

# Pharmacologic Characterization of BMS-191095, a Mitochondrial $K_{ATP}$ Opener with No Peripheral Vasodilator or Cardiac Action Potential Shortening Activity

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## ABSTRACT

Previous work described ATP-sensitive  $K^+$  channel ( $K_{ATP}$ ) openers (e.g., BMS-180448), which retain the cardioprotective activity of agents such as cromakalim while being significantly less potent as vasodilators. In this study, we describe the pharmacologic profile of BMS-191095, which is devoid of peripheral vasodilating activity while retaining glyburide-reversible cardioprotective activity. In isolated rat hearts subjected to 25 min of global ischemia and 30 min of reperfusion, BMS-191095 increased the time to onset of ischemic contracture with an  $EC_{25}$  of 1.5  $\mu$ M, which is comparable to 4.7  $\mu$ M and 3.0  $\mu$ M for cromakalim and BMS-180448, respectively. Comparisons of cardioprotective and vasorelaxant potencies in vitro and in vivo showed BMS-191095 to be significantly more selective for cardioprotection with virtually no effect on peripheral smooth muscle, whereas cromakalim showed little selectivity. In addition to increasing the time to the onset of contracture, BMS-191095 improved postischemic recovery of function and reduced lactate dehydrogenase release in the isolated rat hearts. The cardioprotective effects of BMS-191095 were abolished by glyburide and sodium 5-hydroxydecanoate (5-HD). BMS-191095 did not shorten action potential duration in normal or hypoxic myocardium within its cardioprotective concentration range nor did it activate sarcolemmal  $K_{ATP}$  current ( $\leq 30 \mu$ M). BMS-191095 opened cardiac mitochondrial  $K_{ATP}$  with a  $K_{1/2}$  of 83 nM, and this was abolished by glyburide and 5-HD. These results show that the cardioprotective effects of BMS-191095 are dissociated from peripheral vasodilator and cardiac sarcolemmal  $K_{ATP}$  activation. Agents like BMS-191095 may owe their cardioprotective selectivity to selective mitochondrial  $K_{ATP}$  activation.

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$K_{ATP}$  may serve an endogenous cardioprotective function, because myocardial preconditioning is thought to be mediated by activation of these channels (Gross and Auchampach, 1992; Tomai et al., 1994). Pharmacologic openers of  $K_{ATP}$  are cardioprotective in numerous experimental models of ischemia and reperfusion, suggesting they may simulate preconditioning (Ohta et al., 1991; Grover, 1994; Mizumura et al., 1995). These protective effects are exerted directly on ischemic myocardium and are independent of vasodilator activity, as well as APD shortening activity (Grover, 1994; Yao and Gross, 1994; Grover et al., 1995a,b). This vasodilator activity may be contraindicated, because blood pressure re-

ductions can compromise diseased myocardium (Belin et al., 1996). The lack of correlation between APD shortening and cardioprotection also suggests that the relevant  $K_{ATP}$  for cardioprotection is distinct from cardiac sarcolemmal channels and may involve activation of mitochondrial  $K_{ATP}$  (Garlid et al., 1996, 1997; Liu et al., 1998). Involvement of mitochondrial  $K_{ATP}$  was deduced from the cardioprotective effects of diazoxide, which opens cardiac mitochondrial  $K_{ATP}$  while having little effect on cardiac sarcolemmal channels. Interestingly, diazoxide is a potent vasorelaxant, suggesting that it opens plasmalemmal channels in vascular smooth muscle and that tissue subtypes for  $K_{ATP}$  exist.

Structure-activity determinations in our laboratories showed a poor correlation between cardioprotective and vasorelaxant activities of  $K_{ATP}$  openers (Atwal et al., 1993). Further studies led to the identification BMS-180448, which

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**ABBREVIATIONS:**  $K_{ATP}$ , ATP-sensitive potassium channels; APD, action potential duration; EDP, end-diastolic pressure; LVDP, left ventricular diastolic pressure; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase;  $APD_{90}$ , action potential duration at 90% repolarization; MAP, monophasic action potential; PBFI,  $K^+$  binding benzofuran isophthalate;  $TEA^+$ , tetraethylammonium; 5-HD, sodium 5-hydroxydecanoate; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

shows greater selectivity for ischemic myocardium (lower vasodilator activity), compared with first generation agents such as cromakalim (Grover et al., 1995b). Interestingly, BMS-180448 is also relatively devoid of APD shortening activity in ischemic myocardium (Grover et al., 1995a), even though its cardioprotective effects are abolished by  $K_{ATP}$  blockers. Although BMS-180448 has a lower propensity to vasodilate at cardioprotective doses when compared with currently existing agents, we continued our efforts to further reduce vasodilator activity in novel agents. In addition to reducing vasodilator activity, it was also desirable to eliminate APD shortening activity to reduce proarrhythmic potential.

Further structure-activity studies confirmed that cardioprotection and vasorelaxant effects of  $K_{ATP}$  openers are not correlated (Rovnyak et al., 1997). These studies led to the identification of 4-(*N*-aryl)-substituted benzopyran derivatives having poor vasorelaxant potency, but retaining cardioprotective potency. One compound in this series, BMS-191095 (see structure in Fig. 1), has little measurable peripheral vasorelaxant activity up to 30  $\mu$ M while retaining cardioprotective activity that was comparable with BMS-180448 and cromakalim. Due to its superior in vitro pharmacologic profile, BMS-191095 was selected for detailed pharmacologic studies. The goal of these studies was to elucidate the cardioprotective profile for BMS-191095. In addition to determining cardioprotective and vasodilator profile, we also determined if BMS-191095 can selectively open cardiac mitochondrial  $K_{ATP}$ .

## Materials and Methods

### Cardioprotective Profile in Isolated Rat Hearts

Male Sprague-Dawley rats (400–500 g) were anesthetized using 100 mg/kg sodium pentobarbital (i.p.). The trachea was intubated, and then the jugular vein was injected with heparin (1000 U/kg). While being mechanically ventilated, their hearts were perfused in situ via retrograde cannulation of the aorta. The hearts were then excised and quickly moved to a Langendorff apparatus where they were perfused with oxygenated Krebs-Henseleit solution containing (in mM): 112 NaCl, 25  $\text{NaHCO}_3$ , 5 KCl, 1.2  $\text{MgSO}_4$ , 1  $\text{KH}_2\text{PO}_4$ , 1.2  $\text{CaCl}_2$ , 11.5 glucose, and 2 pyruvate at a constant perfusion pressure (85 mm Hg). A water-filled latex balloon attached to a metal cannula was then inserted into the left ventricle and connected to a Statham pressure transducer for measurement of left ventricular pressure. The hearts were allowed to equilibrate for 15 min, at which time EDP was adjusted to 5 mm Hg, and this balloon volume was maintained for the duration of the experiment. Preischemia or predrug function, heart rate, and coronary flow (extracorporeal electromagnetic flow probe; Carolina Medical Electronics, Inc., King, NC) were then measured. Contractile function was calculated by subtracting EDP from left ventricular peak systolic pressure, resulting in LVDP. Cardiac temperature was maintained throughout the experiment by submerging the hearts in 37°C buffer, which was allowed to accumulate in a stoppered, heated chamber.

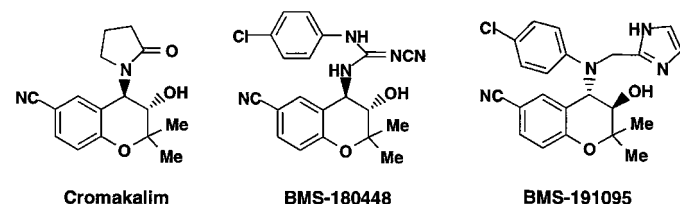


Fig. 1. Chemical structures of the three  $K_{ATP}$  openers used in this study.

After equilibration, the hearts were subjected to one of several treatments. Hearts were treated with vehicle (0.04% DMSO,  $n = 8$ ), 0.3 to 10  $\mu$ M BMS-191095 ( $n = 5$  per group), 0.3 to 10  $\mu$ M BMS-180448 ( $n = 5$  per group), 1 to 10  $\mu$ M cromakalim ( $n = 5$  per group), 6  $\mu$ M BMS-191095 + 1  $\mu$ M glyburide ( $n = 5$ ), 6  $\mu$ M BMS-191095 + 100  $\mu$ M 5-HD, 1  $\mu$ M glyburide + 10  $\mu$ M BMS-180448 ( $n = 5$ ), 1  $\mu$ M diltiazem ( $n = 5$ ), and 1  $\mu$ M glyburide + 1  $\mu$ M diltiazem ( $n = 5$ ). The respective drug treatments were given for 10 min before ischemia and were included in the perfusate. Diltiazem was used to confirm that  $K_{ATP}$  blockers are specific for abolishing the protective effects of  $K_{ATP}$  openers. After 10 min of drug treatment, the hearts were subjected to 25 min of global ischemia and 30 min of reperfusion. Ischemia was initiated by completely shutting off perfusate flow. At the end of the reperfusion period, contractile function, coronary flow, and LDH release were measured. The respective drugs were given only before global ischemia and were not given during reperfusion. Severity of ischemia was determined from time to the onset of ischemic contracture, recovery of contractile function at 30 min into reperfusion, and LDH release into the perfusate. Time to the onset of ischemic contracture was defined as the time (min) during global ischemia in which the first 5 mm Hg increase in EDP was observed. Cardioprotective potency was calculated as the  $\text{ED}_{25}$  for increasing time to contracture.  $\text{ED}_{25}$  is the concentration of drug causing a 25% increase in time to contracture relative to vehicle-treated hearts. Because a detailed cardioprotective profile for cromakalim and BMS-180448 has previously been published (Grover, 1994; Grover et al., 1995b), we are only showing the time to contracture and coronary flow data for this compound. For coronary vasodilator potency determinations, extra hearts were prepared to obtain complete concentration responses: 0.03, and 0.1  $\mu$ M concentrations were added as necessary for each drug ( $n = 5$  for each group done). The respective drug treatment was given for 10 min, and coronary flow was recorded as described above, except that the hearts were removed after the coronary flow determination and ischemia was not instituted.

### Effect of BMS-191095 on APD in Guinea Pig Papillary Muscles and Isolated Hearts

**Guinea Pig Papillary Preparation.** Male guinea pigs (450–600 g) were sacrificed by cervical dislocation. Hearts were rapidly removed and rinsed in Krebs-Henseleit bicarbonate buffer solution (room temperature) equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . The Krebs-Henseleit solution for all electrophysiologic studies was composed of the following (mM): 112 NaCl, 5.0 KCl, 11.5 glucose, 25  $\text{NaHCO}_3$ , 1.2  $\text{MgSO}_4$ , 1.25  $\text{CaCl}_2$ , 5.0 mM HEPES, and 1.0  $\text{KH}_2\text{PO}_4$  at pH 7.4. A posterior papillary muscle, 3 to 5 mm in length and 1 mm or less in diameter, was removed from the right or left ventricle and was pinned to the base of a 5 ml tissue chamber. The papillary muscle was continuously stimulated through a pair of platinum wires with 1-ms square wave pulses set at 200% of threshold voltage. The frequency of stimulation was held constant at 6 Hz during the first hour of equilibration and then paced at 1 Hz during the remaining equilibration period. Muscles were allowed 2 to 3 h to equilibrate while being superfused with oxygenated buffer solution (15–25 ml/min at  $37 \pm 0.2^\circ\text{C}$ ).

Standard microelectrode techniques were used to impale single myocardial cells in a multicellular preparation and to record transmembrane action potentials. Microelectrodes were filled with a solution containing 3 M KCl, 500  $\mu$ M EDTA, and 10 mM HEPES. Electrodes with tip resistances greater than 10 M $\Omega$  were coupled to an amplifier (Axoclamp-2A; Axon Instruments, Burlingame, CA). Electrical potentials were displayed on a digital oscilloscope (Yokogawa model D1200; Yokogawa, Newnan, GA). Signals were recorded on a chart recorder (Gould model 2400S; Gould, Cleveland, OH) and analyzed with a computer using the digital data obtained from the oscilloscope and a customized BASIC program.

Following equilibration, transmembrane action potentials were recorded, and resting membrane potentials, amplitudes,  $V_{\text{max}}$ , and

action potential durations at 90% levels of repolarization ( $APD_{90}$ ) were measured at a stimulating frequency of 2 Hz. Either vehicle (<0.1% DMSO,  $n = 12$ ), BMS-191095 (6  $\mu$ M,  $n = 10$ ) or cromakalim (10  $\mu$ M,  $n = 13$ ) were added to the buffer solution, and tissues allowed 30 min for equilibration. Action potential measurements were repeated. At this time, tissues were exposed to an hypoxic buffer solution (Krebs-Henseleit solution bubbled with 95%  $N_2/5\%$   $CO_2$  for 15 min either containing vehicle, BMS-191095, or cromakalim). Action potential parameters were measured at 1, 3, 5, 7, 9, 11, 13, and 15 min of hypoxia. Oxygenated buffer solution containing the appropriate compounds was returned to the bath (reoxygenation) and action potentials measured at 3, 5, 7, 9, 11, 15, 20, 25, and 30 min.

**Electrophysiologic Determinations in Isolated Perfused Hearts.** Guinea pigs were anesthetized and hearts removed as described above. Hearts were quickly connected to a Langendorff apparatus where they were perfused with oxygenated Krebs-Henseleit solution without the addition of HEPES. Hearts were perfused horizontally with buffer at a constant pressure (85 mm Hg) and temperature (37°C).

Atria were removed and hearts instrumented with a surface electrode (Inapres, Norwich NY) and ECG leads. The surface electrode was used for pacing. ECG as well as an epicardial MAP (Franz epicardial probe; EP Technologies, Sunnyvale, CA) were continuously recorded throughout the experiment. ECG and MAP signals were routed to a chart recorder (TA4000; Gould) and oscilloscope (DL1200; Yokogawa). The ambient temperature around the preparation was maintained by a heated vessel (37°C; FE 2; Haake, Germany).

Electrophysiologic measurements were made twice before drug administration and following sequential administration of test substances. Determinations of APD at the 90% repolarization level were made from the plateau region of the MAP, and QT intervals were determined at a ventricular pacing rate of 4 Hz with single pulses of 2-ms duration at twice the threshold current.

Each heart was given 20 min equilibration time. Following equilibration, two control electrophysiologic readings were taken. Hearts were then given vehicle (0.1% DMSO), cromakalim (10  $\mu$ M) or BMS-191095 (6  $\mu$ M). At the end of 10 min of compound administration, electrophysiologic determinations were repeated. At this time, tissues were exposed to an hypoxic solution of Krebs-Henseleit buffer bubbled with 95%  $N_2/5\%$   $CO_2$  for 7 min and measurements repeated.

### Electrophysiological Recording: Whole-Cell $K^+$ Currents

In addition to cardioprotection studies, the relative effect of BMS-191095 on  $K^+$  currents in guinea pig ventricular myocytes was studied to determine if a dissociation between cardioprotection and sarcolemmal currents could be observed. Guinea pig ventricular myocytes were dissociated using previously described methods (Hamill et al., 1981; Lodge and Smith, 1996). Currents were recorded using the whole-cell configuration of the patch-clamp technique. Electrodes (2–5 M $\Omega$ ) were fabricated from borosilicate glass. Voltage-clamp protocols were generated and data acquired using Pulse software (HEKA) in conjunction with a HEKA EPC9 amplifier (Lambrecht, Germany). Voltage ramps from –100 to 40 mV were applied from a holding potential of –40 mV (to inactivate sodium current). Resultant currents were filtered at 3 KHz using an analog 4-pole Bessel filter. The bath solution contained (in mM): NaCl, 140; KCl, 4;  $MgCl_2$ , 1;  $CaCl_2$ , 2; glucose, 10; HEPES, 5; pH 7.4. Nisoldipine (1  $\mu$ M) was added to the bath solution to inhibit L-type calcium current. The pipette solution contained (in mM): KCl, 125;  $MgCl_2$ , 2;  $CaCl_2$ , 2; NaCl, 10; EGTA, 10; HEPES, 5; glucose, 10; ATP, 1; pH 7.2 with KOH. All experiments were performed at 33 to 34.5°C. Stock solutions of BMS-191095 or cromakalim (0.01–0.03 M) were prepared using DMSO and then diluted in bath solution as required.

$K^+$  currents were also recorded using the perforated patch technique (Horn and Marty, 1988; Korn and Horn, 1989). The patch pipette contained (in mM): 55 KCl, 75  $K_2SO_4$ , 2  $MgCl_2$ , pH 7.3, and

150 to 225  $\mu$ g/ml nystatin. The external bathing solution contained in mM: 140 NaCl, 4.6 KCl, 1  $MgCl_2$ , 10 dextrose, 10 HEPES, and 1.5  $CaCl_2$ , pH 7.4 (28–31°C). Nifedipine (3  $\mu$ M) was used to block calcium currents. Currents were activated by applying slow voltage ramps from –100 to 40 mV over a period of 33.75 s (holding potential = –40 mV).

### Effect of BMS-191095 on $K^+$ Flux through Mitochondrial $K_{ATP}$ and on the Matrix Volume of Isolated Rat Heart Mitochondria

The purpose of this part of the study was to determine the effect of BMS-191095 on mitochondrial  $K_{ATP}$  in rat heart. Mitochondria were isolated by differential centrifugation from hearts of Sprague-Dawley rats, as described by Saks et al. (1986). The final mitochondrial pellet was resuspended at 20 mg protein/ml in 0.22 M mannitol and 0.07 M sucrose and buffered with 5 mM TES ( $K^+$  salt), pH 7, and maintained at 0°C. For assays of matrix volume, stock mitochondria were transferred to medium containing  $K^+$  salts of 130 mM chloride, 20 mM succinate, 5 mM phosphate, 0.5 mM EGTA, and 1 mM  $MgCl$ , buffered to pH 7.2, 25°C. Changes in matrix volume, secondary to net salt transport across the inner membrane, were followed using a quantitative light-scattering technique, as described in Jaburek et al. (1998). This technique is based on the principal that reciprocal absorbance of the mitochondrial suspension, when corrected for the extrapolated absorbance at infinite protein concentration, is linear with matrix volume within well defined regions (Beavis et al., 1985).

Submitochondrial particles were also prepared, and mitochondrial  $K_{ATP}$  was extracted, purified, and reconstituted into lipid vesicles containing the potassium-sensitive fluorescent probe PBF1, as previously described (Paucek et al., 1992; Garlid et al., 1996). Electrophoretic  $K^+$  flux was catalyzed by carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone addition, to provide a counterion, and the resulting rates were obtained from calibrations. *Internal medium* contained 100 mM TEA- $SO_4$ , 1 mM EDTA, 25 mM TEA-HEPES, pH 6.8, and 300  $\mu$ M PBF1. Kinetic studies were performed on proteoliposomes suspended at 0.4 mg lipid/ml of *external medium* containing 150 mM KCl, 1 mM EDTA, 1 mM  $MgCl$ , and 25 mM TEA-HEPES, pH 7.2, at 25°C.

### Determination of Vasorelaxant Activity

**In Vivo Vasorelaxant Activity.** Adult mongrel dogs of either sex weighing 12 to 19 kg were anesthetized with intravenous pentobarbital sodium (30 mg/kg), intubated, and respired with room air to maintain eucapnic conditions using a Harvard respirator (Harvard Apparatus, South Natick, MA). The right femoral artery was cannulated for measurement of blood pressure via a pressure transducer (Gould Statham, Oxnard, CA). The right femoral vein was cannulated for drug infusion. Arterial blood was sampled before and after drug administration for blood gas determinations using an ABL 4 blood gas analyzer (Radiometer, Westlake, OH) to ensure eucapnia and normoxia were maintained. A Millar Mikro-Tip pressure transducer (Millar Instruments, Houston, TX) was inserted into the left carotid artery and advanced into the left ventricle for the measurement of left ventricular pressure and dp/dt. A lead II ECG was also monitored. A left thoracotomy was performed at the 5th intercostal space, and a pericardial cradle was formed.

Electromagnetic flow probes (Carolina Medical Electronics, Inc.) were placed on the left femoral artery, left carotid artery, left circumflex coronary artery, and the ascending aorta for the measurement of blood flow. All waveforms were recorded on a Grass model 7D polygraph (Grass Instruments, Quincy, MA).

Vehicle (PEG 200,  $n = 5$ ), BMS-180448 ( $n = 5$ ), cromakalim ( $n = 5$ ), or BMS-191095 ( $n = 5$ ) were administered intravenously in a cumulative manner over 5 min at 15-min intervals (5 min of drug followed by 10 min of recovery) using an infusion pump (Harvard model 22, Harvard Apparatus). Recordings were taken at 0, 5 (time point shown in data figures for this study), and 10 min after the

cessation of each dose. The concentrations of drugs used were 20 mg/ml for the 1 and 3 mg/kg doses, and 200 mg/ml for the remaining higher doses. Intravenous glyburide at 1 mg/kg was administered after the highest dose of the respective drug to determine if any of the hemodynamic effects elicited by the compounds could be reversed. Data were presented as the percent change from predrug baseline values (each animal served as its own control).

**In Vitro Vasorelaxant Activity.** In vitro vasorelaxant activity was determined using rat aortas. IC<sub>50</sub> values for relaxation of methoxamine (3 μM)-contracted aortas was used to compare with in vitro cardioprotective potency (time to onset of ischemic contracture). The experimental details of determination of vasorelaxant potency in vitro have been previously described (Grover et al., 1995b). The compounds tested were cromakalim, BMS-180448, and BMS-191095.

## Results

### Cardioprotective Profile of Action of K<sub>ATP</sub> Openers

The effect of increasing concentrations of BMS-191095 on cardiac function is shown in Table 1. Before ischemia, BMS-191095 had a slight cardiodepressant effect, which was only significant at the 10 μM concentration. Because we had difficulty keeping BMS-191095 in solution at the 10 μM concentration, the reliability of the data at this concentration may be questionable. During reperfusion, LVDP was significantly reduced in vehicle-treated hearts, indicating severe ischemic/reperfusion damage. BMS-191095 significantly improved reperfusion function starting at the 1 μM concentration, and the peak effect was observed at 6 μM. This protective effect seemed to decrease at the 10 μM concentration, although this may have been due to the poor solubility at this concentration. The effects of BMS-191095 on LDH release are shown in Fig. 2. As can be seen in this figure, LDH release was reduced in a concentration-dependent manner by BMS-191095. Similar results were obtained for reperfusion EDP, although some of the protective effect of BMS-191095 was lost at 10 μM (data not shown). These data were comparable with data generated for cromakalim. BMS-191095 did not exert protective effects when given only during reperfusion (data not shown).

TABLE 1  
Effect of BMS-191095 on pre- and postischemic cardiac function in isolated rat hearts  
All values are mean ± S.E.

	Predrug	Postdrug	Reperfusion 30 min
Heart rate (beats/min)			
Vehicle	305 ± 10	293 ± 8	272 ± 21
0.3 μM BMS-191095	298 ± 13	290 ± 8	285 ± 24
1 μM BMS-191095	309 ± 9	289 ± 6	299 ± 6
3 μM BMS-191095	305 ± 8	293 ± 10	301 ± 10
6 μM BMS-191095	309 ± 20	300 ± 18	310 ± 13
10 μM BMS-191095	297 ± 9	268 ± 21	270 ± 23
6 μM BMS-191095 + 1 μM glyburide	282 ± 8	280 ± 13	273 ± 15
6 μM BMS-191095 + 100 μM 5-HD	294 ± 5	282 ± 2	278 ± 10
LVDP (mm Hg)			
Vehicle	127 ± 4	117 ± 4	11 ± 2 <sup>a</sup>
0.3 μM BMS-191095	131 ± 6	116 ± 4	19 ± 3 <sup>a</sup>
1 μM BMS-191095	131 ± 4	121 ± 4	35 ± 6 <sup>a,b</sup>
3 μM BMS-191095	130 ± 4	119 ± 4	52 ± 6 <sup>a,b</sup>
6 μM BMS-191095	129 ± 10	116 ± 11	60 ± 8 <sup>a,b</sup>
10 μM BMS-191095	133 ± 3	103 ± 8 <sup>a</sup>	31 ± 4 <sup>a,b</sup>
6 μM BMS-191095 + 1 μM Gly	135 ± 5	138 ± 7	12 ± 2 <sup>a</sup>
6 μM BMS-191095 + 100 μM 5-HD	138 ± 4	136 ± 4	15 ± 5 <sup>a</sup>

<sup>a</sup> Significantly different than its respective predrug value (*P* < 0.05).

<sup>b</sup> Significantly different from its respective vehicle group value (*P* < 0.05).

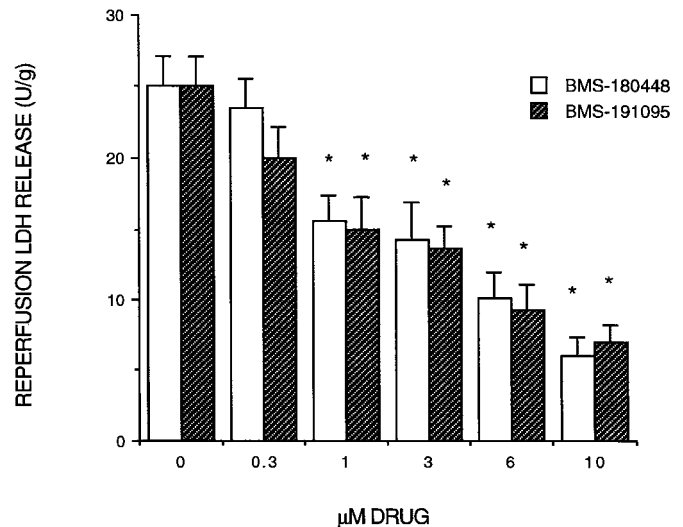
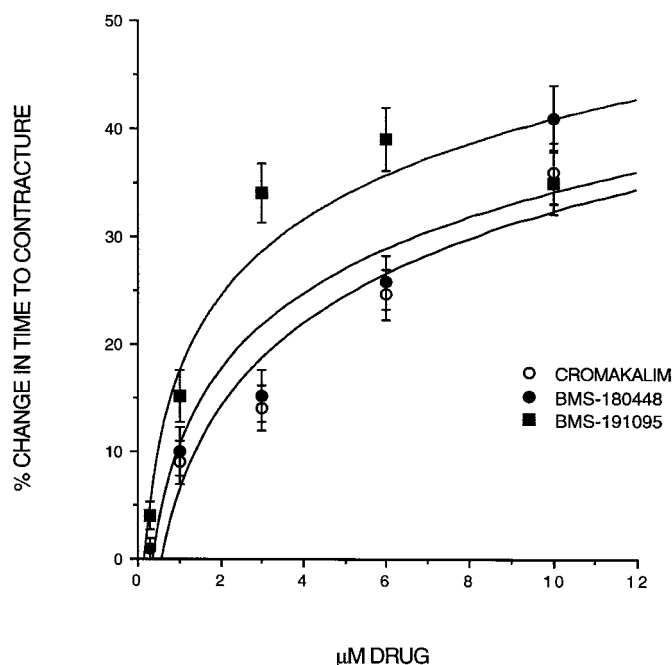


Fig. 2. Effect of increasing concentrations of BMS-180448 and BMS-191095 on reperfusion LDH release in isolated rat hearts. The hearts were treated for 10 min with their respective drug and were then rendered globally ischemic for 25 min and reperfused for 30 min. LDH values are cumulative over the 30-min reperfusion period. BMS-180448 and BMS-191095 significantly reduced LDH release relative to vehicle-treated hearts (\**P* < 0.05).

The effects of cromakalim, BMS-180448, and BMS-191095 on time to the onset of ischemic contracture are shown in Fig. 3. All compounds increased time to contracture in a concentration-dependent manner and were similar in potency. The EC<sub>25</sub> for the percent increase in time to contracture was 3.0 μM for BMS-180448, 1.4 μM for BMS-191095, and 5.0 μM for cromakalim. The data are expressed as the percent change in time to contracture relative to vehicle values, and EC<sub>25</sub> is the concentration of drug causing a 25% increase in time to contracture relative to vehicle-treated groups.

We compared the cardioprotective effects of these agents with their vasorelaxant activity, and these data are shown in Table 2. Vasorelaxant potencies in vitro for the K<sub>ATP</sub> openers were measured using methoxamine-contracted rat aortas (IC<sub>50</sub>). Cromakalim was a potent vasorelaxant in this model



**Fig. 3.** Effect of cromakalim, BMS-180448, and or BMS-191095 on the time to the onset of ischemic contracture during global ischemia in isolated rat hearts. The values are expressed as percent change from vehicle-treated group values. All agents increased the time to the onset of contracture and the cardioprotective potencies were similar.

and was significantly more potent than BMS-180448 and BMS-191095. Using this methodology, BMS-191095 did not show vasodilator activity. By taking the ratio of cardioprotective versus vasorelaxant potencies ( $EC_{25}/IC_{50}$ ), the relative selectivities of these compounds for ischemic heart can be ascertained. The ratio for cromakalim, BMS-180448, and BMS-191095 are 15.7, 1.0, and  $<0.05$ , respectively. Therefore, BMS-191095 is significantly more selective for ischemic myocardium (compared with vascular smooth muscle) relative to cromakalim. Similar results were observed in anesthetized dogs using blood pressure as an index of peripheral vasorelaxation (Table 2). While being significantly less potent as a peripheral vasorelaxant compared with BMS-180448, BMS-191095 was equivalent as a coronary dilator both in vitro and in vivo. The coronary dilator effect of BMS-191095 and BMS-180448 was inhibited by glyburide (data not shown). Although there appears to be some coronary selectivity for BMS-191095, it is nevertheless significantly less potent as a coronary dilator compared with cromakalim.

Data for the effect of  $K_{ATP}$  blockers on the cardioprotective action of BMS-191095 are shown in Fig. 4 and Table 1. The protective effect of BMS-191095 on reperfusion function was abolished by glyburide as was its weak preischemic coronary dilator effect (not shown). The preischemic coronary dilator effect of BMS-191095 was not abolished by 5-HD (data not shown), but the cardioprotective effects of this agent were abolished by 5-HD. These results are consistent with previously published data showing 5-HD to selectively block the cardioprotective effects of  $K_{ATP}$  openers (McCullough et al., 1991). Similar results were seen for BMS-180448 (data not shown). The preischemic and postischemic (Fig. 4) effects of diltiazem were not affected by glyburide as expected.

## Effect of $K_{ATP}$ Openers on Electrophysiologic Parameters

**Effect on Guinea Pig Papillary Muscles.** Baseline electrophysiologic data for all groups were similar. Average baseline resting membrane potential was  $-90 \pm 2$  mV,  $V_{max}$  was  $202 \pm 20$  V/s, and  $APD_{90}$  was  $144 \pm 7$  ms. APD values expressed as percent change from baseline are shown in Fig. 5. Guinea pig papillary muscles treated with vehicle alone displayed no change in action potential characteristics under normoxic conditions. In the presence of hypoxia, a slight reduction in  $V_{max}$  (data not shown) and  $APD_{90}$  was observed. Values returned to control levels during the re-oxygenation period. BMS-191095 did not significantly change action potential characteristics under normoxic conditions. It is important to note that BMS-191095 was evaluated up to  $6 \mu M$  due to problems associated with solubility at higher concentrations, although  $6 \mu M$  BMS-191095 and  $10 \mu M$  cromakalim displayed similar cardioprotective activity. During hypoxia, BMS-191095 did not significantly alter  $APD_{90}$  values relative to vehicle.  $APD_{90}$  values decreased approximately 25% in the vehicle-treated muscles, compared with 20% in the BMS-191095 treated muscles at 15 min of hypoxia. In contrast, cromakalim ( $10 \mu M$ ) significantly reduced  $APD_{90}$  values under normoxic conditions from  $140 \pm 9$  to  $105 \pm 9$  ms (25%). During hypoxia, cromakalim reduced  $APD_{90}$  values by 80% at 15 min of hypoxia. Following reoxygenation, both vehicle-treated and BMS-191095-treated muscles returned to near control levels. There were no changes in either resting membrane potential or action potential overshoots in any treatment group (data not shown).

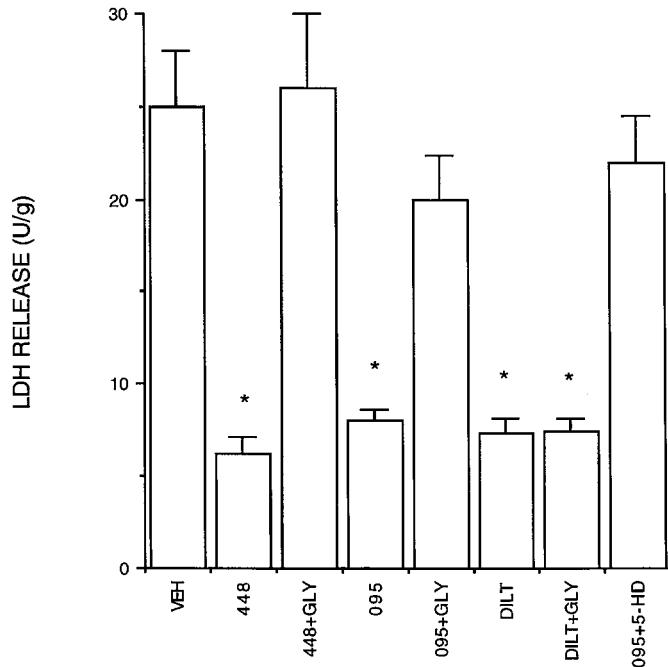
**Effect on Perfused Guinea Pig Hearts.** Isolated perfused guinea pig hearts demonstrated no significant differences in  $APD_{90}$  or QT interval between groups at control readings. Following administration of cromakalim ( $10 \mu M$ ), there was a significant reduction in  $APD_{90}$  and QT interval of 16% and 11%, respectively (Fig. 6). These changes were also significantly different from vehicle-treated hearts. BMS-191095 caused no significant change in  $APD_{90}$  or QT-interval values. In contrast, BMS-180448 caused a slight but significant increase in  $APD_{90}$  and QT-interval values of 9% and 11%, respectively. Under hypoxic conditions,  $APD_{90}$  and QT intervals in vehicle-treated hearts decreased 25% and 28%, respectively. Cromakalim caused a precipitous decrease in  $APD_{90}$  and QT-interval values during hypoxia of 66% and 58%, respectively. Unlike cromakalim, BMS-191095 in the presence of hypoxia had no effect on  $APD_{90}$  (29%) and QT interval (30%), compared with vehicle-treated hearts. Similar results were observed for BMS-180448.

**Effect of BMS-191095 on Cardiac Myocyte Whole-Cell  $K^+$  Currents.** Experimental conditions were controlled to exclude  $Na^+$  and  $Ca^{2+}$  currents from  $K^+$  currents. Using the standard whole-cell patch-clamp technique, BMS-191095 ( $\leq 30 \mu M$ ) failed to activate  $K_{ATP}$  current ( $n = 9$  cells) (Fig. 7). Interestingly, BMS-191095 partially inhibited delayed rectifier potassium currents at voltages greater than approximately  $-25$  mV. Using the perforated patch technique, 3 and  $10 \mu M$  BMS-191095 ( $n = 6$  and 5 cells, respectively) failed to produce a detectable increase in current. Again, BMS-191095 partially inhibited the delayed rectifier current. Cromakalim activated  $K_{ATP}$  currents as previously described. BMS-191095 had no effects on either  $Na^+$  or  $Ca^{2+}$  ( $IC_{50} \sim 30 \mu M$ ) currents.

TABLE 2

Comparison of the anti-ischemic and vasodilating potencies of cromakalim, BMS-180448, and BMS-191095

	Time to Contracture (Rat Heart, $EC_{25}$ , $\mu M$ ) <sup>a</sup>	Methoxamine-Contracted Rat Aorta ( $IC_{50}$ , $\mu M$ ) <sup>b</sup>	Blood Pressure- Lowering, Dogs ( $ED_{20}$ , $\mu mol/kg$ ) <sup>c</sup>	Coronary Flow	
				Rat Heart ( $EC_{10}$ , $\mu M$ ) <sup>d</sup>	Dogs ( $ED_{150}$ , $\mu mol/kg$ ) <sup>e</sup>
Cromakalim	4.7	0.3	0.15	0.03	0.1
BMS-180448	3.0	3.1	10.3	0.9	10.3
BMS-191095	1.4	>30	>100	1.1	25.5

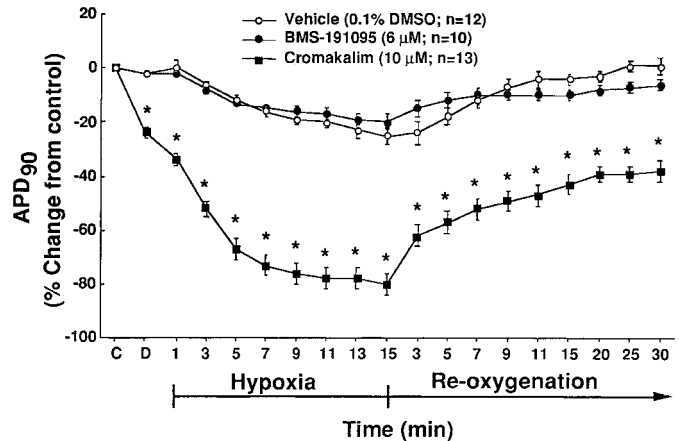
<sup>a</sup>  $EC_{25}$  for time to contracture is defined as the concentration necessary for a 25% increase in time to contracture.<sup>b</sup>  $IC_{50}$  is defined as the concentration necessary for relaxation by 50% of methoxamine-contracted rat aorta.<sup>c</sup>  $ED_{20}$  is defined as the intravenous dose-lowering mean arterial blood pressure by 20% in anesthetized dogs.<sup>d</sup>  $EC_{10}$  is defined as the concentration necessary to increase coronary flow 10% in isolated rat hearts.<sup>e</sup>  $ED_{150}$  is defined as the dose necessary for a 150% increase in coronary blood flow in dogs.

**Fig. 4.** Effect of 10  $\mu M$  BMS-180448 (448), 6  $\mu M$  BMS-191095 (095), or 1  $\mu M$  diltiazem (DILT) with or without glyburide (GLY) or 5-HD on reperfusion LDH release in isolated rat hearts. The hearts were treated for 10 min with their respective drug and were then rendered globally ischemic for 25 min and reperfused for 30 min. LDH values are cumulative over the 30-min reperfusion period. Diltiazem, BMS-180448, and BMS-191095 were all significantly cardioprotective, compared with vehicle (VEH) ( $*P < 0.05$ ), and the effects produced by the  $K_{ATP}$  openers were abolished by the  $K_{ATP}$  blockers, unlike diltiazem.

#### Effect of BMS-191095 on Reconstituted Mitochondrial $K_{ATP}$

Since little effect on sarcolemmal  $K^+$  currents was observed, we then tested the hypothesis that BMS-191095 is selectively opening mitochondrial  $K_{ATP}$ . We used two independent protocols to explore the pharmacology of BMS-191095 in mitochondrial  $K_{ATP}$ .

Figures 8 and 9 contain data from  $K^+$  flux measurements performed on proteoliposomes reconstituted with mitochondrial  $K_{ATP}$  and suspended in assay medium containing  $Mg^{2+}$  and ATP to inhibit the channel (Garlid et al., 1996). The data in Fig. 8 show that BMS-191095 is a potent opener of mitochondrial  $K_{ATP}$  ( $K_{1/2} = 83$  nM,  $n_H = 1$ ). The data in Fig. 9 show that the open state induced by BMS-191095 is inhibited by either glyburide ( $K_{1/2} = 77$  nM,  $n_H = 1$ ) or 5-HD ( $K_{1/2} = 83$   $\mu M$ ,  $n_H = 1$ ). Similar results were obtained in three independent experiments. To verify these reconstitution results, BMS-191095 was evaluated for its effects on matrix volume



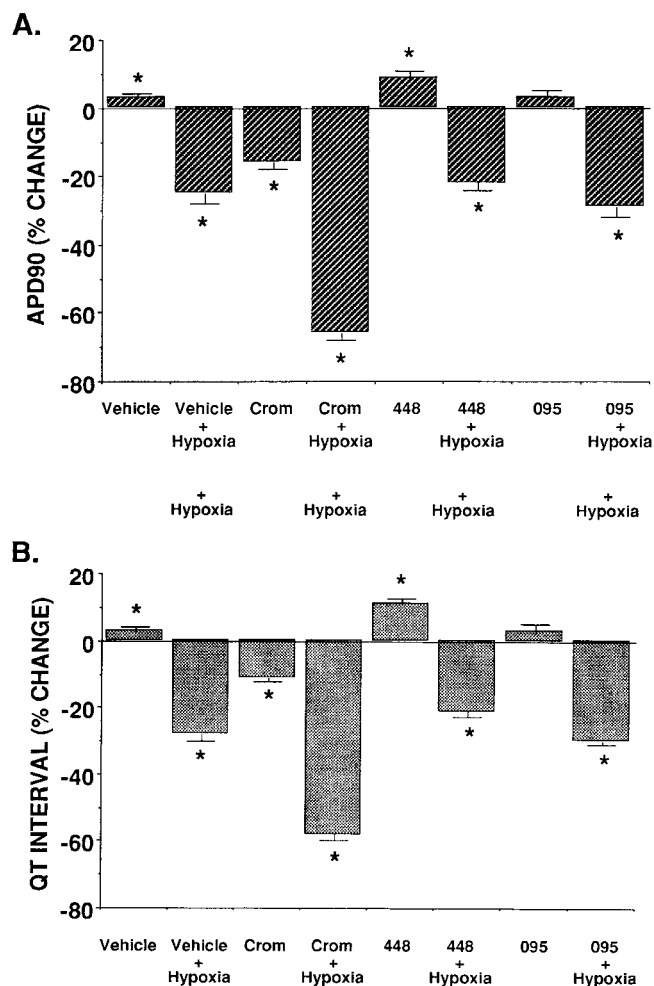
**Fig. 5.** Effects of vehicle, BMS-191095, or cromakalim on  $APD_{90}$  values recorded intracellularly from guinea pig papillary muscles. Measurements of  $APD_{90}$  were taken at control (C), postdrug (D), during 15 min of hypoxia, and 30 min of reoxygenation. Values were expressed as a percent change from control, and statistical significance was determined at the  $P < 0.05$  level and indicated with an asterisk. No change in  $APD_{90}$  was noted between vehicle and BMS-191095, whereas cromakalim produced significant shortening of  $APD_{90}$  following the drug.  $APD_{90}$  values further shortened during hypoxia and recovery were incomplete to post-drug levels.

of mitochondria (0.2 mg/ml) respiring in buffered KCl-succinate medium. Kinetic experiments gave results similar to those reported in Figs. 8 and 9 for the reconstituted channel (data not shown).

The trace in Fig. 10 shows the effect of BMS-191095 on mitochondrial steady-state volume during oxidative phosphorylation. Mitochondria were first brought to steady-state volume in  $K^+$  medium containing 0.5 mM ATP. Initiation of oxidative phosphorylation by addition of ADP caused a significant matrix contraction. This is caused by a normal depolarization under conditions of high electron flow, which perturbs the balance between diffusive  $K^+$  influx and  $K^+$  efflux via the mitochondrial  $K^+/H^+$  antiporter (Garlid et al., 1996). Matrix volume was partly restored after addition of 3  $\mu M$  BMS-191095, and this effect on matrix volume was reversed by 10  $\mu M$  glyburide. The trace in Fig. 10 is representative of four independent experiments, in which the extent of restoration of matrix volume induced by BMS-191095 ranged between 50 to 80%.

## Discussion

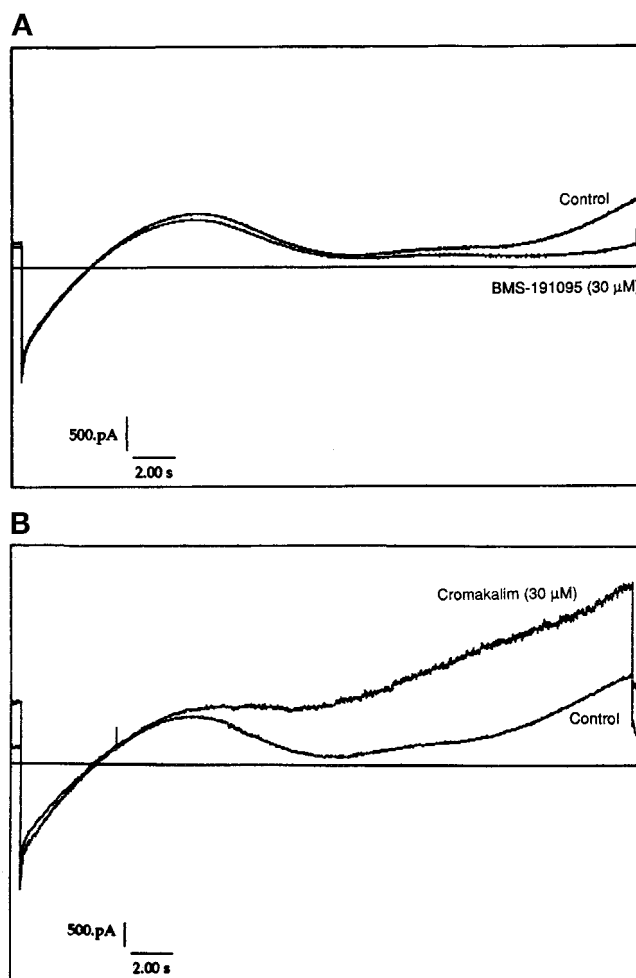
Pharmacologic openers of  $K_{ATP}$  have been shown by numerous investigators to exert protective effects in experimental models of myocardial ischemia and reperfusion (Aucham-



**Fig. 6.** The effects of vehicle (0.1% DMSO), cromakalim (CROM; 10  $\mu$ M), BMS-180448 (448; 10  $\mu$ M), or BMS-191095 (095; 6  $\mu$ M) on APD<sub>90</sub> values (A) recorded epicardially and QT intervals (B) taken from isolated perfused hearts are expressed as a percent change from control. Statistical significance was determined at the  $P < 0.05$  level and indicated with an asterisk. Reading were taken under normoxic and hypoxic conditions. BMS-191095 results were nearly identical to the vehicle group for both APD<sub>90</sub> and QT-interval values.

pach et al., 1991; Yao and Gross, 1994; Armstrong et al., 1995). The protective effects of these agents are characterized by conservation of ATP during ischemia, enhanced recovery of contractile function, and reduction of necrosis (Grover, 1994). The conservation of ATP is seen despite a lack of cardioplegic effects of K<sub>ATP</sub> openers (Grover and Sleph, 1995). The cardioprotective effect is also independent of vasodilator activity and is most likely due to a direct protective effect on myocytes (Armstrong et al., 1995). Interest in K<sub>ATP</sub> has been further heightened by the findings that preconditioning may be mediated by K<sub>ATP</sub> opening (Gross and Auchampach, 1992; Tomai et al., 1994).

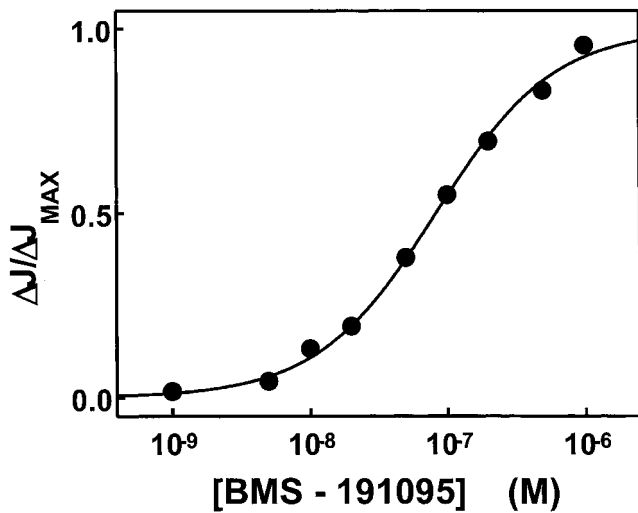
Since the protective effects of K<sub>ATP</sub> openers are exerted directly on ischemic myocardium, their vasodilator effects are undesirable. A recent study showed that the vasodilator activity of aprikalim caused toxic effects (cardiac lesions) in animal models (Belin et al., 1996). Potent hypotensive activity during acute myocardial ischemia could further compromise coronary blood flow. With this in mind, we performed detailed structure-activity studies to determine whether cardioprotective activity could be separated from vasodilator



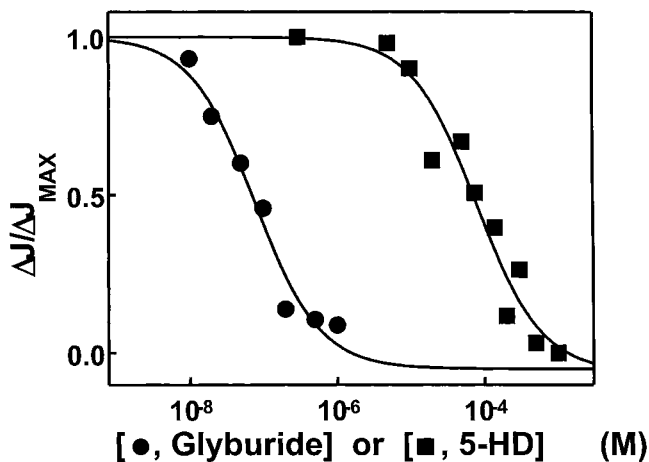
**Fig. 7.** Top panel, effect of BMS-191095 (30  $\mu$ M) on cell currents measured in isolated ventricular myocytes. Bottom panel, effect of cromakalim (30  $\mu$ M) on whole-cell currents. For both, currents were evoked by a slow voltage ramp from  $-100$  to  $+40$  mV. Holding potential =  $-40$  mV using the standard whole-cell (open patch) recording technique.

activity for K<sub>ATP</sub> openers and whether distinct structure-activity relationships exist for these two activities (Atwal et al., 1993; Rovnyak et al., 1997). Further investigation led to the discovery of BMS-180448, which is approximately 100-fold more selective for ischemic myocardium relative to vascular smooth muscle (D'Alonzo et al., 1995; Grover et al., 1995b). Although BMS-180448 could be successfully infused in vivo at cardioprotective doses without hemodynamic consequences, the separation between activities could have been greater (higher doses of this compound did cause hypotension). In this series of selective compounds, shortening of APD was also found to be weak, compared with cromakalim (Grover et al., 1995a). Further structure-activity studies revealed cardioprotective K<sub>ATP</sub> openers that are devoid of peripheral vasodilator activity (Rovnyak et al., 1997), including BMS-191095.

BMS-191095 exerted concentration-dependent cardioprotective effects in isolated rat hearts, which were not accompanied by reduced cardiac work. Increased time to onset of contracture, however, is suggestive of ATP conservation consistent with findings for other K<sub>ATP</sub> openers (McPherson et al., 1993; Grover, 1994). While being slightly more potent as a cardioprotectant relative to cromakalim, BMS-191095 was



**Fig. 8.** BMS-191095 activated ATP-inhibited  $K^+$  flux in the reconstituted cardiac mitochondrial system. The channel-active DEAE fraction was reconstituted into vesicles containing the fluorescent probe PBF1, and the  $K^+$  flux was monitored upon addition of 15  $\mu$ l of proteoliposomes into 2 ml of assay medium. This graph shows the dose response for BMS-191095 activation in the presence of 0.5 mM ATP. Linear regression of Hill plots of these data (not shown) yielded a Hill slope of 1 and a  $K_{1/2}$  of 83 nM.

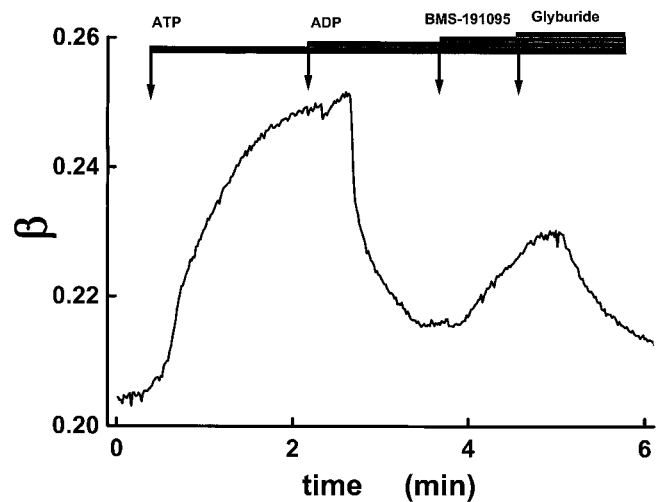


**Fig. 9.** The pharmacologically open state, with ATP (0.5 mM) and BMS-191095 (0.5 mM), was inhibited by either glyburide ( $K_{1/2} = 77$  nM,  $n_H = 1$ ) or 5-HD ( $K_{1/2} = 83$  nM,  $n_H = 1$ ).

devoid of peripheral vasorelaxant activity within the concentration range tested. Cromakalim was more potent as a vasorelaxant than as a cardioprotectant.

Although BMS-191095 is not a peripheral vasorelaxant, it retained some coronary dilator activity albeit significantly less, compared with cromakalim, suggesting that coronary arteries may respond differently to  $K_{ATP}$  openers. Although the data were not shown, carotid blood flow and femoral blood flow were not affected by BMS-191095, similar to results for BMS-180448 (Weselcouch and Gomoll, 1997).

Since earlier studies showed a lack of correlation between APD shortening and cardioprotection for  $K_{ATP}$  openers (Yao and Gross, 1994; Hamada et al., 1998), we examined this for BMS-191095. As we showed for BMS-180448 (Grover et al., 1995a; D'Alonzo et al., 1996), BMS-191095 was devoid of APD shortening activity within its cardioprotective concentration range. Cromakalim slightly shortened APD under normoxic conditions, but this activity was potentiated during



**Fig. 10.** Opening effect of BMS-191095 in intact mitochondria. Rat heart mitochondria (0.2 mg/ml) were assayed in  $K^+$  salts of 50 mM chloride, 10 mM succinate, 100  $\mu$ M EGTA, 5 mM inorganic phosphate, MOPS pH 7.2 buffer, and 500  $\mu$ M ATP + 1 mM  $MgCl_2$ . In the presence of ATP (control trace), the mitochondrial  $K_{ATP}$  channel was closed, but when BMS-191095 was added, the channel was fully opened. This pharmacologic open state was reversed by glyburide (6  $\mu$ M).  $\beta = P(A^{-1} - a)/P_s$ , where  $P$  is mitochondrial protein concentration (mg/ml) in the assay medium,  $A$  is the absorbance ( $\lambda = 520$  nm),  $a$  is a machine constant (equal to the y-intercept of a plot of  $A^{-1}$  versus  $P^{-1}$ ), and  $P_s$  is defined as 1 mg/ml and is a normalizing constant to make  $\beta$  a dimensionless quantity

hypoxia which is consistent with previous reports (Cole et al., 1991; D'Alonzo et al., 1992). In addition to APD studies, whole-cell myocyte patch-clamp studies showed a lack of effects for BMS-180448 (Grover et al., 1995b) and BMS-191095 on sarcolemmal channels.

Despite the lack of correlation between APD shortening, sarcolemmal  $K_{ATP}$  activity, and cardioprotection,  $K_{ATP}$  opening appears to be involved in the cardioprotective effects of BMS-191095. Glyburide abolished both the pre- and post-ischemic activity of BMS-191095, whereas 5-HD was selective for abolishing its cardioprotective actions, as seen previously (McCullough et al., 1991). Recent evidence suggests that 5-HD may be a selective mitochondrial  $K_{ATP}$  blocker, and this is consistent with its pharmacologic activities (Garlid et al., 1997). Glyburide had no effect on the cardioprotective action of diltiazem, confirming previously published data (Sargent et al., 1991).

$K_{ATP}$  are expressed in mitochondria (Inoue et al., 1991), and Garlid's laboratory showed that  $K_{ATP}$  openers such as cromakalim and diazoxide activate this channel (Garlid et al., 1996). Diazoxide opens mitochondrial  $K_{ATP}$  with low micromolar potency, while being relatively devoid of sarcolemmal  $K_{ATP}$  activity. Diazoxide was cardioprotective at 1 to 10  $\mu$ M, while having no effect on sarcolemmal  $K_{ATP}$  current (Garlid et al., 1997). Since BMS-191095 was devoid of APD shortening activity, we determined its effect on mitochondrial  $K_{ATP}$ . BMS-191095 opened mitochondrial  $K_{ATP}$  with a potency comparable with values previously published for diazoxide and cromakalim (Garlid et al., 1997). This mitochondrial  $K^+$  flux was inhibited by both 5-HD and glyburide. Therefore, the pharmacologic profile of BMS-191095 in reconstituted mitochondrial  $K_{ATP}$  is consistent with the profile in isolated hearts and intact animals. These data strongly suggest BMS-191095 is protecting ischemic myocardium through selective activation of mitochondrial  $K_{ATP}$ . Although



these data are consistent with the hypothesis that BMS-191095 protects through mitochondrial  $K_{ATP}$  activation, further work is still needed to prove this definitively.

BMS-191095 is devoid of peripheral vasodilator activity, which should increase its therapeutic window, compared with agents such as cromakalim. The lack of APD shortening by BMS-191095 may be advantageous because of a reduced propensity for reentrant arrhythmias. Our data also suggest that BMS-191095 may be a selective mitochondrial  $K_{ATP}$  opener. Cloning of various  $K_{ATP}$ s, suggest tissue heterogeneity that is consistent with pharmacologic data (Ashcroft, 1996; Inagaki et al., 1996; Wellman and Quayle, 1997). As we learn more about the regulation of mitochondrial  $K_{ATP}$ , the combination of pharmacologic and molecular tools may enable us to elucidate the cardioprotective mechanism of  $K_{ATP}$  openers. A clear understanding of the molecular mechanism for protection by  $K_{ATP}$  will enable us to develop structurally novel and selective agents for protecting ischemic myocardium.

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