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[Original Article]

INHIBITORY EFFECT OF β-HYDROXYBUTYRIC ACID ON L-TYPE Ca^{2+} CURRENT UNDER β-ADRENERGIC STIMULATION IN GUINEA PIG CARDIAC VENTRICULAR MYOCYTES

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Abstract : Severe ketoacidosis induces heart failure and cardiac arrest, but its mechanism is unknown. Recently, hydroxy-carboxylic acid receptor 2 (HCA_2) was found to be a receptor for a ketone body, β-hydroxybutyric acid (BHB), and is coupled with Gi-GTP binding protein. HCA_2 expression was reported in the guinea pig heart. Therefore, using guinea pig cardiac myocytes, we investigated effects of BHB on L-type Ca^{2+} current pre-augmented with β-adrenoceptor agonist, isoproterenol under the whole-cell voltage clamp. BHB significantly reduced the Ca^{2+} current pre-augmented with isoproterenol. The effect of BHB was concentration dependent with IC_{50} of 1.1 mM. Nicotinic acid (NA), another ligand for HCA_2, also exerted an effect on the Ca^{2+} current similar to that of BHB. The effects of BHB and NA were reduced by a specific Gi inhibitor, pertussis toxin in the pipette solution. Our results suggest that BHB activates Gi-coupled signal transduction pathway via HCA_2 in guinea pig cardiac myocytes. The HCA_2-mediated signal transduction may be associated with ketoacidosis-induced cardiac suppression.

Key words : β-hydroxybutyric acid, ketone body, L-type Ca^{2+} current, cardiac myocyte, HCA_2 receptor

INTRODUCTION

Ketoacidosis is a life-threatening complication of both type1 and type2 diabetes[1-3]. However, the mechanism of ketoacidosis affecting cardiac function has not been fully understood. Ketone bodies include β-hydroxybutyric acid (BHB), acetoacetic acid, and acetone. In diabetic ketoacidosis, the ratio of BHB : acetone is elevated from normal (1 : 1) to values as high as (10 : 1)[4]. The serum BHB is 0.1 mM or less in normal humans, but could increase to a level higher than 25 mM during ketoacidosis[5]. Recently, hydroxy-carboxylic acid receptor 2 (HCA_2) was identified as a receptor for BHB[6]. This receptor was formally called GPR109A, which was initially reported as a receptor for nicotinic acid in adipocytes[7,8], and was coupled with Gi-GTP-binding-protein[9]. GPR109A expression was found not only in adipocytes but also in guinea pig ventricular myocytes[10].

To assess the mechanism of ketone body affecting cardiac function, we attempted to reveal that BHB stimulates Gi-coupled signal transduction in the heart. To do so, we investigated the effect of BHB on L-type Ca^{2+} current pre-augmented by β-adrenergic agonist, isoproterenol under the whole-cell voltage clamp in guinea pig ventricular myocytes.

MATERIALS AND METHODS

All experiments were performed with the approval of the Animal Research Committee of Fukushima Medical University.

The method of cell isolation and the whole cell-voltage clamp were described previously[11]. Briefly

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the heart was isolated from guinea pig under pentobarbital-anesthesia. Single ventricular myocytes were isolated by perfusing the heart with collagenase (5 mg/50 ml ; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and protease (1 mg/50 ml ; Sigma, St. Louis, MO, USA) on Langendorff apparatus. Tyrode solution contained (in mM) 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.33 NaH_2PO_4, 5.5 glucose, 5 HEPES (pH 7.4 with NaOH). The pipette solution contained (in mM) 30 CsCl, 100 CsOH, 3 $MgCl_2$, 100 aspartic acid, 5 MgATP, 20 BAPTA, 20 HEPES (pH 7.2 with aspartic acid). Membrane currents were recorded by the whole-cell voltage clamp with a patch-clamp amplifier (CEZ2400, Nihon Kohden, Tokyo, Japan). The temperature of the bath solution was kept at 35±0.5°C with a water jacket connected to a thermostat incubator (HAAKE, E15, Berlin, Germany). Patch pipette resistance was 2-4 MΩ when filled with the pipette solution. Recording signals were filtered at 2.5-kHz bandwidth, stored online and analyzed with pClamp Version 9 (Axon Instruments, Union City, CA, USA).

D-(−)beta-hydroxybutyrate was purchased from MP Biomedicals, LLC (California, USA). BHB was directly dissolved in Tyrode solution. Pertussis toxin (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) at 5 μg was activated by mixing with 50 μl dithiothreitol (100 μM) and 450 μl pipette solution and incubated at 37°C for 15-20 min. Then, 4.5 ml pipette solution was added, so that the final pertussis toxin (PTX) concentration of in the pipette solution was 1 μg/ml[12,13]. Each experiment was repeated 4 to 5 times. The data were expressed as the means±S.E.M. Statistical significance between two groups was evaluated using Welch's *t*-test.

RESULTS

Effect of BHB on L-type Ca^{2+} current pre-augmented by isoproterenol

First we found that BHB alone did not affect the L-type Ca^{2+} current in single ventricular myocytes of guinea pig (data not shown). Then, we tested BHB on the L-type Ca^{2+} current pre-augmented by isoproterenol. A family of depolarizing square voltage pulses were given from the holding potential of −40 mV to +40 mV for 400 ms duration (Fig. 1A inset). Isoproterenol at 0.1 μM in the extracellular Tyrode solution increased L-type Ca^{2+} current (Fig. 1A and 1B). When 10 mM BHB was added to isoproterenol, the Ca^{2+} current was gradually decreased (Fig. 1C). Finally, 1 μM nifedipine was added to completely inhibit the Ca^{2+} current (Fig. 1D). In some cells, positive background currents increased as shown in Fig. 1C and Fig. 1D. These currents were most likely isoproterenol-induced Cl^- currents[14]. We measured the magnitude of the Ca^{2+} current between the peak and at the inactivated level at the end of 400 ms depolarizing pulse at each voltage in order to avoid contamination of the isoproterenol-induced Cl^- current. The I-V curves of the Ca^{2+} current were plotted (Fig. 1E). Since the peak potential of the Ca^{2+} current varied between −20 mV and 10 mV among different cells, we employed the value at 10 mV for further analysis.

A concentration-response curve of BHB was obtained (Fig. 2). The peak value of Ca^{2+} current in isoproterenol was set at 100%. BHB at 10 mM significantly inhibited the Ca^{2+} current to 48.9±4.4% ($n=5$, $p<0.001$) of the control in isoproterenol. The inhibitory effect of BHB was concentration dependent, and the IC_{50} value of BHB was approximately 1.1 mM Since the IC_{50} of BHB to HCA_2 receptor was reported to be 0.7 mM[15], our value of 1.1 mM is close enough to that of HCA_2. Therefore, our result suggested that BHB reduced Ca^{2+} current pre-augmented by isoproterenol possibly via HCA_2 receptor in guinea pig ventricular myocytes.

Effect of nicotinic acid on Ca^{2+} current pre-augmented by isoproterenol

If the above result was mediated by HCA_2 receptor, nicotinic acid, another ligand reported for HCA_2[9] should behave similarly to BHB. Therefore, we tested nicotinic acid instead of BHB. When nicotinic acid at 30 μM was added in the external solution, it inhibited the Ca^{2+} current to 73% of isoproterenol-treated control in this cell (Fig. 3). The I-V curves were superimposed in Fig. 3E. In 5 different cells, nicotinic acid inhibited the Ca^{2+} current to 67±6% of the isoproterenol-treated control. Since the effects of nicotinic acid and BHB were similar, this suggests that the effects may be mediated by HCA_2 receptor.

Effect of pertussis toxin

HCA_2 receptor is coupled with Gi-GTP-binding protein[7]. Pertussis toxin (PTX) is a specific inhibitor of Gi[16]. If the effects of BHB and nicotinic acid on the Ca^{2+} current were mediated by Gi, those effects should be suppressed by PTX. As shown in Fig. 4A, in the presence of activated PTX in the

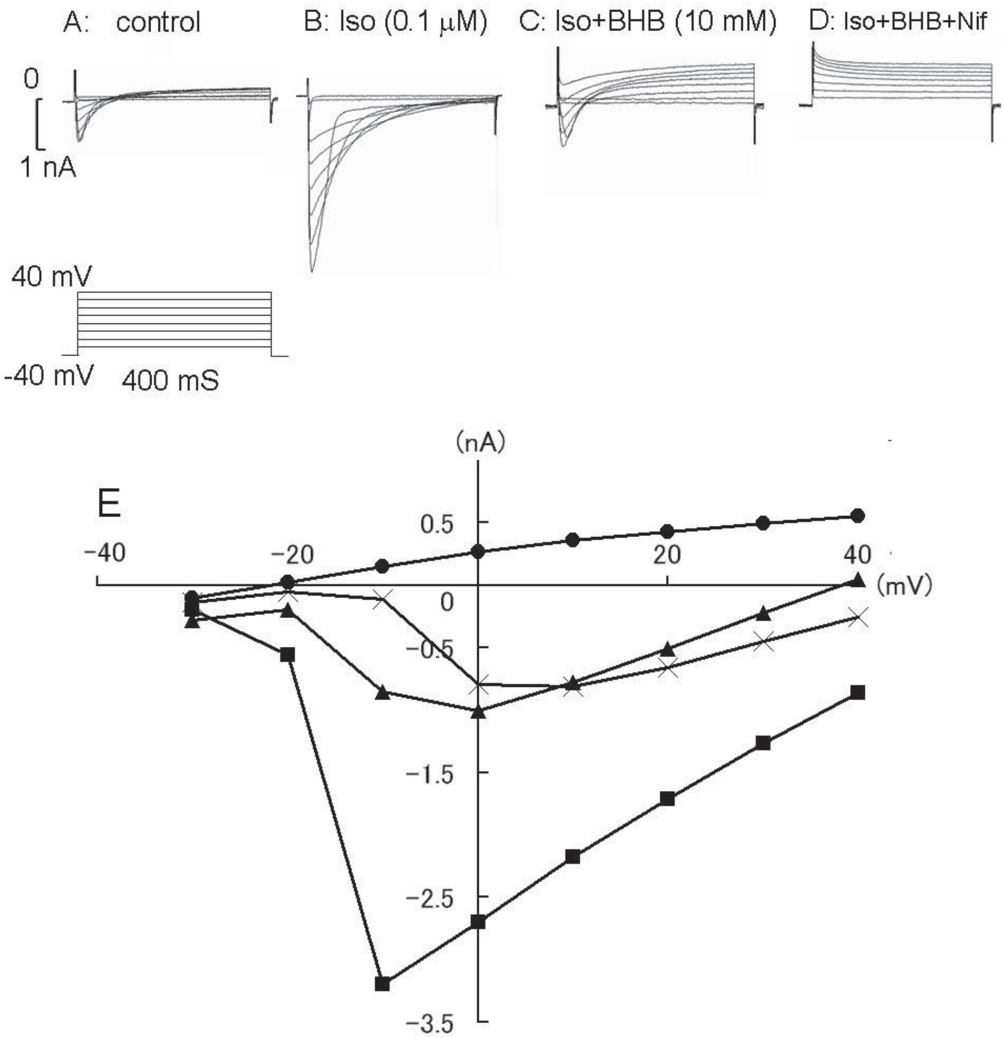

Fig. 1. A representative data of the effect of β-hydroxybutyric acid on the Ca^{2+} current preaugmented by isoproterenol. A : Control Ca^{2+} currents before applying isoproterenol. Voltage pulses are shown in the inset below. B : A set of Ca^{2+} currents augmented by 0.1 μM isoproterenol (Iso). C : Ca^{2+} currents after addition of 10 mM BHB to Iso. D : Nifedipine (Nif) at 1 μM was added and Ca^{2+} currents were completely inhibited. A-D were obtained from the same cell. E : Current-voltage relationships plotted from the data in Fig. 1A-D. Control (X) ; isoproterenol (0.1 μM) (■) ; isoproterenol + BHB (10 mM) (▲) ; isoproterenol + BHB + nifedipine (1 μM) (●).

pipette solution, 10 mM BHB did not significantly decrease the pre-augmented Ca^{2+} current. Similar results were obtained with 30 μM nicotinic acid (Fig. 4B). Nicotinic acid decreased the isoproterenol-augmented Ca^{2+} current only to 90.8±6.3% (n=4) in the presence of PTX (Fig. 4B). These results further suggest that the effects of BHB and nicotinic acid were mediated by Gi-protein.

DISCUSSION

In this study, we obtained the following results. (1) BHB inhibited the L-type Ca^{2+} current pre-augmented by isoproterenol, and this effect was BHB-concentration dependent. (2) Nicotinic acid also inhibited the pre-augmented Ca^{2+} current in a similar manner to BHB. (3) Pertussis toxin significantly inhibited the effects of BHB and nicotinic acid. (4) Estimated IC_{50} value of 1.1 mM BHB was close to a reported value of 0.7 mM for HCA_2 receptor. From these results, we suggest that BHB and nicotinic acid activate Gi-mediated signal transduction via HCA_2 in guinea pig cardiac ventricular cells.

L-type Ca^{2+} channels in cardiac myocytes are regulated dually ; stimulation mediated by Gs-coupled receptors and inhibition via Gi-coupled receptors. The former is represented by β1-adrenergic receptor and the latter by muscarinic M2

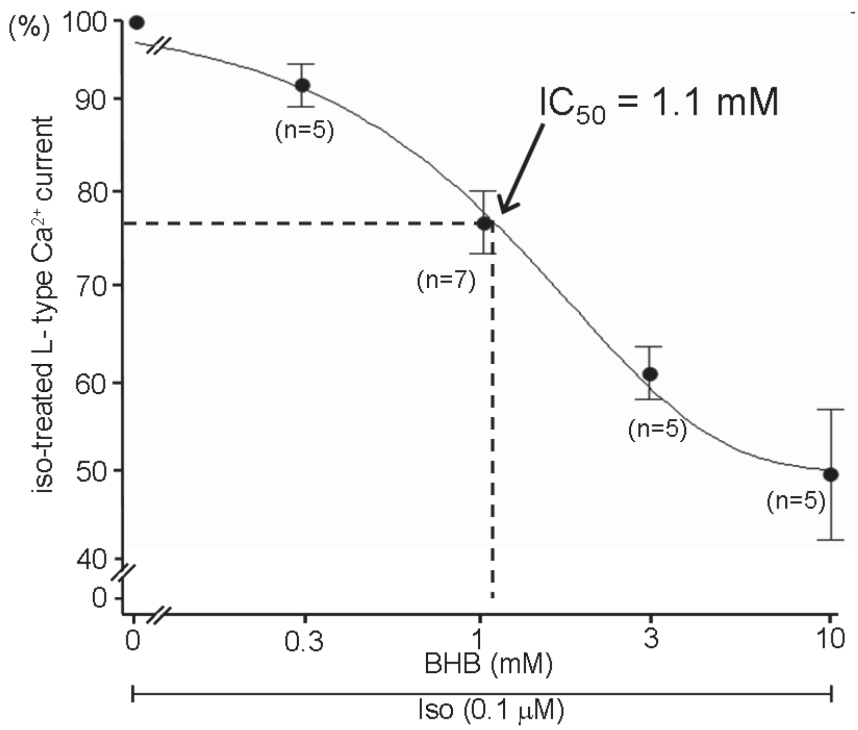

Fig. 2. Concentration-inhibition curve of BHB on the L-type Ca^{2+} current pre-augmented by isoproterenol (Iso). The current magnitude was measured at 10 mV. Data are mean ± S.E.M of 5 to 7 cells.

receptor. Fig. 5 shows a scheme of the L-type Ca^{2+} channel modulation. Activation of a Gi-coupled pathway inhibits adenylyl cyclase, reduce cAMP and protein kinase A activity and inhibit Ca^{2+} channels. This effect cannot be seen in the absence of prior stimulation of Ca^{2+} channels and is therefore called “accentuated antagonism”[17]. In the present study, using this accentuated antagonism we successfully demonstrated that BHB induced Gi-mediated signal transduction pathway possibly via HCA_2 receptor in guinea pig ventricular myocytes.

Isoproterenol is a non-selective agonist of β1 and β2 adrenergic receptors. β2-adrenergic receptor couples to Gq and Gi-proteins in addition to Gs[18]. In canine heart failure model, β2-adrenergic receptor stimulation activated Gi, and inhibited L-type Ca^{2+} channel[19]. However, in guinea pig cardiac myocytes, the percentage of β2 receptor is only 7% and is significantly less than 27% in human hearts[20]. Therefore, we estimated that the involvement of β2-receptor mediated activation of Gi must be negligibly small in guinea pig myocytes we used in this study.

HCA_2 was discovered as an orphan receptor GPR109A in adipose tissue[7]. In adipose tissue, nicotinic acid stimulates this receptor and lowers LDL cholesterol[21]. An exogenous ligand of this receptor is acipimox, which is carboxylic acids of N-heterocyclic compounds[22]. BHB was identified as an endogenous ligand for HCA_2[23], which is coupled with PTX-sensitive Gi[24]. In this study, PTX in the pipette solution diminished the inhibitory effects of BHB and nicotinic acid on the Ca^{2+} current. This result strongly indicates that Gi is involved in the effect of BHB and nicotinic acid in guinea pig ventricular myocytes, and therefore, supports our view that the receptors for BHB and nicotinic acid are HCA_2.

Because catecholamine levels are elevated in the blood in diabetic ketoacidosis[25], patients often exert tachycardia[26]. In rats, however, diabetic ketoacidosis decreased the heart rate and contractility[27]. In cardiac sinus node, atrium and atrio-ventricular node, Gi activation leads to opening of K^{+}_{ACh} channels and decreases the heart rate[28].

In the treatment of ketoacidosis, catecholamines such as dopamine are often used to maintain circulation[29]. However, our results indicated that BHB higher than 3 mM may attenuate Ca^{2+} current pre-augmented by endogenous or exogenous catecholamines and thus may reduce myocardial contraction. So far, sodium bicarbonate administration has been proposed in ketoacidosis only under severe acidosis such as pH <7.1[30]. However, since sodium bicar-

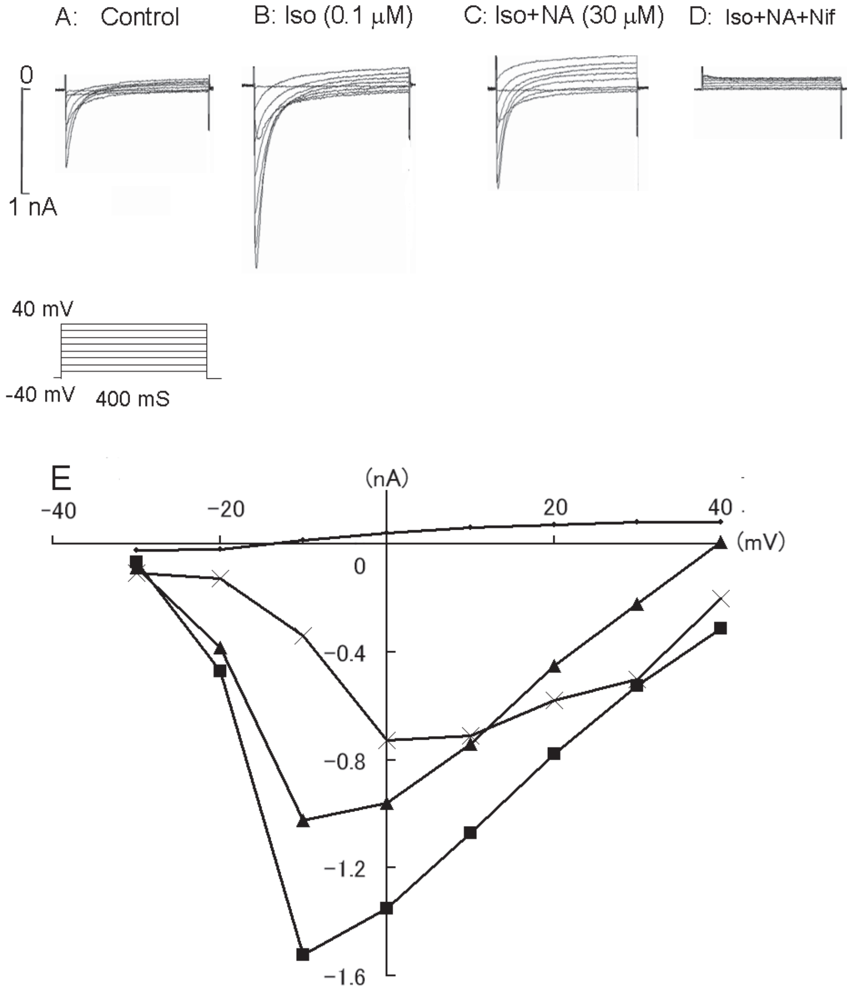

Fig. 3. Inhibitory effect of nicotinic acid (NA) on the Ca^{2+} current pre-augmented with isoproterenol. A : Control Ca^{2+} currents before isoproterenol. Voltage pulses are the same as those shown in Fig. 1. B : Ca^{2+} currents augmented by 0.1 μM isoproterenol (Iso). C : Ca^{2+} currents in the presence of 30 μM nicotinic acid (NA) added to Iso. D : Nifedipine (Nif) at 1 μM was added to completely inhibit the Ca^{2+} current. A-D were obtained from the same cell. E : Current-voltage relations obtained from the data in Fig. 3A-D. Control (X) ; 0.1 μM isoproterenol (■) ; isoproterenol + nicotinic acid (30 μM) (▲) ; isoproterenol + nicotinic acid + nifedipine (1 μM) (●).

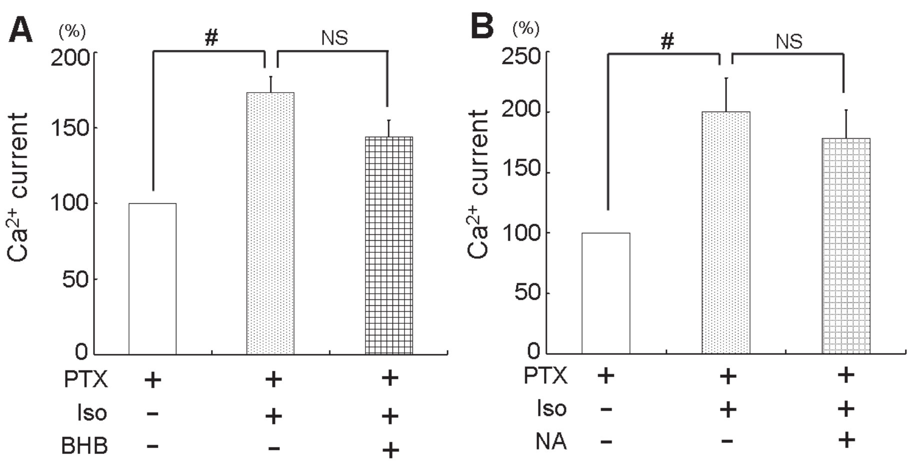

Fig. 4. Effects of BHB (A) and niconitic acid (NA) (B) in the presence of pertussis toxin (PTX) in the pipette solution. Control Ca^{2+} currents before isoproterenol was set as 100%. The current magnitude was measured at 10 mV. BHB : $n=5$ # ; $p < 0.05$ vs. Iso 0.1 μM NS ; no significance. NA : $n=4$ # ; $p < 0.05$ vs. Iso 0.1 μM.

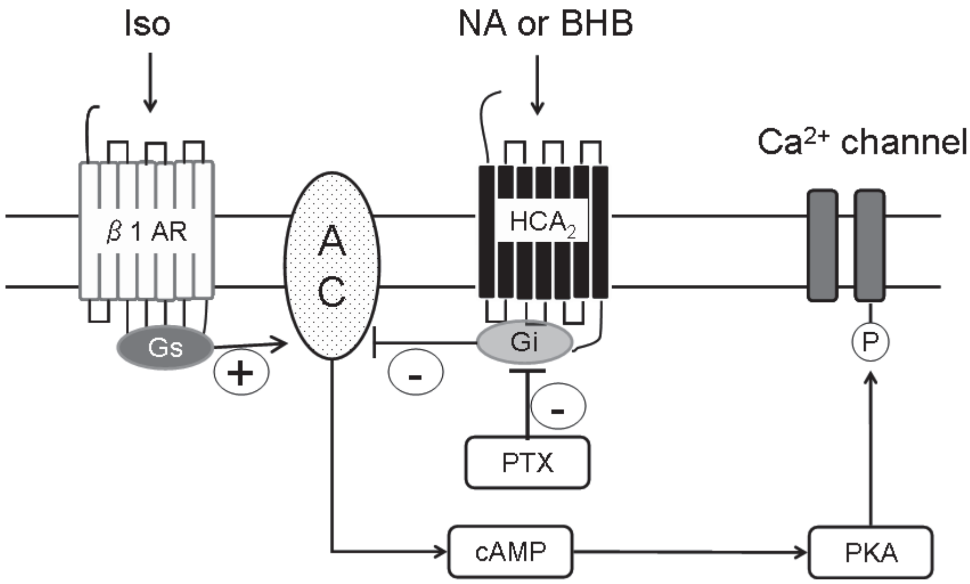

Fig. 5. A schematic representation of the effects of BHB and nicotinic acid (NA) on L-type Ca^{2+} channel. Isoproterenol (Iso) binds to β1 adrenergic receptor and activates adenylate cyclase (AC) via activation of Gs. HCA_2 receptor activates Gi and suppresses AC. cAMP activates protein kinase A (PKA) and phosphorylates Ca^{2+} channel. Phosphorylation of Ca^{2+} channel enhances the Ca^{2+} current in cardiac myocytes. Pertussis toxin (PTX) inhibits Gi.

bonate decreases ketone bodies including BHB[31]. More aggressive administration of sodium bicarbonate may be revalidated in severe ketoacidosis with high levels of catecholamine and BHB in the blood.

Limitations of the present study include the following. Whether our results obtained from guinea pig cardiac myocytes are applicable to human heart should be evaluated. It is not so common to see ketoacidosis so advanced to accompany cardiac dysfunction.

In conclusion, BHB stimulates Gi signaling pathway possibly via HCA_2 receptors and decrease the Ca^{2+} current pre-augmented by adrenergic β stimulation in guinea pig cardiac ventricular myocytes. This BHB effect may be responsible for suppression of cardiac function in ketoacidosis. Further investigation of the BHB effect on cardiac function is necessary.

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