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BMS-191095, a Cardioselective Mitochondrial K_{ATP} Opener, Inhibits Human Platelet Aggregation by Opening Mitochondrial K_{ATP} Channels

Mi-Ra Cho, Jung-Woo Park[1], In-Sang Jung[1], Kyu-Yang Yi[2], Sung-Eun Yoo[2], Hun-Jong Chung[3], Yeo-Pyo Yun, Suk-Hyung Kwon[4], and Hwa-Sup Shin[1]

College of Pharmacy, Chungbuk National University, Cheongju 361-763, Korea, [1]Division of Life Science, College of Natural Sciences, Konkuk University, Chungju 380-701, Korea, [2]Medical Science Division, Korea Research Institute of Chemical Technology, Daejon 305-600, Korea, [3]Pediatric Department, Chungju Hospital, Konkuk Medical School, Konkuk University, Chungju 380-701, Korea, and [4]Rexgene Biotech Co. Ltd., Seoul 137-070, Korea

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We evaluated the antiplatelet effects of two classes of ATP-sensitive potassium channel openers (K_{ATP} openers) on washed human platelets, and the study's emphasis was on the role of mitochondrial K_{ATP} in platelet aggregation. Collagen-induced platelet aggregation was inhibited in a dose dependent manner by lemakalim and SKP-450, which are potent cardio-nonselective K_{ATP} openers, and also by cardioselective BMS-180448 and BMS-191095 (IC_{50}: 1,130, >1,500, 305.3 and 63.9 μM, respectively), but a significantly greater potency was noted for the cardioselective K_{ATP} openers. The latter two K_{ATP} openers also inhibited platelet aggregation induced by thrombin, another important blood-borne platelet activator, with similar rank order of potency (IC_{50}: 498.0 and 104.8 μM for BMS-180448 and BMS-191095, respectively). The inhibitory effects of BMS-191095 on collagen-induced platelet aggregation were significantly blocked by a 30-min pretreatment of platelets with glyburide (1 μM) or sodium 5-hydroxydecanoate (5-HD, 100 μM), a nonselective and selective mitochondrial K_{ATP} antagonist, respectively, at similar magnitudes; this indicates the role of mitochondrial K_{ATP} in the antiplatelet activity of BMS-191095. However, glyburide and 5-HD had no effect when they were added to the platelet cuvette immediately prior to the addition of BMS-191095. These findings indicate that cardioselective mitochondrial K_{ATP} openers like BMS-191095 are able to exert cardioprotective effects in cardiac ischemia/reperfusion injury via dual mechanisms directed at the inhibition of platelet aggregation and the protection of cardiomyocytes, and both these mechanisms are mediated by mitochondrial K_{ATP}.

Key words: Platelet, BMS-180448, BMS-191095, ATP-Sensitive K^+ Channel opener, Cardioprotection, Mitochondrial K_{ATP}

INTRODUCTION

It has been shown that human platelets, which belong to the family of non-excitable cells, contain three types of K^+ channels. These channels are sensitive to apamin (the small-conductance calcium-dependent potassium channels, SK_{Ca}), charybdotoxin (K_{Ch}, probably being equal to intermediate conductance calcium-dependent potassium channels, IK_{Ca}), or alpha-dendrotoxin (the voltage-gated K_v channels) (de Silva *et al.*, 1997, 1998). Although certain potassium channels, especially SK_{Ca} and K_{Ch}, were demonstrated to be abnormal in the platelets of patients with Alzheimer's disease (de Silva *et al.*, 1998), and voltage-gated K_v channels were involved in stabilization of the resting membrane potential in platelets and their progenitors megakaryocytes (Maruyama, 1987; Kawa, 1990; Kapural *et al.*, 1995), the role of potassium channels in platelet reactivity is still controversial (Andersson and Vinge, 1991; Kapural and Fein, 1997).

Cardiac ischemia followed by reperfusion is well known to severely damage the function of cardiomyocytes, and recent studies have further demonstrated that myocardial ischemia-reperfusion injury extends to the vascular wall

Correspondence to: Hwa-Sup Shin, Division of Life Science, College of Natural Sciences, Konkuk University, Chungju 380-701, Korea
Tel: 82-43-840-3575, Fax: 82-43-851-4169
E-mail: hsshin@kku.ac.kr

and especially to the endothelial cells. Myocardial ischemia-reperfusion was shown to induce marked structural injury to endothelial cells (Kaeffer *et al.*, 1996); this was accompanied by decreased endothelium-dependent relaxation of isolated large or medium size coronary arteries to thrombin (Ku, 1982), acetylcholine (Van Benthuysen *et al.*, 1987), and aggregating platelets (Pearson *et al.*, 1990). More recently, it has been shown that either a homologous or heterologous population of platelets was able to induce myocardial dysfunction in the ischemic and reperfused guinea pig heart *via* the release of free radicals (Seligmann *et al.*, 2000, 2002).

It has also been repeatedly reported that ischemic preconditioning not only protected various aspects of myocardial function in different species including humans (Li and Kloner, 1992; Yellon *et al.*, 1992, 1993), but it also protected the cardiac endothelium (Laude *et al.*, 2002). Pharmacological activation of K_{ATP} channels in cardiomyocytes by various types of K_{ATP} openers is associated with cardioprotection and this may simulate some aspects of ischemic preconditioning (Armstrong *et al.*, 1995). Intensive research on the types of K_{ATP} channels and the development of their modulators has led to the discovery that sarcolemmal K_{ATP} openers and mitochondrial K_{ATP} openers subserve different functions in various cells such as vascular smooth muscle cells, cardiomyocytes, cerebellar granule neurons, and pancreatic beta-cells (Grover *et al.*, 2001; Teshima *et al.*, 2003).

Despite the studies on K_{ATP} channels in these cells, less is known about the role of K_{ATP} channels in human platelets, except for the discovery that glyburide, an inhibitor of K_{ATP} channels, inhibited the thrombin-stimulated efflux of $^{86}Rb^+$ (a marker for K^+) from human platelets, but only when it was applied at a high concentration (de Silva *et al.*, 1997). The goal of this study was to compare the inhibitory effects of two classes of K_{ATP} openers (cardioselective BMS-180448 (Grover *et al.*, 1995; D'Alonzo *et al.*, 1996) and BMS-191095 (Grover *et al.*, 2002), and nonselective lemakalim (Kinoshita *et al.*, 1999) and SKP-450 (Shin *et al.*, 1998a, b); the chemical structures are shown in Fig. 1) on the aggregation of washed human platelets that was induced by platelet agonists. We will put the study's emphasis on the role of BMS-191095, which is one of the most cardioselective, mitochondrial K_{ATP} openers, for platelet aggregation.

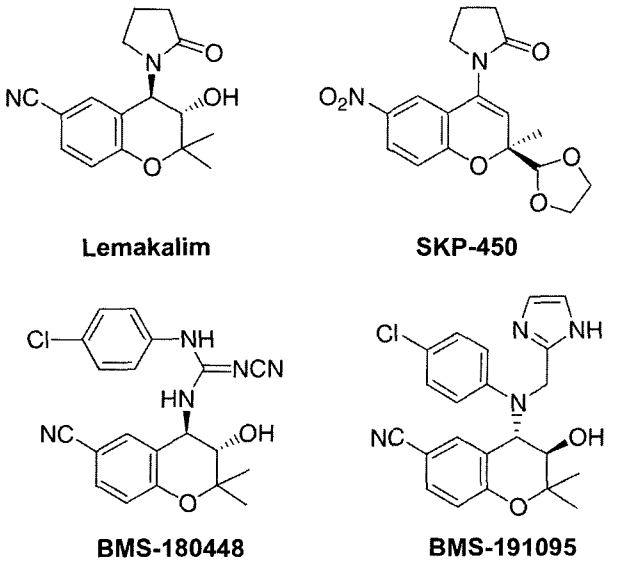

Fig. 1. The chemical structures of lemakalim, SKP-450, BMS-180448, and BMS-191095

MATERIALS AND METHODS

Preparation of washed human platelets

We collected blood by venupuncture from healthy volunteers who had not taken any medical drugs for at least 15 days, and the blood was put in a plastic flask containing 3.15% sodium citrate solution (1:9 v/v). The platelet-rich plasma was prepared by centrifugation of the blood at 120 g for 15 min. Washed platelets were prepared from the platelet-rich plasma. The supernatants were pooled and next centrifuged at 600 g for 15 min at room temperature. The platelet pellets were washed with modified Tyrode-*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (HEPES) buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 2 mM egtazic acid, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4) and they were centrifuged at 600 g for 15 min. Finally the platelets were gently resuspended in Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 1 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4); they were counted using a Coulter counter (Coulter Electronics, Hialeah, FL, U.S.A.), and then adjusted to a concentration of 3×10^8 platelets/mL.

Aggregation of the washed human platelets

Platelet aggregation was measured using an aggregometer (Chrono-Log Co., Havertown, PA, U.S.A.) according to the turbidimetry method of Born and Cross. Briefly, the washed human platelet suspensions (400 μL) were incubated at 37 °C for 4 min in the aggregometer with stirring at 1,000 rpm before aggregation was induced by the addition of either collagen (50 μg/mL) or thrombin (0.5 U/mL). The resulting aggregation was measured as the change in light transmission, that was recorded for 6 min. The anti-aggregatory effects of 4 kinds of ATP-sensitive potassium channel (K_{ATP}) openers were studied by treating the washed platelets with the K_{ATP} openers for 3 min before the addition of the platelet activating agents. Platelet aggregation was used as an indicator of Ca^{2+}

influx: the effect of K_{ATP} openers on Ca^{2+} influx was estimated from the level of inhibition of platelet aggregation. Each inhibition rate was obtained from the maximal aggregation induced by the respective agonists, and the 50% inhibitory concentration (IC_{50}) was calculated

Chemicals and drugs

Lemakalim, SKP-450, BMS-180448 and BMS-191095 were synthesized at the Medical Science Division, Korea Research Institute of Chemical Technology (Daejeon, Korea). Collagen, bovine serum albumin (BSA), and U46619 were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and glyburide and sodium 5-dydroxydecanoate were purchased from Biomol (Plymouth Meeting, PA, USA). Lemakalim, KR-30450, BMS-180448, BMS-191095, glyburide, and sodium 5-dydroxydecanoate were dissolved in dimethyl sulfoxide (DMSO). The other chemicals were dissolved in distilled water and further dilution was made with PSS. All the other chemicals used in the experiments were of the highest analytical grade.

Statistical analysis

The antiplatelet aggregation effects of the K_{ATP} openers were expressed as the percentage inhibition of platelet aggregation that was induced by the platelet aggregating agents. The IC_{50} values (the concentrations that produce 50% inhibition of platelet aggregation induced by the platelet activators) were calculated by linear regression analysis of log concentration-response curves by using data points in the range of 20 to 80% inhibition. The results were presented as means ± S.E.M. of the experiments. The differences between the control and treated groups were evaluated by Student's *t* test.

RESULTS

Effect of two classes of K_{ATP} openers on the aggregation of human washed platelets induced by collagen

The antiplatelet effects of the two classes of ATP-sensitive potassium channel openers (K_{ATP} openers) against collagen-induced platelet aggregation are shown in Table I. Lemakalim and SKP-450, two potent nonselective K_{ATP} openers, inhibited collagen (50 μg/mL)-induced platelet aggregation in a concentration-dependent manner. Lemakalim's and SKP-450's IC_{50} values are 1,130 ± 4.78 and >1,500 μM, respectively. Cardioselective K_{ATP} openers, BMS-180448 and BMS-191095, inhibited the collagen-induced aggregation in a concentration-dependent manner, but they had significantly greater potencies (IC_{50} values: 305.3 ± 4.4 and 63.9±4.4 μM, respectively) than did lemakalim and SKP-450 (Table I, Fig. 2). The antiplatelet effects of these cardioselective K_{ATP} openers, especially BMS-191095, a mitochondrial K_{ATP} opener with the highest currently known cardioselectivity, were much greater than that of aspirin, a prototype cyclooxygenase inhibitor (IC_{50} value for aspirin: 378 μM).

Table I. IC_{50} values of four different K_{ATP} openers for their inhibitory effects on the human platelet aggregation that was induced by the platelet aggregating agents collagen and thrombin

Drug	IC_{50} (μM)	
	Collagen	Thrombin
Lemakalim	1,130±4.78	-
SKP-450	>1,500	-
BMS-180448	305.3±4.4*	498.0±5.6
BMS-191095	63.9±4.4*#	104.8±1.1#
Aspirin	378.9	>2,000

Data represent the means ± S.E.M. calculated from the dose-response curve. IC_{50} values were calculated from at least three separate experiments. *P <0.05 and #P <0.05, as compared with the lemakalim group and BMS-180448 group, respectively, by Students *t*-test.

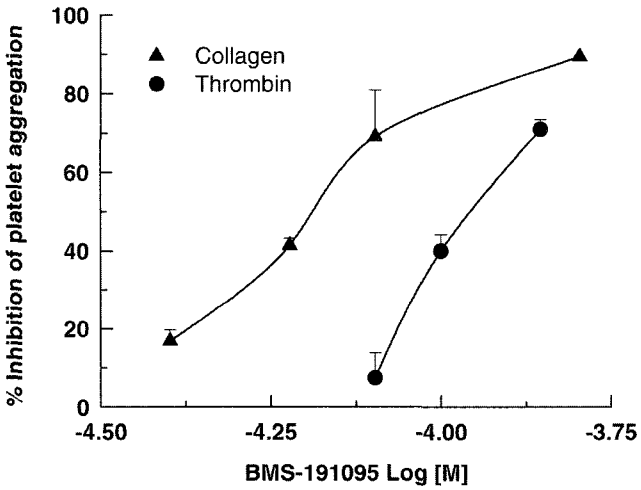

Fig. 2. Inhibitory effects of mitochondrial K_{ATP} opener BMS-191095 on human platelet aggregation that was induced by collagen and thrombin. The results are expressed as means ± S.E.M. (n=4). BMS-191095 exerted a more potent antiplatelet activity against collagen-induced platelet aggregation than thrombin-induced platelet aggregation.

Effect of cardioselective K_{ATP} openers on aggregation of washed human platelets induced by thrombin

The cardioselective K_{ATP} openers, BMS-180448 and BMS-191095, also inhibited the platelet aggregation induced by thrombin (0.5 U/mL), another important blood-borne platelet aggregating agent, and they had markedly greater potencies than aspirin (IC_{50} values: 498.0 ± 5.6, 104.8 ± 1.1 and >2,000 μM for BMS-180448, BMS-191095 and aspirin, respectively) (Table I, Fig. 2). BMS-191095

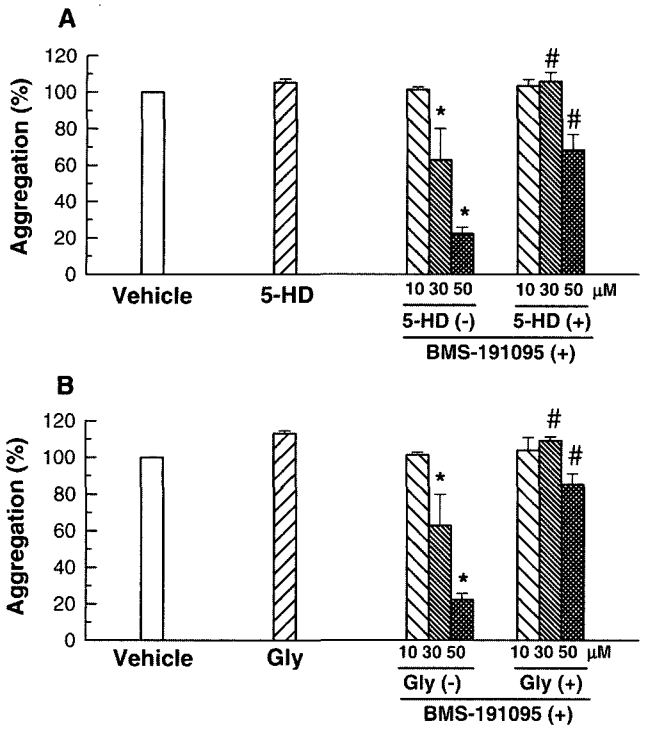

Fig. 3. Inhibitory effects of BMS-191095 (10, 30, 50 μM) on collagen-induced human platelet aggregation in the presence of two different K_{ATP} blockers that were added to the cuvette containing the platelets 30 min prior to the addition of BMS-191095. A: The effect of 5-HD, a selective mitochondrial K_{ATP} blocker; B: The effect of glyburide, a nonselective K_{ATP} blocker, n=4, $^{*}P$ <0.05 versus vehicle group, $^{\#}P$ <0.05 versus the antiplatelet activity of BMS-191095 in the absence of 5-HD (A) or glyburide (B). 5-HD, sodium 5-hydroxydecanoate; Gly, glyburide.

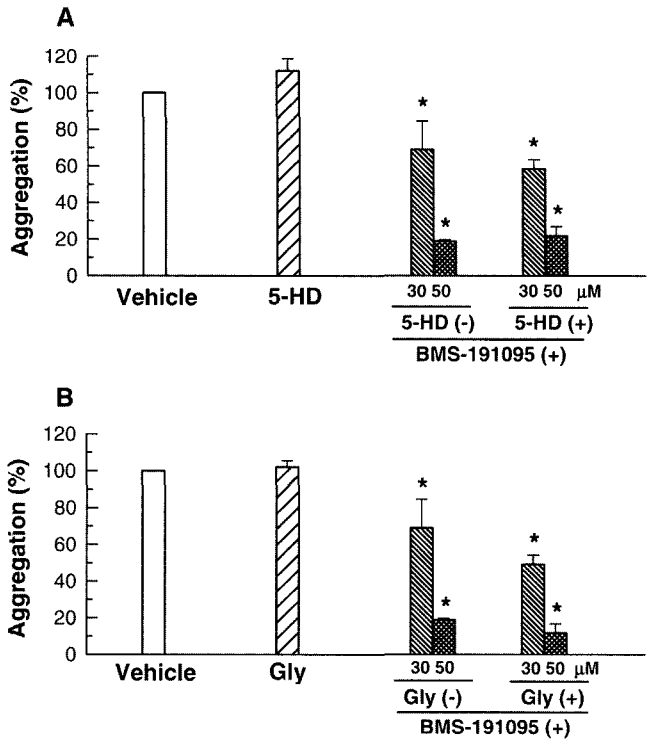

Fig. 4. Inhibitory effects of BMS-191095 (30 and 50 μM) on collagen-induced human platelet aggregation in the presence of two different K_{ATP} blockers blockers that were added to the cuvette containing the platelets immediately prior to the addition of BMS-191095. A: The effect of 5-HD, B: The effect of glyburide, a nonselective K_{ATP} blocker, n=4, $^{*}P$ <0.05 versus vehicle group. 5-HD, sodium 5-hydroxydecanoate; Gly, glyburide.

was 5-fold more potent than BMS-180448 in inhibiting the platelet aggregation induced by both collagen and thrombin.

Blockade of antiplatelet activity of BMS-191095 by glyburide and 5-hydroxydecanoate

A separate series of experiments were conducted to demonstrate the antagonistic effects of glyburide, a non-selective antagonist of plasmalemma and mitochondrial K_{ATP}, and 5-hydroxydecanoate (5-HD), a selective antagonist of mitochondrial K_{ATP}. The inhibitory effect of BMS-191095 on washed human platelet aggregation induced by collagen was significantly blocked at a similar magnitude with a 30-minute pretreatment of the platelets with glyburide (1 μM), or sodium 5-hydroxydecanoate (100 μM) (Fig. 3). However, the antagonistic effects of glyburide and 5-HD were not noted when these chemicals were added immediately prior to the addition of BMS-191095 to the platelet cuvette (Fig. 4).

DISCUSSION

The findings from the present study with washed human platelets clearly demonstrated that a broad class of K_{ATP} openers was able to exert inhibitory effects on the aggregation of platelets induced by various platelet activators, and the potency of the effect varied among the K_{ATP} channel openers. Thus, the potency rank order for the antiplatelet activities of K_{ATP} openers we studied was BMS-191095 > BMS-180448 > lemakalim ≥ SKP-450, indicating that the antiplatelet activity increased with the cardioselectivity of the K_{ATP} openers. These observations appear quite intriguing when they are examined in the light of accumulating evidences from the recent studies on the differentiation of the pharmacological and physiological roles of sarcolemma K_{ATP} openers and mitochondrial K_{ATP} openers.

The K_{ATP} openers we used were known to represent the nonselective K_{ATP} openers (Lemakalim and SKP-450) and the cardioselective K_{ATP} openers (BMS-180448 and BMS-191095). We have previously shown that SKP-450, a benzopyran derivative closely related to lemakalim, caused a significant increase in the ^{86}Rb efflux from canine coronary artery, and SKP-450 also exerted potent vasorelaxant effects on canine coronary artery, rabbit basilar artery and

vertebral artery, and potent vasodepressor effects in various rat models of hypertension; it did so with a greater potency than lemakalim in most of these models (Shin *et al.,* 1998a), and it also did so in anesthetized and conscious beagle dogs (Shin *et al.,* 1998b). In these studies, the vasorelaxant and vasodepressor effects of SKP-450 were all antagonized by glyburide, and this confirmed the classification of SKP-450 (and lemakalim) as first generation K_{ATP} openers. We further showed that SKP-450 exerted cardioprotective effects in isolated rat heart that was subjected to global ischemia/reperfusion (Jung *et al.,* 1998). Nonselective first generation K_{ATP} openers relax smooth muscle, and shorten the cardiac action potential duration by acting on sarcolemma K_{ATP}, and they exert cardioprotective effects within the vasorelaxant dose ranges by acting on mitochondrial K_{ATP} (Edwards and Weston, 1997; Garlid *et al.,* 1996). However, a clear pharmacological distinction between smooth muscle relaxation and cardioprotection has been reported for several K_{ATP} openers such as BMS-180448, BMS-191095 and KR-31378, and they have been classified as cardioselective K_{ATP} openers (Atwal *et al.,* 1993; Grover *et al.,* 2001; Rovnyak *et al., 1997;* Lee *et al.,* 2001). BMS-180448 was developed and recognized as a prototype cardioselective K_{ATP} opener having a markedly reduced vasorelaxant activity over the first generation K_{ATP} openers, and this led to new era of discovery and development of cardioselective antiischemic agents with improved pharmacological profiles based on BMS-180448's novel mechanism of action (Grover and Atwal, 2002). This compound still retained a significant vasorelaxant activity due to various mechanisms including the opening of sarcolemmal K_{ATP}, as shown in our recent studies (Park *et al.,* 2003).

In particular, BMS-191095 has been reported to be very selective; it has no vasorelaxant activity while it retained the cardioprotective activity of the nonselectivefirst generation K_{ATP} openers, and recent data has shown that BMS-191095 selectively opens mitochondrial K_{ATP} without affecting sarcolemma channels in vascular smooth muscle, heart or pancreatic beta β-cells (Grover *et al.,* 2001). The absence of cardiac electrophysiological and hemodynamic effects for BMS-191095 has been demonstrated in vivo (Grover *et al.,* 2002; Grover and Atwal, 2002). Mitochondiral K_{ATP} has been shown to be important in mediating pharmacological cardioprotection and for the ischemic preconditioning phenomenon (Nakai *et al.,* 2001). Our findings, that BMS-191095 exerted antiplatelet efficacy with the potency 5-fold and over 20-fold greater than BMS-180448 and lemakalim/KP-450, respectively, appear to be in line with the data published on the cardioprotective activities of sarcolemma and mitochondrial K_{ATP} openers. This indicates the important role of mitochondrial K_{ATP} in antiplatelet activity. The role of mitochondrial K_{ATP} in antiplatelet activity was further supported by the observation that the inhibitory effect of BMS-191095 on washed human platelet aggregation induced by collagen was significantly blocked at a similar magnitude by a 30-min pretreatment of the platelets with glyburide (1 μM), a nonselective antagonist of plasmalemma and mitochondrial K_{ATP}, and sodium 5-hydroxydecanoate (100 μM), a selective antagonist of mitochondrial K_{ATP}. It is worthwhile to note the absence of any antagonistic effects of glyburide and 5-HD when they were added immediately prior to the addition of BMS-191095 to the platelet-containing cuvette, thus indicating the importance of an adequate period for the incubation of antagonists with the platelets in our experimental setup.

It has been repeatedly reported that ischemia/reperfusion-induced heart injury contributes to platelet aggregation and to the endothelial dysfunction of large and small coronary arteries. This indicates the formation of a vicious cycle in the ischemic hearts (Van Benthuysen *et al.,* 1987; Pearson *et al.,* 1990; Seligmann *et al.,* 2000). Therefore, the pharmacological approach toward the total protection of platelets, cardiomyocytes and coronary arteries from ischemia/reperfusion injury may be a promising therapeutic strategy. This perspective appears to be partly supported by the finding that blockade of platelet aggregation may lead to the preservation of myocardial post-ischemic function, as was demonstrated by antibody to platelet fibrinogen receptor GPIIb/IIIa (Habazettl *et al.,* 2000; Kleiman, 1999). The findings from the present study indicates the presence of mitochondrial K_{ATP} in human platelets and their role in platelet aggregation. The above results further indicate that cardioselective mitochondrial K_{ATP} openers like BMS-191095 are able to exert cardioprotective effects in cardiac ischemia/reperfusion heart injury *via* dual mechanisms. These effects are directed at the inhibition of platelet aggregation induced by the various platelet activators that are released during cardiac ischemia/reperfusion, and they are also directed at the enhancement of the endogenous cardioprotective capability involving the mitochondrial K_{ATP} in cardiomyocytes.

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