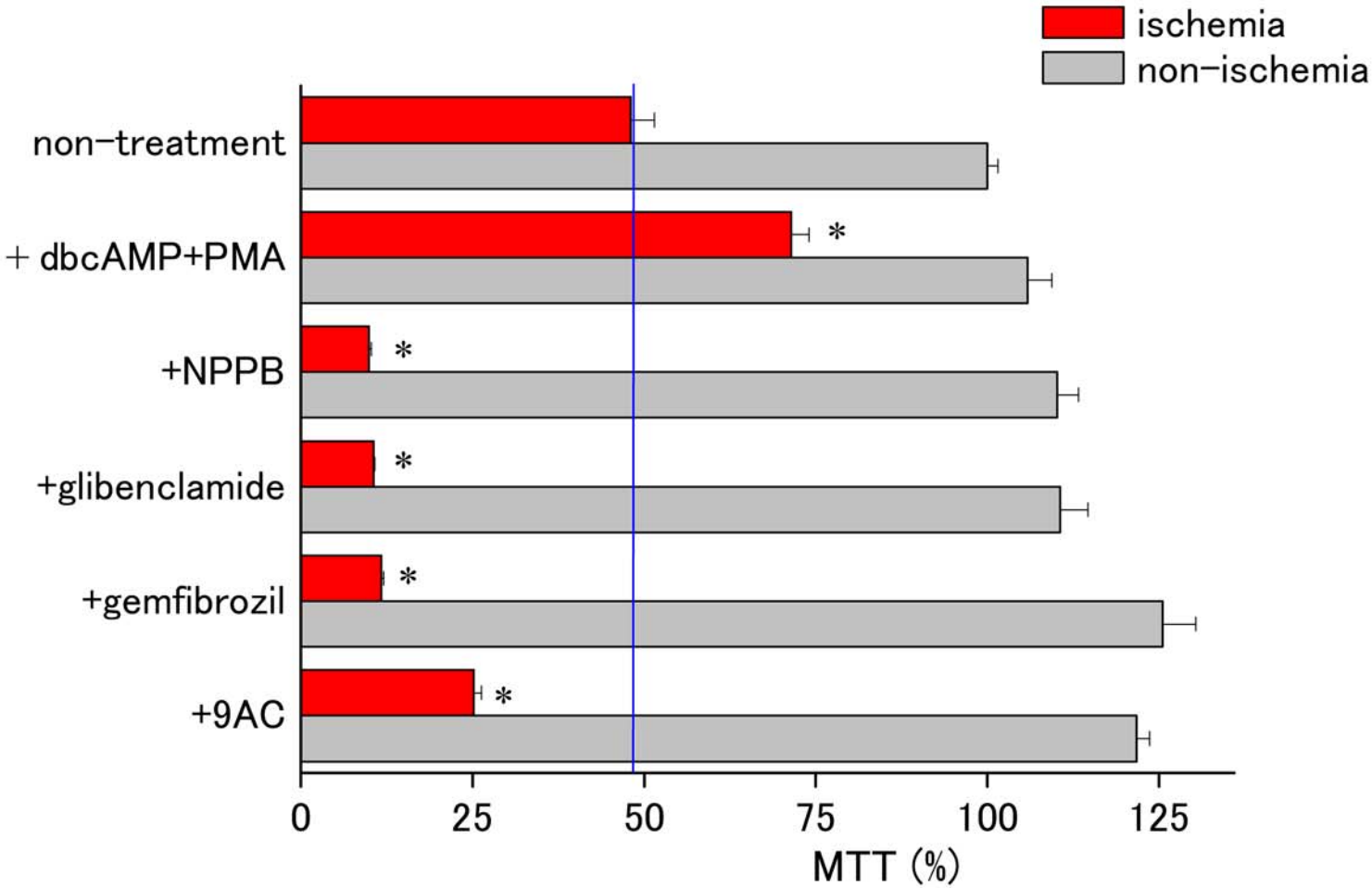

ischemia
non-ischemia
non-treatment
+ dbcAMP+PMA
+NPPB
+glibenclamide
+gemfibrozil
+9AC
*
*
*
*
*
0
25
50
75
100
125
MTT (%)

Fig. 14

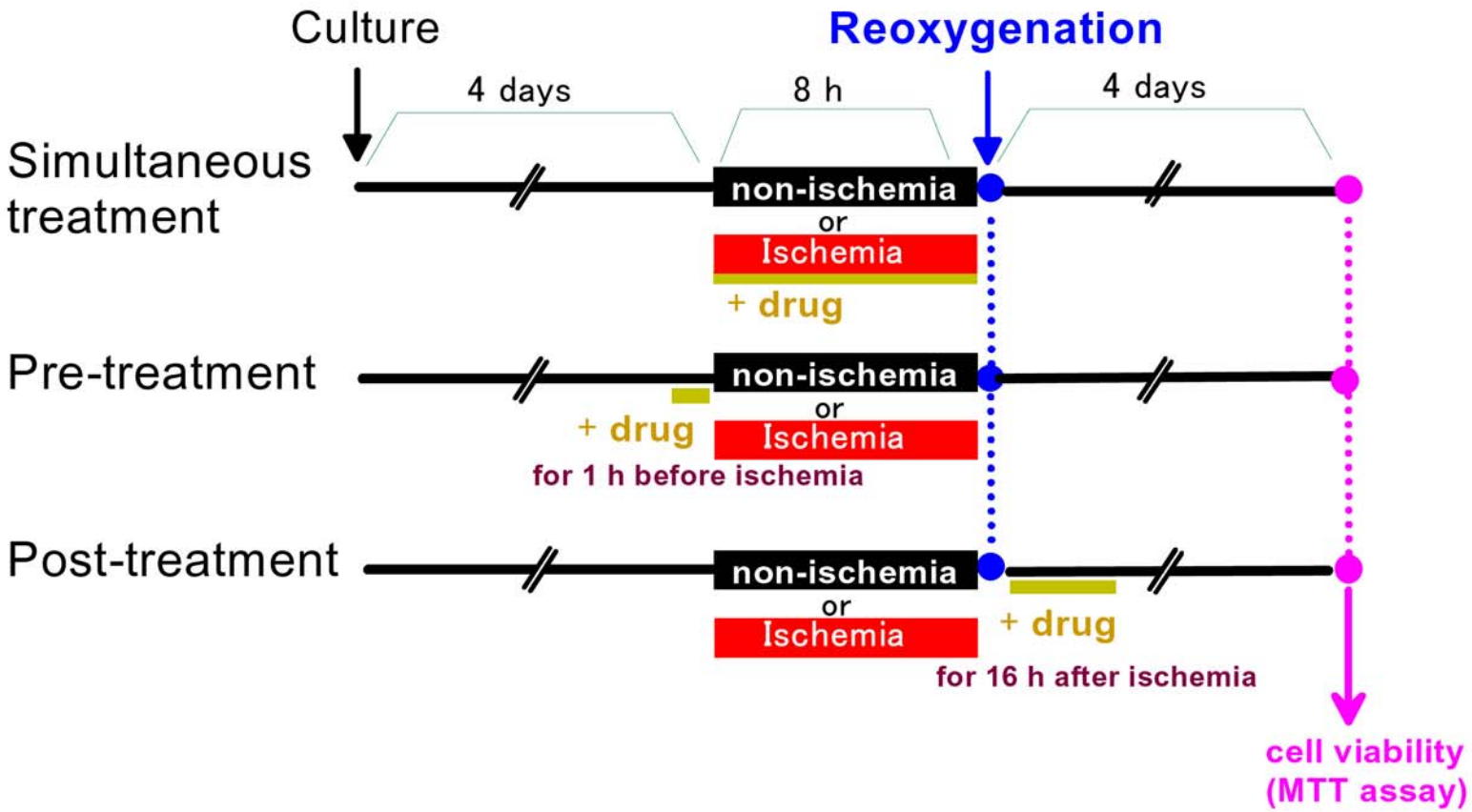

Culture
Reoxygenation
4 days
8 h
4 days
Simultaneous treatment
non-ischemia
or
Ischemia
+ drug
Pre-treatment
non-ischemia
or
Ischemia
+ drug
for 1 h before ischemia
Post-treatment
non-ischemia
or
Ischemia
+ drug
for 16 h after ischemia
cell viability
(MTT assay)

Fig. 15

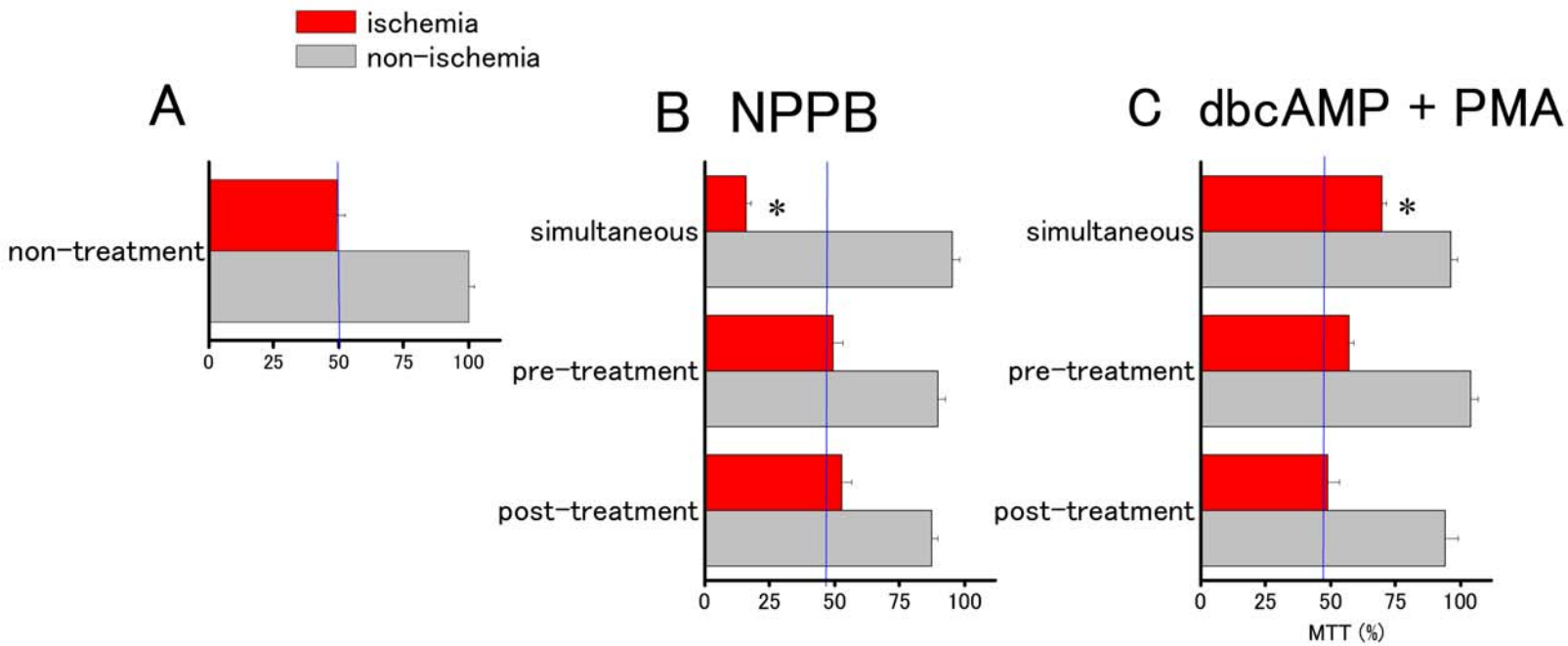

ischemia
non-ischemia
A
non-treatment
0
25
50
75
100
B NPPB
simultaneous
pre-treatment
post-treatment
*
0
25
50
75
100
C dbcAMP + PMA
simultaneous
pre-treatment
post-treatment
*
0
25
50
75
100
MTT (%)

Fig. 16

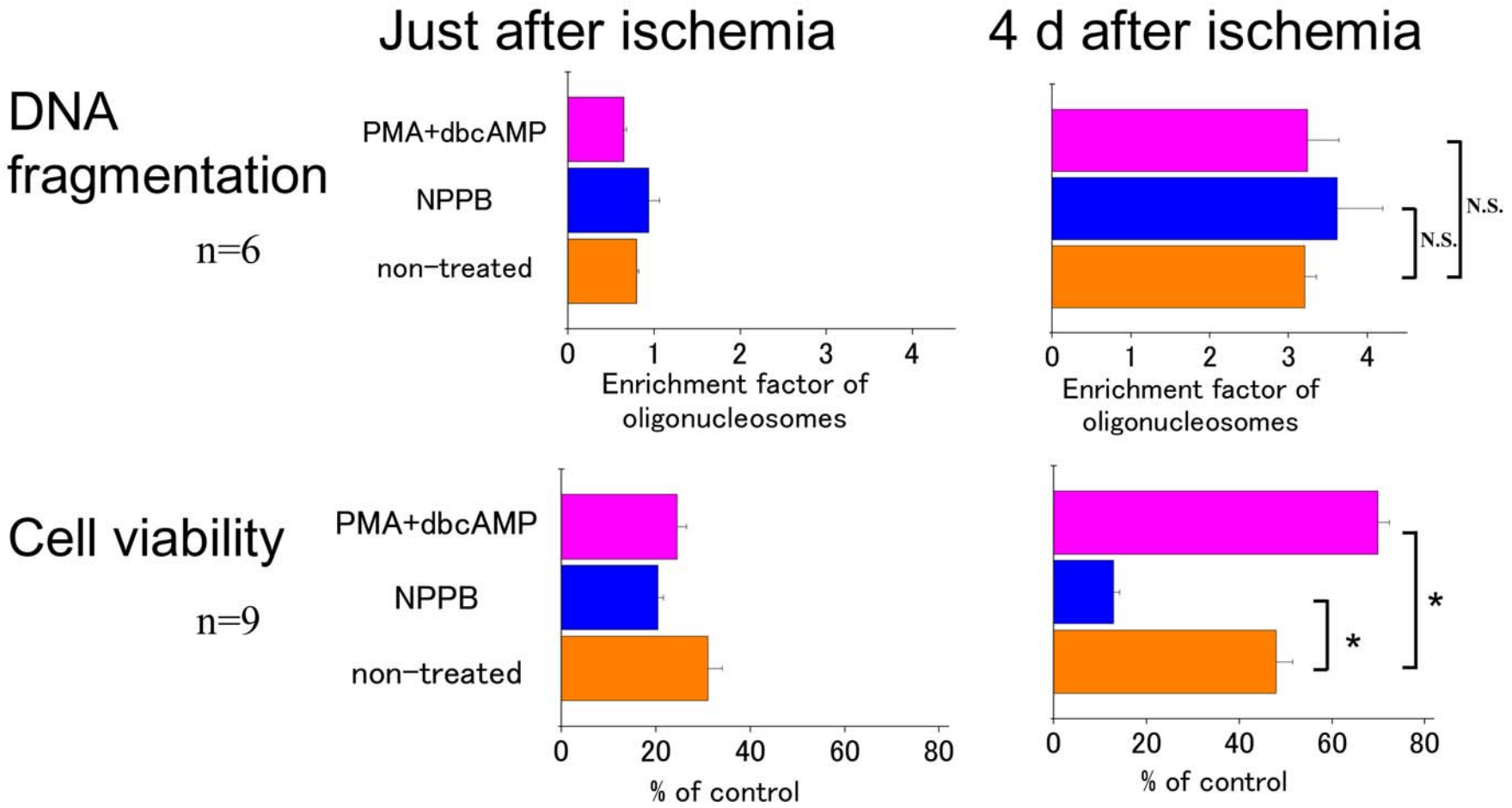

Just after ischemia
4 d after ischemia
DNA fragmentation
n=6
PMA+dbcAMP
NPPB
non-treated
0
1
2
3
4
Enrichment factor of oligonucleosomes
N.S.
N.S.
Cell viability
n=9
PMA+dbcAMP
NPPB
non-treated
0
20
40
60
80
% of control
*
*

Fig. 17