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Diclofenac, a Non-steroidal Anti-inflammatory Drug, Inhibits L-type Ca^{2+} Channels in Neonatal Rat Ventricular Cardiomyocytes

Oleg V. Yarishkin[1], Eun Mi Hwang[1], Donggyu Kim[1], Jae Cheal Yoo[1], Sang Soo Kang[2], Deok Ryoung Kim[3], Jae-Hee-Jung Shin[4], Hye-Joo Chung[4], Ho-Sang Jeong[4], Dawon Kang[1], Jaehee Han[1], Jae-Yong Park[1,*], and Seong-Geun Hong[1,*]

Departments of [1]Physiology, [2]Anatomy, [3]Biochemistry, Institute of Health Sciences, and Medical Research Center for Neural Dysfunction, Gyeongsang National University School of Medicine, Jinju 660-751, [4]Division of Molecular Pharmacology, National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul 122-704, Korea

A non-steroidal anti-inflammatory drug (NSAID) has many adverse effects including cardiovascular (CV) risk. Diclofenac among the nonselective NSAIDs has the highest CV risk such as congestive heart failure, which resulted commonly from the impaired cardiac pumping due to a disrupted excitation-contraction (E-C) coupling. We investigated the effects of diclofenac on the L-type calcium channels which are essential to the E-C coupling at the level of single ventricular myocytes isolated from neonatal rat heart, using the whole-cell voltage-clamp technique. Only diclofenac of three NSAIDs, including naproxen and ibuprofen, significantly reduced inward whole cell currents. At concentrations higher than 3 μM, diclofenac inhibited reversibly the Na^+ current and did irreversibly the L-type Ca^{2+} channels-mediated inward current (IC_{50}=12.89±0.43 μM) in a dose-dependent manner. However, nifedipine, a well-known L-type channel blocker, effectively inhibited the L-type Ca^{2+} currents but not the Na^+ current. Our finding may explain that diclofenac causes the CV risk by the inhibition of L-type Ca^{2+} channel, leading to the impairment of E-C coupling in cardiac myocytes.

Key Words: Diclofenac, L-type Ca^{2+} current, Rat cardiac myocytes, NSAID

INTRODUCTION

Diclofenac, a nonselective non-steroidal anti-inflammatory drug (nonselective NSAID), has been widely used as an anti-inflammatory, analgesic, and antipyretic drug. Medication with diclofenac has many adverse effects on gastrointestinal, renal, hepatic, and the cardiovascular (CV) system (Bort et al., 1998; Kearney et al., 2006). Clinical observations have shown that long-term treatment with diclofenac correlates with the onset or aggravation of the congestive heart failure (CHF), which can cause serious CV thromboembolic events, such as myocardial infarction and stroke (Hudson et al., 2007; Waksman et al., 2007). A recent systemic study claimed that diclofenac has the highest CV risk score of the nonselective NSAIDs (McGettigan and Henry, 2006).

Heart failure (HF) is an impairment of cardiac pumping, rendering in insufficient to meet the body's demand. This is frequently associated with electrical instability and reduced contractile force in the ventricles (Bodi et al., 2005; Dalla Libera et al., 2008; Hombach, 2008). Changes in the Na^+ current can slow myocardial conduction and cause conduction defects and reentrant arrhythmia (Pinto and Boyden, 1999; Tan et al., 2001). Reduced systolic Ca^{2+} with prolonged Ca^{2+} transient can result in a decreased generation capacity and a reduction in the decay rate of the contraction force in the failing heart (Pieske, 1999). This has been demonstrated by the decreasing Na^+ and Ca^{2+} current densities in experimentally induced CHF in dog's heart and in ventricular myocytes from patients with terminal heart failure (Lindner et al., 1998; Maltsev et al., 2002; Cha et al., 2004).

In normal cardiac muscle, Ca^{2+} influx through the L-type Ca^{2+} channels (LCC) is a key to initiate the excitation-contraction (E-C) coupling via Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR). The impairment of LCC function is a potential mechanism for altered CICR and E-C coupling disorders (McGettigan and Henry, 2006). Therefore, altered LCC activity can be a serious factor in heart failure. Little is known about interference of NSAIDs with function of heart. Some NSAIDs were found to impair normal activity of cardiac pacemaker cells by inhibiting LCC (Morales et al., 1992; Morales et al., 1993).

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Corresponding to: Seong-Geun Hong, Department of Physiology, Institute of Health Sciences, and Medical Research Center for Neural Dysfunction, Gyeongsang National University School of Medicine, 92, Chilam-dong, Jinju 660-751, Korea (Tel) 82-55-751-8741, (Fax) 82-55-759-0169, (E-mail) hong149@gnu.ac.kr
*Jae-Yong Park and Seong-Geun Hong contributed equally to this work as co-corresponding authors.

ABBREVIATIONS: APs, action potentials; CHF, congestive heart failure; CICR, Ca^{2+}-induced Ca^{2+} release; CV, cardiovascular; E-C coupling, excitation-contraction coupling; LCC, L-type Ca^{2+} channel; NSAIDs, non- steroidal anti-inflammatory drugs.

Considering its ability to modulate several ion channels, diclofenac also may modulate functioning of excitable membranes. Diclofenac can inhibit voltage-dependent Na^+ channels in cardiac myoblasts and neurons (Lee et al., 2003; Yang and Kuo, 2005; Fei et al., 2006). It also activates neuronal K^+ channels, such as the transient outward K^+ currents and the ATP-sensitive potassium (K_{ATP}) channel (Tonussi and Ferreira, 1994; Asomoza-Espinosa et al., 2001; Alves and Duarte, 2002; Ortiz et al., 2002; Liu et al., 2005). However, to date, there has been no evidence of a suppressive effect of diclofenac on LCC, which is critical in working myocytes.

In our preliminary study to test the adverse effects of three nonselective NSAIDs, diclofenac, naproxen, and ibuprofen, we found that only diclofenac inhibited the inward currents in single myocytes, whereas the others did not. In this study, we focused on the effects of diclofenac on ion channels, in particular, its modulation of LCC. We found that diclofenac inhibits LCC and the Na^+ current in neonatal rat cardiomyocytes. Our findings may provide some clues to the diverse adverse effects of diclofenac on the heart, such as diclofenac-associated high risk for heart failure.

METHODS

Cells

This study was performed in accordance with the Gyeongsang National University Institutional Guidelines for the Care and Use of Laboratory Animals. Neonatal rat ventricular cardiomyocytes were isolated from rat pups on postnatal day 1 and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and supplemented with 100 units/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidity-controlled incubator with 5% CO_2 (Fu et al., 2005). The experiments began the next day after plating.

Electrophysiology

The standard extracellular (bath) solution for whole-cell current measurements contained, (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 5.5 glucose, 5 $BaCl_2$, 10 HEPES, and was adjusted to pH 7.35 with HCl. The standard pipette solution contained, (in mM): 100 K-glutamate, 5 NaCl, 5 KCl, 1 $MgCl_2$, 23/10 KOH/EGTA, 10 HEPES, 4 ATP potassium salt and adjusted at pH 7.20. For the measurement of L-type Ca^{2+} currents the Na-free bath solution was used contained, (in mM): 140 TEA-Cl, 5 KCl, 1 $MgCl_2$, 5.5 glucose, 5 $BaCl_2$, 10 HEPES, and was adjusted at pH 7.35 with HCl. The pipette solution was 50 CsOH, 80 CsCl, 40 aspartate, 5 HEPES, 10 EGTA, 4 MgATP (pH 7.2). Diclofenac, naproxen, and ibuprofen were purchased from Sigma (St. Louis, MO, USA).

Whole-cell currents were recorded with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, USA). The current-voltage (I-V) relationship was measured by applying step pulses from a holding potential (HP) of −100 or −50 mV. In particular, an HP of −50 mV was applied to isolate LCC Ca^{2+} currents. Step pulses were up to +60 mV in 10 mV increments. The duration of the step pulses was 200 ms. The recorded currents were filtered at 5 kHz and sampled at 5 kHz. Currents were analyzed with Clampfit software (Axon Instruments, USA). Statistical analysis was performed with Origin 7.5 software. Data are given as mean values±SE. Cell membrane capacitance were 13.14± 0.97 pF (n=18). All experiments were performed at room temperature.

RESULTS

In our preliminary study, we examined the effects of three NSAIDs, diclofenac, naproxen, and ibuprofen, on ion currents in single cardiac myocytes. These drugs are known

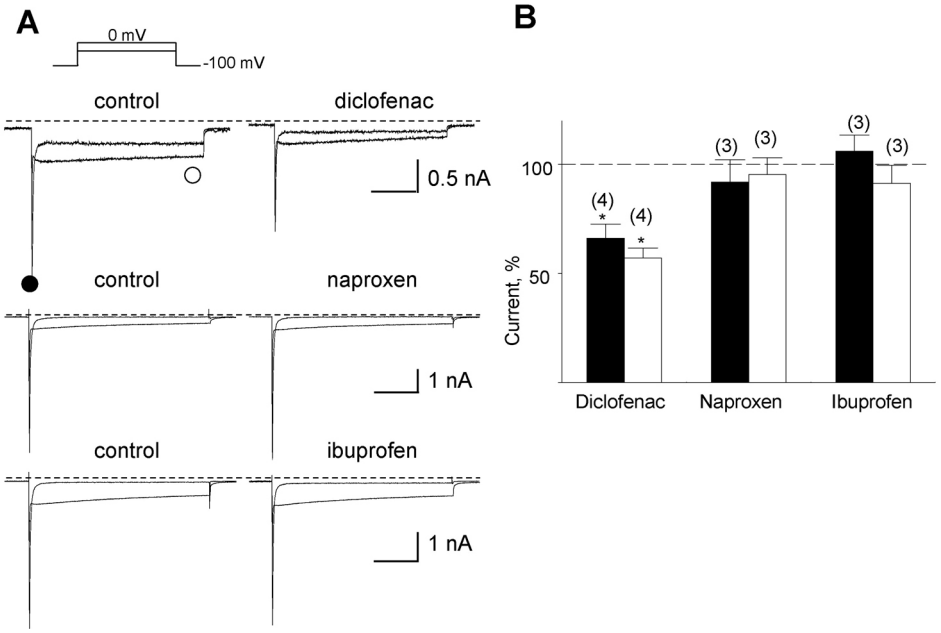

Fig. 1. Inhibition of the Na+- and the Ca^{2+}-sensitive inward current by three NSAIDs. (A) Representative currents before and after application of the drugs denoted above the corresponding trace. The drugs were applied at a concentration of 10 μM each. The amplitudes of the initial transient component and the slowly decayed components were measured at positions marked by closed (●) and open circles (○), respectively. (B) Summary of the normalized data for the effect of drugs on two components. Relative inhibition (%) for the Na^+-sensitive initial transient (black bar) and the nifedipine-sensitive slowly decayed components (open bar) are shown with number of observations. Data were normalized to currents measured before application of each drug.

as nonspecific cyclooxygenase (COX) I and II inhibitors. Only diclofenac significantly inhibited the inward currents elicited by step depolarization, whereas the other drugs did not (Fig. 1), suggesting that current inhibition by diclofenac is COX-independent.

The whole-cell currents elicited by depolarization steps from −100 mV of HP to +60 mV were characterized by the rapid transients (−340.7±53.4 pA/pF, n=5) with the peak current at −40 mV, followed by a slowly decayed component (−31.7±4.3 pA/pF, n=5; left in Fig. 2A) with the peak at 0 mV (Fig. 2C). Diclofenac (100 μM) irreversibly inhibited the second component. However, the rapid transient current was restored upon the removal of diclofenac (right in Fig. 2A).

To investigate the ionic nature of both components, we used a Na^+-free solution for the bath. The rapid component was abolished but the slowly decayed component was still observed in Na^+-free solution, indicating that the initial inward current was carried by Na^+ through voltage-dependent Na^+ channels (data not shown). To examine whether the second component is permeable to Ba^{2+} through the LCC, step depolarization from HP of −50 mV to 0 mV was applied. The slowly decayed component was still observed under these conditions (Fig. 2B, 2C). This component was completely blocked with 1 μM nifedipine, a specific LCC blocker, strongly suggesting that the slowly decayed component is the LCC-mediated Ba^{2+} current (I_{Ba}). As shown in Fig. 2B and 1C, 100 μM diclofenac drastically inhibited I_{Ba}.

The transient low-voltage activated (T-type) Ca^{2+} current can be transiently activated by depolarizations from HP of −100 mV (Perez-Reyes, 1998). The initial transient inward

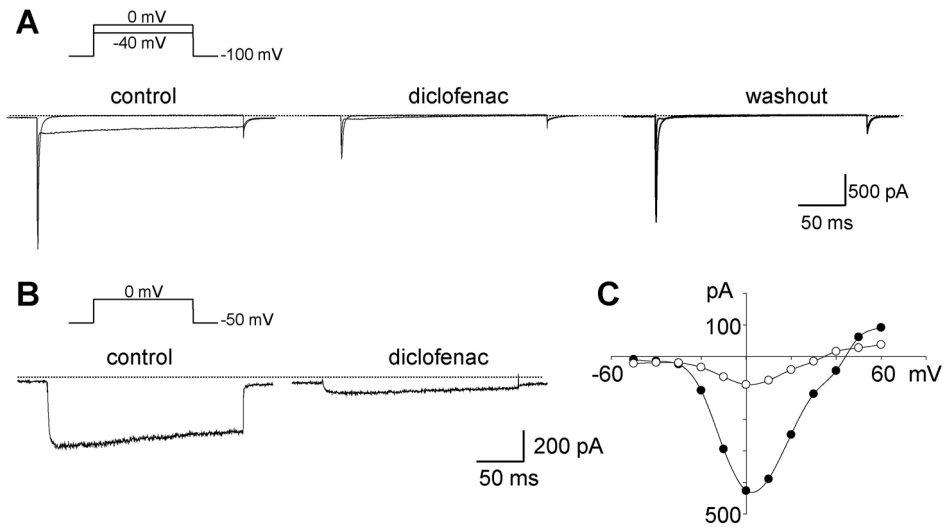

Fig. 2. Representative traces of whole-cell currents elicited by step depolarizations in single cardiac myocytes. (A) Inhibition of the inward current induced by diclofenac. Changes in whole-cell currents evoked at −40 and 0 mV from a holding potential of −100 mV in bath solution containing 140 mM Na^+ before (left) and after adding diclofenac (middle), and following washout (right), respectively. Dotted lines in A and B indicate the zero current level. (B) Currents induced by depolarization as indicated above the traces, in Na^+-free bath solution before and after the addition of diclofenac. (C) Current-voltage relationship measured from the peak current of the traces in panel B. Diclofenac of 100 μM was applied. Outward components were not detected due to the presence of Ba^{2+}, instead of Ca^{2+} in the bath.

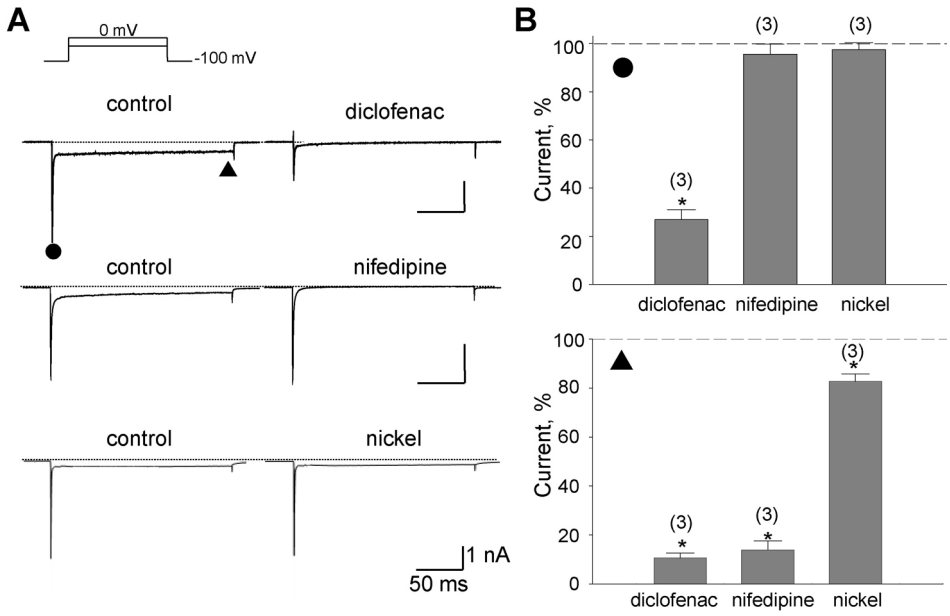

Fig. 3. Inhibition of the Na^+ and the Ba^{2+} components by diclofenac. (A) Representative currents inhibited by drugs denoted above the right trace. With the application of diclofenac (100 μM), nifedipine (1 μM), or nickel (300 μM), reduced currents were shown on the right. The amplitudes of the initial transient component and the slowly decayed components were measured at the positions marked by the closed circle (●) and triangle (▲), respectively. (B) Summary of the normalized data for the effect of drugs on the two components. Relative inhibitions (%) of the Na^+-sensitive initial transient and the nifedipine-sensitive components are shown in upper and lower panel with number of observations, respectively. Data were normalized to currents measured before application of each drug. Scale bars are equal to 1 nA and 50 ms.

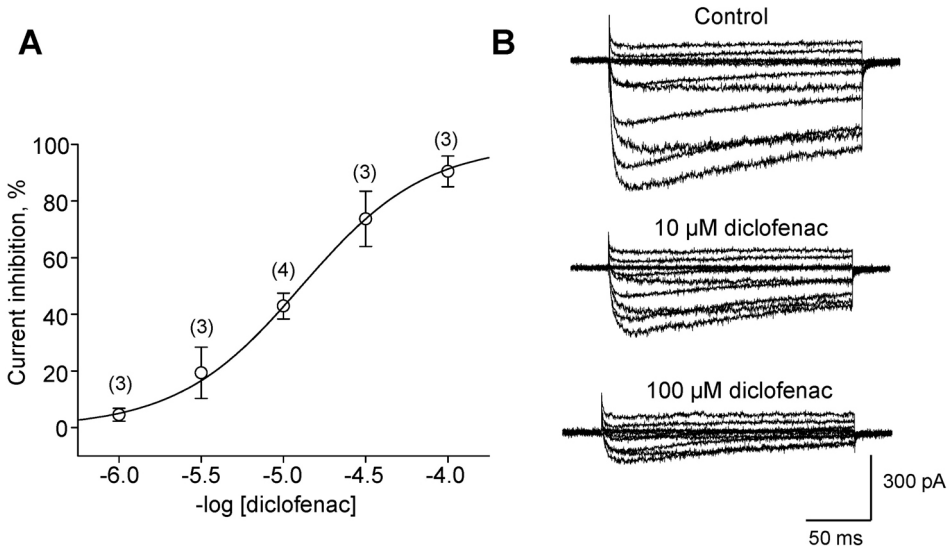

Fig. 4. Dose-dependent inhibition of the L-type current by diclofenac. (A) Dose-response relationship of the inhibitory effect of diclofenac on peak L-type (I_{Ba}) currents in cardiomyocytes, with the numbers of cells. The molar concentration of diclofenac is given. (B) Representative traces of L-type currents reduced by diclofenac. Step depolarizations were applied from HP of −50 mV to +60 mV in 10 mV increments.

component is possibly mingled with the T-type Ca^{2+} current. Thus, we examined whether T-type Ca^{2+} current might be activated by step depolarization from −100 mV and sensitive to diclofenac. This was done by comparing the effects of diclofenac with those of nifedipine and Ni^{2+}, which is known to be more specific blocker of T-type Ca^{2+} channel (Lee et al., 1999; Perez-Reyes et al., 1999; Doering and Zamponi, 2003).

In this experiment using Ba^{2+} instead of Ca^{2+}, step depolarization from −100 mV to 0 mV elicited both initial rapidly transient and sustained inward currents (I_{Ba}). As shown in Fig. 3, diclofenac significantly reduced the rapidly transient component by 74.6±4.8% (n=3) and the I_{Ba} component by 89.5±2.7% (n=3). Nifedipine (1 μM) and Ni^{2+} (300 μM) reduced I_{Ba} component by 85.2±4.5% (n=3) and by 17.6±3.3% (n=3), respectively. However neither had any effect on the initial transient component and blocking potency of Ni^{2+} for I_{Ba} was negligible (lower bar chart in Fig. 3B), suggesting that the T-type Ca^{2+} channels were not detected especially in the initial transient inward currents. These results confirmed again that diclofenac inhibits the current through LCC as well as the Na^+ current.

In Na^+-free bath solution, diclofenac dose-dependently inhibited the LCC-mediated I_{Ba} with an IC_{50}=12.89±0.43 μM (Fig. 4A). Diclofenac reduced the current amplitude without changing its kinetics in inactivation process (see current traces in Fig. 4B). This led to an implication that diclofenac did not at least play as an open channel blocker which remarkably accelerates inactivation process or decay phase (Nawrath et al., 1998). Although not shown here, diclofenac also depressed Ca^{2+} transients elicited by high K^+ (25 mM)-induced depolarization. This depression was done with a similar fashion introduced by nifedipine.

DISCUSSION

Recommendation of diclofenac for patients with CHF or other cardiac problems has been under debate, remaining unknown about the molecular mechanism of its adverse effects. The present study focused side effects of diclofenac on ion channels since heart problems such as CHF could be initiated or triggered commonly by ionic disturbances. Here we provided the first finding that diclofenac dose-dependently suppressed LCC which is crucial to excitation-contraction coupling. In addition, diclofenac reversibly inhibitied the voltage-activated Na^+ currents, which is consistent with other studies (Lee et al., 2003; Yang and Kuo, 2005; Fei et al., 2006).

In heart cells, L-type Ca^{2+} channels are major source to increase intracellular Ca^{2+} level ($[Ca^{2+}]_i$) via CICR. Restriction of Ca^{2+} entry by blocking LCC should reduce $[Ca^{2+}]_i$ and weaken the cardiac muscle contraction. This could be confirmed with the result that diclofenac (30 μM), as well as nifedipine (10 μM), significantly suppressed Ca^{2+} transients elicited by high (25 mM) K^+-induced depolarization, although not shown as data in the present study. Since diclofenac inhibited the LCC activity, it could disturb the muscle contraction for the efficient pumping as did by other LCC blockers such as verapamil, Cd^{2+}, nifedipine, and Ni^{2+} (Ferrier and Howlett, 1995; Hobai et al., 1997; Howlett et al., 1998; Ferrier et al., 2000; Zhu and Ferrier, 2000). The voltage-dependent Na^+ channel is essential to generate the cardiac action potentials (APs) and its propagation throughout the whole heart. Due to its inhibitory action on Na^+ currents as shown in Fig. 2A and 3A, diclofenac might fail to, or generate APs inadequate to conduct the electrical excitation, which can induce arrhythmia. These combined effects observed at the cellular level can at least partly explain why diclofenac reveals severe cardiac risks such as the congestive heart failure.

In this study, we have examined the effect of diclofenac mainly on the LCC present in ventricular myocytes isolated from one-day old rat hearts. The quantitative analysis of the expression and distribution of Ca^{2+} channels demonstrated the expression of four types of Ca^{2+} channels in rat hearts, Cav1.2, Cav2.3, Cav3.1, and Cav3.2. The level of Cav3.1 and Cav3.2, the phenotypes of the T-type Ca^{2+} channel, is not changed significantly during development and become undetectable at five weeks postpartum. Cav2.3, an R-type Ca^{2+} channel, gradually declines after four weeks, when it reaches its peak expression. Of the four channel types, the phenotype of LCC, Cav1.2 is 10∼100 times more abundant than other types and remains steadily its ex-

pression throughout development (Larsen et al., 2002). In accordance with others, the LCC density in neonatal rat cardiomyocytes corresponds to ca. 85% of that in adult rats (Katsube et al., 1998). Diclofenac can therefore induce cardiac problems to the adult from the neonates, due to its inhibitory effect on LCC.

During short-term therapeutic intake of diclofenac, its plasma concentration has been reported to reach 1.50～3.0 μg/ml (corresponding to 5～10 μM (Willis et al., 1979; Leucuta et al., 2004), which is close in the range of the concentrations effective on the LCC block in this study (refer to Fig. 4A). Because of its irreversible action, repeated intakes of diclofenac may progressively aggravate the LCC function. Diclofenac more than 10 μM also blocks the Na^+ channels which are responsible to generate action potentials. Combined together, diclofenac may depress cardiac excitability and the contractility simultaneously. Therefore, its dual effects provide insight into how to bring about side effects on the heart and why it is more critical to patients with heart problems. To explain clearly, one should explore whether diclofenac suppresses Ca^{2+} transients induced by Ca^{2+} entry (i.e. CICR) and is sensitively offensive to the patients. The present study could not address the differences in the sensitivity to diclofenac between normal and cardiac cells from the heart with impaired function, since we could not find the appropriate rat model with experimentally induced heart failure.

In conclusion, this study showed that diclofenac reversibly inhibited the Na^+ currents and irreversibly the L-type Ca^{2+} channel currents in cardiac muscle cells as our first finding. This finding provides a clue to explain at least partly why diclofenac play as a critical risk factor on heart as well as smooth muscle cells at the cellular/molecular level. The further study is required to assay the effects of long-term administration of therapeutic concentrations of diclofenac on the L-type Ca^{2+} channel and the E-C coupling in muscle cells.

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