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Ouabain protects rat hearts against ischemia-reperfusion injury via pathway involving src kinase, mitoK_{ATP}, and ROS

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Pasdois P, Quinlan CL, Rissa A, Tariosse L, Vinassa B, Costa AD, Pierre SV, Dos Santos P, Garlid KD. Ouabain protects rat hearts against ischemia-reperfusion injury via pathway involving src kinase, mitoK_{ATP}, and ROS. *Am J Physiol Heart Circ Physiol* 292: H1470–H1478, 2007. First published November 10, 2006; doi:10.1152/ajpheart.00877.2006.—We showed recently that mitochondrial ATP-dependent K^+ channel (mitoK_{ATP}) opening is required for the inotropic response to ouabain. Because mitoK_{ATP} opening is also required for most forms of cardioprotection, we investigated whether exposure to ouabain was cardioprotective. We also began to map the signaling pathways linking ouabain binding to Na^+-K^+-ATPase with the opening of mitoK_{ATP}. In Langendorff-perfused rat hearts, 10–80 μM ouabain given before the onset of ischemia resulted in cardioprotection against ischemia-reperfusion injury, as documented by an improved recovery of contractile function and a reduction of infarct size. In skinned cardiac fibers, a ouabain-induced protection of mitochondrial outer membrane integrity, adenine nucleotide compartmentation, and energy transfer efficiency was evidenced by a decreased release of cytochrome *c* and preserved half-saturation constant of respiration for ADP and adenine nucleotide translocase-mitochondrial creatine kinase coupling, respectively. Ouabain-induced positive inotropy was dose dependent over the range studied, whereas ouabain-induced cardioprotection was maximal at the lowest dose tested. Compared with bradykinin (BK)-induced preconditioning, ouabain was equally efficient. However, the two ligands clearly diverge in the intracellular steps leading to mitoK_{ATP} opening from their respective receptors. Thus BK-induced cardioprotection was blocked by inhibitors of cGMP-dependent protein kinase (PKG) or guanylyl cyclase (GC), whereas ouabain-induced protection was not blocked by either agent. Interestingly, however, ouabain-induced inotropy appears to require PKG and GC. Thus 5-hydroxydecanoate (a selective mitoK_{ATP} inhibitor), *N*-(2-mercaptopropionyl)glycine (MPG; a reactive oxygen species scavenger), ODQ (a GC inhibitor), PP2 (a src kinase inhibitor), and KT-5823 (a PKG inhibitor) abolished preconditioning by BK and blocked the inotropic response to ouabain. However, only PP2, 5-HD, and MPG blocked ouabain-induced cardioprotection.

Na^+-K^+-ATPase; inotropy; bradykinin; signaling pathway; reactive oxygen species

PRECONDITIONING THE HEART before prolonged ischemia results in protection against ischemia-reperfusion injury. Preconditioning begins with activation of cell surface receptors, which leads in turn to initiation of intracellular signal transduction pathways. These have been extensively studied and reviewed by Downey and coworkers (6, 11, 31) and by Gross and Gross (17). The signaling cascades lead to mitochondria, where they cause opening of mitochondrial ATP-dependent K^+ channel (mitoK_{ATP}) and subsequent release of reactive oxygen species (ROS) from mitochondria (2). Further downstream effects occur, including inhibition of the mitochondrial permeability transition and prevention of cellular necrosis (8).

Ouabain, a member of the oldest class of drugs used in the treatment of heart failure, has generally not been viewed as a potential cardioprotective agent. Indeed, Ishida et al. (19) used high concentrations of ouabain to induce Ca^{2+} overload in the heart and to show that the toxic effects of Ca^{2+} overload were blunted by the mitoK_{ATP} opener diazoxide. Nevertheless, several lines of evidence raised the possibility that ouabain may be cardioprotective in the therapeutic dose range. First, it has been known for several years that ouabain interaction with the Na^+-K^+-ATPase activates a cellular signaling cascade (reviewed in Ref. 47) that resembles the signaling cascade of cardioprotection (13). Second, we showed (41) that low concentrations of ouabain stimulated mitochondrial production of ROS in rat cardiac myocytes and that this ROS production was blocked by 5-hydroxydecanoate (5-HD). Third, we reported (15) that mitoK_{ATP} is required for increased contractility by various positive inotropic agents including ouabain, further suggesting that wired mechanisms link the activation of Na^+-K^+-ATPase signaling complex to mitoK_{ATP} opening. Because mitoK_{ATP} opening and increased mitochondrial ROS production are critical for cardioprotection, we decided to investigate whether ouabain is capable of preconditioning the isolated rat heart.

At first glance, the physiology of inotropy and the pathophysiology of cardioprotection appear to have little in common, but a closer analysis of mitochondrial bioenergetics revealed that mitoK_{ATP} opening should be critically important in both mechanisms. During both inotropic stimulation and ischemia, the mitochondrial membrane potential decreases. As a consequence, K^+ diffusion into the matrix decreases and the mitochondrial matrix contracts, resulting in an expansion of the mitochondrial intermembrane space (IMS). IMS expansion disrupts the coupling between the outer membrane voltage-dependent anion channel, the inner membrane adenine nucleotide translocase (ANT), and the mitochondrial creatine kinase (miCK), leading to increased outer membrane permeability to ADP and ATP and decreased efficiency of energy transfer (9, 10, 13). Thus energy transfer from mitochondria to cytosolic

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ATPases is impaired when it is most needed, whether during inotropic stimulation or reperfusion following ischemia. This effect is prevented by mitoK$_{ATP}$ opening, which prevents IMS expansion by adding a parallel K^+ conductance to compensate for the decrease in K^+ driving force (9, 10, 13).

The present study focuses on ouabain-induced cardioprotection and begins an investigation of the signaling pathway involved. We show that preconditioning with ouabain protects against infarction, protects heart function, and preserves adenine nucleotide compartmentation in mitochondria. We compared ouabain cardioprotection with the well-characterized cardioprotection by bradykinin (BK) and found, through the use of inhibitors, that both pathways depend on src kinase, mitoK$_{ATP}$, and ROS. We also observed important differences in the signaling pathways for ouabain cardioprotection. First, ouabain protection was not affected by inhibitors of cGMP-dependent protein kinase (PKG) or guanylyl cyclase, whereas BK protection was abolished by these agents. Second, cardioprotection was more sensitive to ouabain than was the inotropic response; that is, protection was observed at ouabain concentrations that induced little or no inotropic response. Third, whereas inhibitors of PKG or guanylyl cyclase had no effect on ouabain-induced cardioprotection, they abolished the inotropic response to ouabain. These divergences suggest that the intracellular signaling steps leading to mitoK$_{ATP}$ opening differ between ouabain and BK and, moreover, that ouabain-induced increases in contractility require an additional signaling pathway in parallel with that which opens mitoK$_{ATP}$.

A preliminary report of these findings has been presented in abstract form (37).

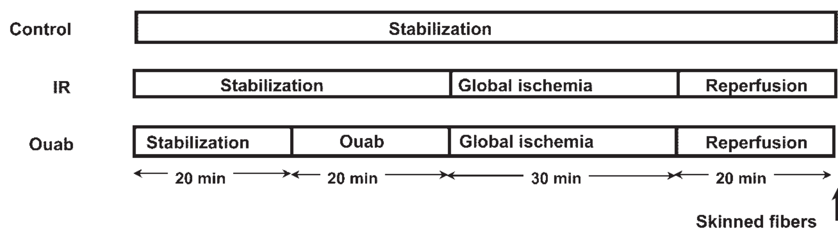
MATERIALS AND METHODS

Chemicals and reagents. 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), KT-5823 (KT), and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) were purchased from Calbiochem (San Diego, CA). BK, 5-HD, *N*-(2-mercaptopropionyl)-glycine (MPG), and all other reagents were all purchased from Sigma-Aldrich (St. Louis, MO). 8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (CPT-cGMP) was purchased from BIOLOG (Hayward, CA).

Langendorff perfusion. Different perfusion protocols were conducted in Bordeaux and Portland (Fig. 1). They differed slightly based on established practices in each laboratory and required adaptations for preparation and analysis of skinned fibers. The experimental protocols used in these studies were performed in compliance with the American Physiological Society's "Guiding Principles in the Care and Use of Animals" and were approved by the Institutional Animal Care and Use Committee at Portland State University.

Assessment of ouabain-induced cardioprotection and effects on mitochondrial function. These experiments were conducted in Bordeaux. Male Sprague-Dawley rats weighing 250–375 g were anesthetized with 40 mg of pentobarbital sodium injected intraperitoneally. The thorax was opened, and hearts were rapidly excised, immediately cooled in iced Krebs buffer, and perfused by an aortic cannula delivering warm (37°C) buffer at a constant pressure of 100 mmHg. Hearts were perfused with a modified Krebs-Henseleit solution containing (in mM) 118 NaCl, 5.9 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 0.5 EDTA, 25 $NaHCO_3$, and 16.7 glucose. The perfusate was gassed with 95% O_2-5% CO_2, which resulted in a P_{O_2} >600 mmHg at the level of the aortic cannula and a buffer pH of 7.4. The pulmonary artery was transected to facilitate coronary venous drainage, and a left ventricular polyethylene apical drain was inserted through a left atrial incision to allow thebesian venous drainage. Left ventricular pressure was monitored from a water-filled latex balloon placed through the left atrial appendage and connected to a Statham P23Db pressure transducer. The volume of the balloon was adjusted to obtain a left ventricular diastolic pressure around 7 mmHg and kept constant throughout the entire experiment. Hearts were not paced, and mechanical performance was evaluated as the product of heart rate and developed pressure (RPP).

Mitochondrial and heart function were assessed in five groups (n = 6 in each group) as described in *protocol A* in Fig. 1. The control group was perfused under aerobic conditions for 90 min. The ische-



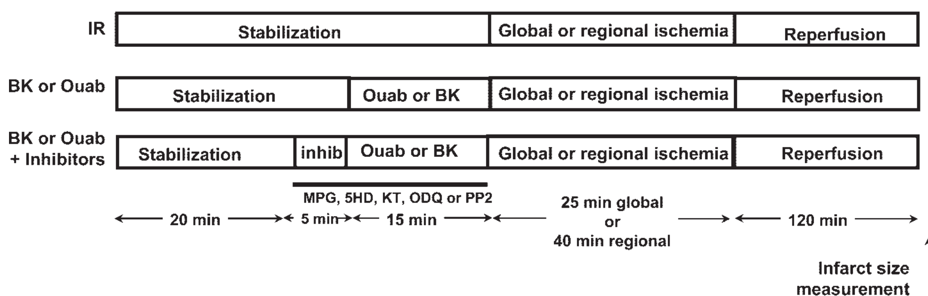


Fig. 1. Rat heart perfusion protocols. Timing of interventions is shown in relation to the zero-flow ischemia. *Protocol A* was used to characterize the protective effect of ouabain on heart function (see Fig. 2), in relation with mitochondrial respiratory function in skinned fibers prepared after 20-min reperfusion (see Figs. 4 and 5). Ouabain concentrations were 10, 20, and 80 μM. *Protocol B* was used to address the question of the role of mitochondrial ATP-dependent K^+ channel (mitoK$_{ATP}$) in ouabain's effect and to compare the mechanism of ouabain preconditioning to bradykinin (BK) preconditioning. After ischemia, all hearts were reperfused with standard Krebs-Henseleit solution for 120 min (see Figs. 3 and 6–8). Ouabain concentrations were 1 nM, 10 μM, 20 μM, 50 μM, 80 μM, and 1 mM. Bradykinin was used at 100 nM, 5-hydroxydecanoate (5-HD) at 300 μM, KT-5823 (KT) at 1 μM, ODQ at 2 μM, *N*-(2-mercaptopropionyl)glycine (MPG) at 1 mM, and PP2 at 1 μM. IR, ischemia-reperfusion group; Ouab, ouabain-treated group.

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mia-reperfusion (IR) group was allowed to stabilize under aerobic conditions over 40 min before a 30-min zero-flow global ischemia period followed by 20 min of reperfusion. The global zero-flow ischemia model was chosen in this case because it is more adapted to the assessment of left ventricular functional alterations and preparation of skinned fibers. Ouabain-treated (Ouab) groups were allowed to stabilize under aerobic condition over 20 min, before 20-min perfusion with a buffer containing ouabain. This was followed by 30-min zero-flow ischemia and 20-min reperfusion without ouabain. Mitochondrial function was assessed on permeabilized fibers of left ventricle obtained immediately at the end of the 20 min of reperfusion. Protocols for their preparation and assay have been extensively described and discussed in earlier studies (9, 15, 43). After incubation with saponin to permeabilize the muscle, fibers were washed in buffer to remove adenine nucleotides, phosphocreatine, and saponin. The oxygen consumption of skinned fibers (0.5–0.75 mg dry wt) was measured polarographically at 25°C with a Clark-type oxygen electrode (Oroboros oxygraph, Paar, Graz, Austria). Data were recorded at sampling intervals of 1 s (Datlab Acquisition Software, Oroboros, Innsbruck, Austria).

To determine control of respiration by ADP and creatine, oxygen consumption of skinned fibers was measured in medium containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA (pCa = 7), 1.38 $MgCl_2$, 0.5 DTT, 100 MES, 20 imidazole, 20 taurine, 3 KH_2PO_4, 10 pyruvate, and 5 malate. pH was adjusted to 7.1 with 10 M KOH at 25°C, and 2 mg/ml of bovine serum albumin was added. To determine the half-saturation constant of respiration for ADP ($K_{1/2}^{ADP}$), the dependence of respiration on ADP concentration in the presence and absence of 20 mM exogenous creatine was calculated by nonlinear regression (Kaleidagraph software).

To examine permeability of the outer mitochondrial membrane to cytochrome *c* after ischemia-reperfusion, respiration was measured in 2 ml of KCl buffer containing (in mM) 125 KCl, 20 HEPES, 10 pyruvate, 5 malate, 3 Mg acetate, 5 KH_2PO_4, 0.4 EGTA, and 0.3 DTT. pH was adjusted to 7.1 with KOH 10 M at 25°C, and 2 mg/ml of bovine serum albumin was added. Respiration was stimulated by the addition of ADP at a final concentration of 1 mM, which induced a maximal activation. Cytochrome *c* was then added at a final concentration of 10 μM to test the intactness of the outer mitochondrial membrane.

Infarct size (IS) was determined in five groups of four to six hearts as described in *protocol B* in Fig. 1. The IR group was allowed to stabilize under aerobic conditions over 40 min before a 40-min regional zero-flow ischemia period followed by 120 min of reperfusion. Ouab groups were allowed to stabilize under aerobic conditions over 20 min, before 15-min perfusion with a buffer containing ouabain. This was followed by 40-min zero-flow ischemia and 120-min reperfusion without ouabain. Regional ischemia was achieved by occlusion of the left anterior descending coronary artery. The model of regional zero-flow ischemia was chosen here to mimic the coronary artery occlusion encountered in the clinical setting at the acute phase of myocardial infarction. The coronary artery was reoccluded at the end of reperfusion, and the area at risk (AAR) was determined by negative staining after perfusion of phthalocyanin blue (SP Chaux, Matière & Couleurs, Bordeaux, France). This allowed for the delineation of the normal, aerobically perfused area (stained blue) versus the AAR (not stained). The heart was then removed from the perfusion apparatus, rinsed of excess blue dye, trimmed of right ventricle and atrial tissue, and cut into six cross-sectional pieces. Slices of the left ventricle were incubated without agitation in 1% (wt/vol) triphenyltetrazolium chloride (TTC) solution for 12 min at 37°C and pH 7.4. TTC stains the viable tissue a brick red color, which allows the discrimination between viable (red) and nonviable (pale yellow) tissue. The samples were then fixed in 3.5% (wt/vol) formalin solution for 24 h at 4°C and weighed. Both sides of each slice were photographed. The areas of each region, delineated with SigmaScan Pro software, were averaged from the photographs of each side for each slice and multiplied by the weight of the slice. IS was finally expressed both as a percentage of total left ventricular mass and as a percentage of AAR.

Comparison of ouabain- and BK-induced cardioprotection. These experiments were conducted in Portland. Perfusion conditions were identical to those in Bordeaux with slight modification in the perfusion buffer, containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.7 $CaCl_2$, 10 glucose, and 1.2 pyruvate, gassed with 95% O_2-5% CO_2. Ten groups of hearts were studied (n = 3–6 in each group) as described in Fig. 1*B*. After stabilization and pretreatment, all groups were exposed to 25-min zero-flow global ischemia followed by 120 min of reperfusion with standard Krebs solution. The IR group was allowed to stabilize under aerobic conditions for 40 min. Before ischemia, the ouabain- and BK-treated groups were allowed to stabilize under aerobic conditions for 25 min, followed by 15-min perfusion with a buffer containing 50 μM ouabain or 100 nM BK, respectively. Where used, ODQ (2 μM), 5-HD (300 μM), KT-5823 (1 μM), MPG (1 mM), or PP2 (1 μM) was added to the perfusate 5 min before ouabain or BK and included in the perfusate during the subsequent 15-min perfusion with ouabain or BK. In the experiments with the PKG activator, CPT-cGMP was added simultaneously with ODQ 5 min before the addition of ouabain.

The hearts in each group were reperfused for 120 min, after which IS was estimated by the method of Ytrehus et al. (49). Fifteen milliliters of 1% (wt/vol) TTC in phosphate-buffered saline pH 7.4 at 37°C was infused into the coronary circulation at a rate of 0.5 $ml \cdot g^{-1} \cdot min^{-1}$. The eluted stain from the cardiac veins was collected and recirculated. After ~15 min of perfusion the epicardial surface was deep red. Hearts were then removed from the cannula and fixed overnight in 10% formalin. Hearts were removed from formalin and sectioned along the atrioventricular plane into ~1-mm sections. Sections were placed between two microscope slides, computerized area analysis was performed with Scion image, and IS of each heart was expressed as a fraction of the total AAR. IS for each heart was determined by averaging the infarct area of five or six sections. The RPP data shown in Fig. 8 were calculated with the average RPP measured during the 15-min drug treatment for each experimental condition (Fig. 1, *protocol B*).

RESULTS

Effects of ouabain concentration on cardiac function. Hemodynamic data shown in Fig. 2 were obtained in experimental conditions described in Fig. 1, *protocol A*. As shown in Fig. 2*A*, the control group was hemodynamically stable, with $<$12% decline in RPP over the 90-min perfusion period. The average control RPP was 33,800 ± 1,200 mmHg·min. Ischemia resulted in immediate cardiac arrest. On reperfusion, a limited recovery of systolic function was observed, with maximal RPP values at the end of the reperfusion period corresponding to 17% of control values. As shown in Fig. 2*B*, diastolic pressure remained constant in the control group. In the IR group, ischemic contracture was observed after 15 min of ischemia with a maximum of 24 ± 4 mmHg (Fig. 2*B*; $P <$ 0.01 vs. control). Overall, these data show the early occurrence of contracture during ischemia and a poor early recovery of systolic function after reperfusion.

Perfusion of ouabain induced a concentration-dependent increase in RPP (Fig. 2*A*), with the maximum increase (57%) being achieved with 80 μM ouabain. The inotropic effect was due exclusively to an increase in developed pressure with no significant associated change in heart rate or left ventricular diastolic pressure (not shown). All three doses of ouabain prevented ischemic contracture during index ischemia (Fig.

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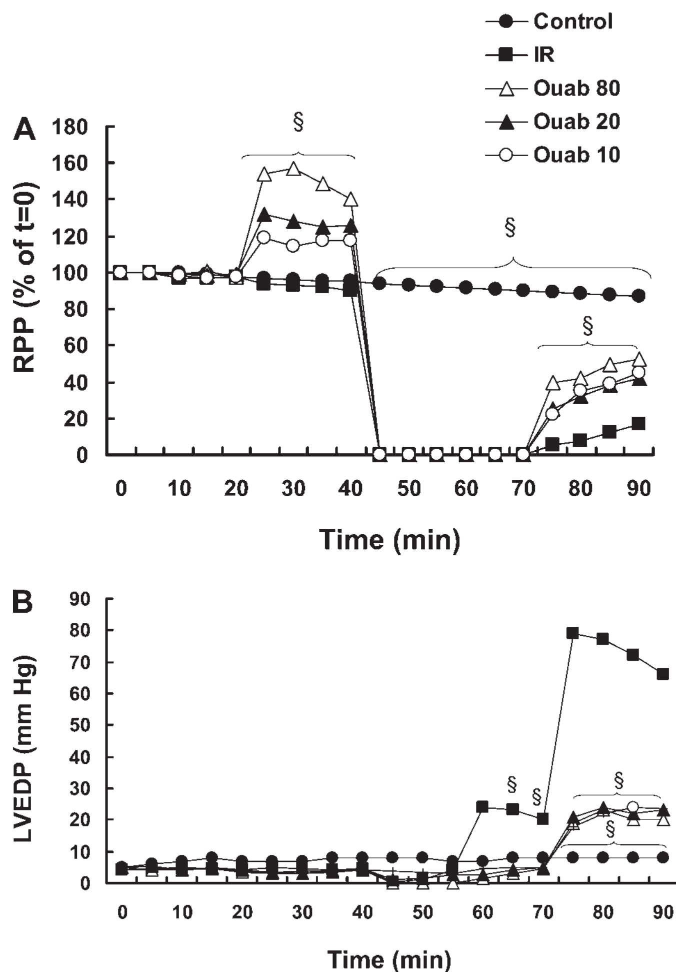

Fig. 2. Effects of ouabain on left ventricular function. Rate-pressure product (RPP; *A*) and left ventricular end-diastolic pressure (LVEDP; *B*) obtained from control, IR, Ouab 10, Ouab 20, and Ouab 80 groups of Langendorff-perfused rat hearts are shown. See MATERIALS AND METHODS and *protocol A* in Fig. 1 for details on perfusion protocols. Values are means of 6 separate experiments. §$P < 0.05$ vs. IR group.

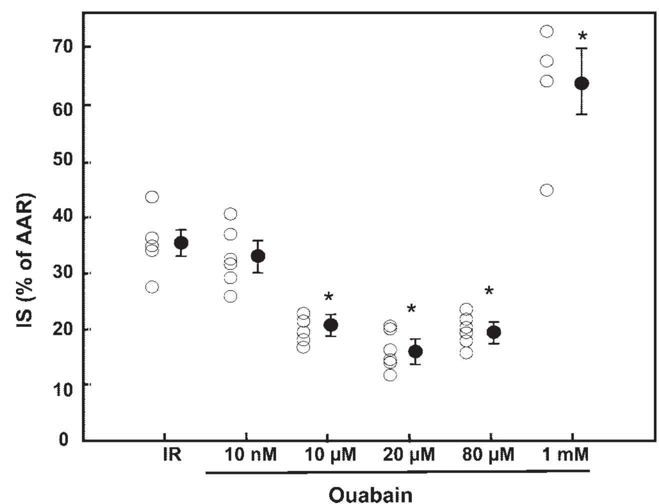

Fig. 3. Effect of ouabain dose on infarct size (IS). IS expressed as % of the area at risk (AAR) in Langendorff-perfused rat hearts subjected to 40-min regional ischemia followed by 120-min reperfusion as described in MATERIALS AND METHODS and *protocol B* in Fig. 1 is shown. *$P < 0.05$ vs. IR group.

2*B*) and significantly improved functional recovery on reperfusion (Fig. 2*A*).

Effects of ouabain on IS. The AAR was identical in each group (Fig. 1, *protocol B*) and averaged 28 ± 2% of left ventricular mass. As shown in Fig. 3, 40-min ischemia followed by 120-min reperfusion resulted in an infarction of 29.8 ± 2.5% of the AAR. Fifteen-minute perfusion of 10, 20, or 80 μM ouabain immediately before ischemia significantly decreased the infarct size to 12 ± 1.7%, 7.4 ± 2.1%, and 11 ± 1.8%, respectively ($P < 0.05$ vs. IR group). Perfusion of 10 nM ouabain did not afford any protection, whereas perfusion of 1 mM ouabain exerted a toxic effect as evidenced by the significant increase in IS to 62 ± 6.4% % of the AAR.

Effects of ouabain on mitochondrial function. Figure 4 contains results from assays of cytochrome *c* release (see MATERIALS AND METHODS). In the control group, maximal respiration was 26 ± 2 nmol $O_2 \cdot min^{-1} \cdot$mg dry wt^{-1}, and addition of cytochrome *c* did not induce any stimulation. After 30-min ischemia followed by 20-min reperfusion, maximal respiration was decreased by 16% compared with control ($P < 0.05$). In these fibers, the addition of exogenous cytochrome *c* accelerated respiration up to the value measured in the control group. These data reflect alterations at the level of the outer mitochondrial membrane leading to a loss of endogenous cytochrome *c*. It should be noted that ischemia followed by reperfusion had no effect on state 2 respiration rates. In the Ouab groups, maximal respiration was not significantly decreased, and addition of cytochrome *c* did not produce any stimulation. These data indicate that ouabain protected against cytochrome *c* loss from mitochondria.

The data in Fig. 5 contain $K_{1/2}^{ADP}$ values, determined in the presence or absence of creatine (Cr), as described in MATERIALS AND METHODS. In fibers from control hearts, the $K_{1/2}^{ADP}$ value was 282 ± 10 μM. This high value reflects restriction of perme-

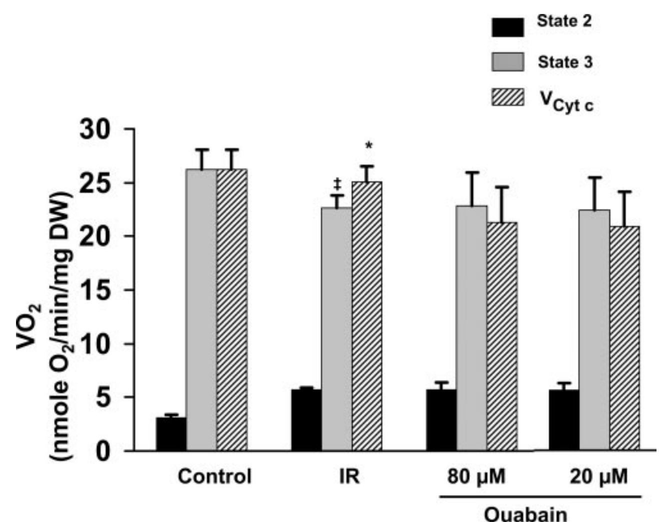

Fig. 4. Effect of ouabain on mitochondrial respiration in skinned fibers. Shown are respiration values measured in KCl medium in the absence of ADP (filled bars, state 2 respiration), in the presence of 2 mM ADP (gray bars, state 3 respiration), and in the presence of 2 mM ADP + 10 μM cytochrome *c* (hatched bars). See MATERIALS AND METHODS and *protocol A* in Fig. 1 for details on perfusion protocols. ‡$P < 0.05$ vs. Control group; *$P < 0.05$ vs. state 3 IR. DW, dry weight. V_{cyt}, maximal respiratory rate in the presence of cytochrome *c*.

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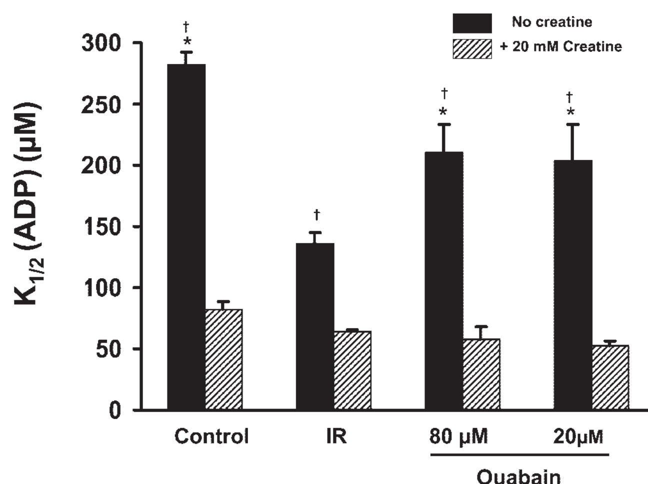

Fig. 5. Effect of ouabain on control of outer membrane permeability to ADP. Half-saturation constant of respiration for ADP ($K_{1/2}^{ADP}$) was assessed as a measure of outer membrane permeability to ADP in the presence or absence of 20 mM creatine. Respiration was evaluated in permeabilized fibers prepared from the left ventricle of isolated rat hearts, as described in MATERIALS AND METHODS and *protocol A* in Fig 1. *$P < 0.05$ vs. IR, †$P < 0.05$ vs. in presence of creatine.

ability to ADP at the level of the outer mitochondrial membrane. In the presence of Cr, this value decreased to 82 ± 7 μM. As previously discussed, the Cr-induced decrease in $K_{1/2}^{ADP}$ reflects functional coupling between ANT and miCK (39). In fibers from the IR group, $K_{1/2}^{ADP}$ was dramatically decreased to 135 ± 8 μM ($P < 0.05$ vs. control) and decreased to 64 ± 2 μM in the presence of Cr. In contrast, $K_{1/2}^{ADP}$ in fibers from the ouabain-treated hearts remained high, with values of 210 ± 23 μM (80 μM ouabain) and 204 ± 29 μM (20 μM ouabain). These values are significantly higher than the value measured in fibers from the IR group, which reflects the preservation of the low permeability of the outer mitochondrial membrane for ADP (9). Fibers obtained from the Ouab 10 group showed similar effects (data not shown). Addition of Cr resulted in a decrease in $K_{1/2}^{ADP}$ to 57 ± 10 and 53 ± 4 μM, respectively, in fibers from the ouabain-treated hearts, reflecting the preservation of functional coupling of ANT and miCK, as observed in the control group.

Ouabain versus BK in cardioprotection. Cardioprotection by ouabain is a newly described phenomenon. We decided to begin an investigation of the signaling pathways involved in ouabain protection by comparing it with the well-characterized effects of BK. BK and ouabain treatment caused improved postischemic functional recovery (Fig. 6) as well as reduced IS (Fig. 7). As shown by Krieg et al. (23, 25), cardioprotection by BK involves src kinase, endothelial nitric oxide synthase, guanylyl cyclase, PKG, mitoK$_{ATP}$, and ROS. Participation of these elements is confirmed by the data in Figs. 6 and 7, in which BK protection against cardiac dysfunction (Fig. 6) and infarction (Fig. 7) is shown to be abolished by PP2, a src kinase inhibitor (18), ODQ, a guanylyl cyclase inhibitor (16), KT-5823, a PKG inhibitor (21), 5-HD, a mitoK$_{ATP}$ blocker (20), and MPG, a free radical scavenger.

Ouabain protection against postischemic cardiac dysfunction (Fig. 6) and IS (Fig. 7) was also abolished by PP2, 5-HD, and MPG, confirming participation of src, mitoK$_{ATP}$, and ROS in ouabain cardioprotection. Interestingly, however, ouabain protection was not abolished by ODQ or KT-5823, suggesting that signaling after administration of ouabain and BK follows different pathways from the plasma membrane to mitochondria.

Ouabain protection versus ouabain-induced inotropy. Although this study was designed primarily to address cardioprotection by ouabain, we were also struck by the effects of drugs on the positive inotropic response to ouabain, which were observed in the preischemic phase (see Fig. 6*A*). As shown in Fig. 8, 5-HD blocked the inotropic response, as shown previously (15). Additionally, the inotropic response to ouabain was blunted by MPG, PP2, KT-5823, and ODQ. These data suggested that the positive inotropic effect of ouabain was mediated by PKG. To test this, we perfused with the cell-permeant cGMP CPT-cGMP in addition to ODQ and ouabain. This reinstated the positive inotropic effect of ouabain that had been inhibited by ODQ, confirming the requirement for PKG in ouabain-induced inotropy.

DISCUSSION

Digitalis-like compounds have long been used in the treatment of heart failure for their inotropic effects. We recently demonstrated (15) that mitoK$_{ATP}$ opening is necessary for the positive inotropic response, including that caused by ouabain. The implication of this result is that ouabain, like ischemic preconditioning (IPC), causes mitoK$_{ATP}$ opening by triggering an intracellular signaling pathway. Because mitoK$_{ATP}$ opening is cardioprotective, we decided to investigate whether ouabain itself is cardioprotective and to begin an investigation of its signaling pathway. The data in Figs. 2–5 show that perfusion of ouabain, in the micromolar range, before ischemia-reperfusion protects against infarction, protects heart function, and preserves adenine nucleotide compartmentation in mitochondria. In this regard, treatment with ouabain reproduces the effects of diazoxide and of IPC previously described (9, 26). Ouabain in the nanomolar range did not induce reduction in IS. This is in line with experiments performed on myocytes showing that ouabain in the nanomolar range does not block, but activates, cardiac Na^+/K^+ pumps (12). On the other hand, ouabain in the millimolar range induced a toxic effect as evidenced by the significant increase in IS shown in Fig. 3. This might be explained by the damaging effect of increased mitochondrial Ca^{2+} overload in this condition (19).

All or most cardioprotective signaling pathways rely on src kinase (5, 24, 44), mitoK$_{ATP}$ (14, 33, 46), and ROS (3, 36, 42, 45), and it is clear from the results in Figs. 6 and 7 that ouabain cardioprotection exhibits the same requirements. Thus the src kinase inhibitor PP2, the mitoK$_{ATP}$ blocker 5-HD, and the ROS scavenger MPG each block protection against contractile failure and infarction after ischemia-reperfusion injury. In these respects, the effects of ouabain were the same as the effects of BK, compared in Figs. 6 and 7.

It is most interesting, however, that the signaling pathways for the two ligands differ in important respects. Thus BK protection depends on PKG and guanylyl cyclase (35), whereas ouabain protection does not. This is manifested in the differential response to KT-5823 and ODQ, inhibitors of PKG and guanylyl cyclase, respectively, and the effect is seen in both functional recovery and protection against infarction (Figs. 6 and 7). Addition of PKG plus cGMP to isolated mitochondria

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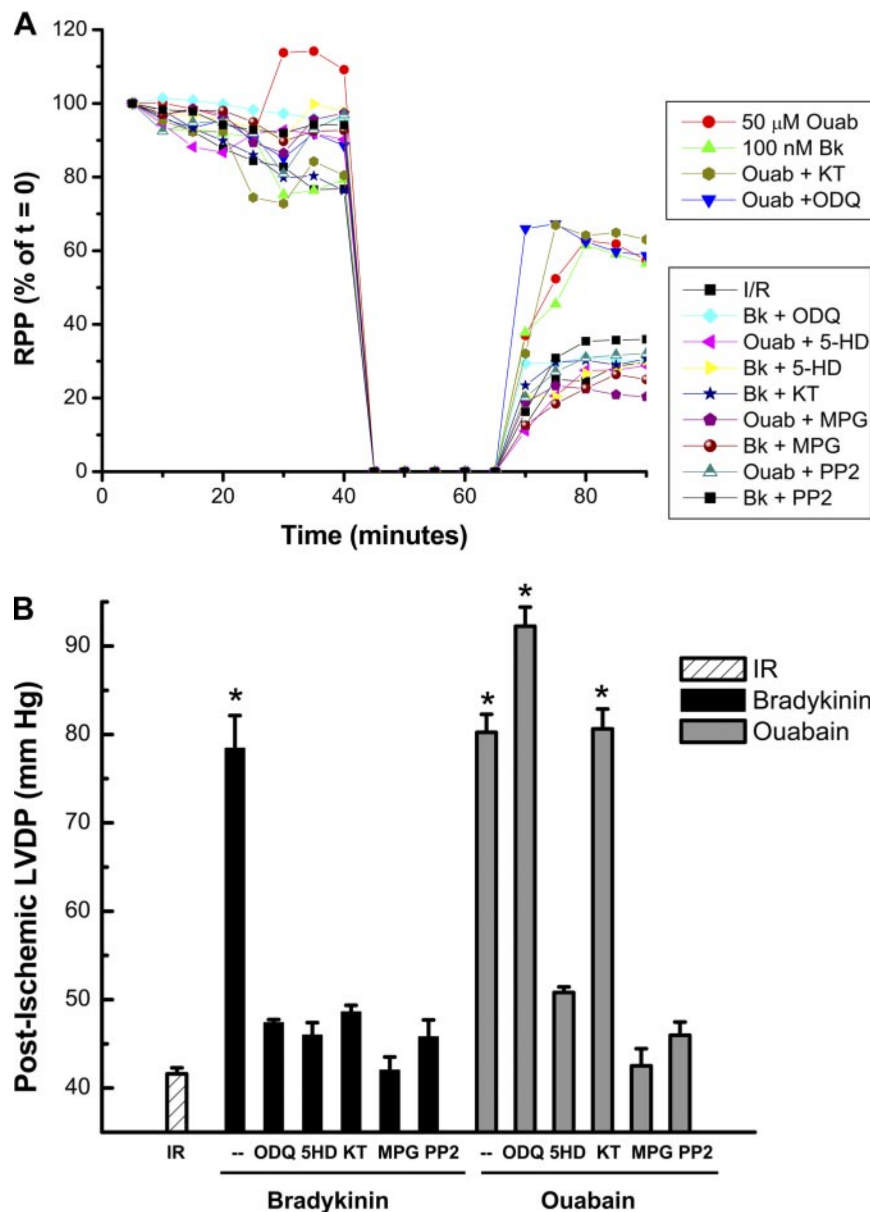

Fig. 6. Effects of ouabain vs. bradykinin on left ventricular function. *A*: functional cardiac recovery expressed as RPP. *B*: postischemic left ventricular developed pressure (LVDP) plotted as the average pressure (mmHg) 30 min after global ischemia, with error bars. See MATERIALS AND METHODS and *protocol B* of Fig. 1 for details on perfusion protocols. Bradykinin (100 nM) protection was blocked by 300 μM 5-HD, 1 μM KT-5823, 1 mM MPG, and 2 μM ODQ. Neither ODQ nor KT-5823 blocked 50 μM ouabain-afforded cardioprotection, but 5-HD, MPG, and PP2 blocked functional recovery. $n \geq 4$ in all trials. *$P < 0.05$ vs. control.

is sufficient to open mitoK$_{ATP}$ (7); however, these ligands appear not to be involved in the ouabain cardioprotective pathway, so there must be more than one mechanism to signal mitoK$_{ATP}$ opening at the level of the mitochondrion. We infer from these studies that mitoK$_{ATP}$ opening is necessary and sufficient for cardioprotection by ouabain and BK.

Another original finding of this study is that KT-5823 and ODQ inhibit the inotropic effect of ouabain (Fig. 8), in contrast to their lack of effect on protection. This indicates that guanylyl cyclase and PKG are required for the inotropic response to ouabain. We showed that ouabain causes mitoK$_{ATP}$ opening and mitochondrial ROS production (41) and, moreover, that mitoK$_{ATP}$ opening is necessary for the inotropic response (15). From these results, we may conclude that mitoK$_{ATP}$ opening is necessary, but not sufficient, for the inotropic response.

On the basis of these findings and those from other studies, we infer that ouabain activates two different signaling platforms, as described in Fig. 9. One platform is the "mitoK$_{ATP}$-ROS platform" (2), which is activated by low concentrations of ouabain, as detected by mitoK$_{ATP}$-dependent cardioprotection. The other is a "Ca^{2+}-regulating platform", such as has been observed in renal cells (29, 50), which is activated by higher concentrations of ouabain, as detected by the concentration dependence of cardiac contractility. The data presented here show that cardioprotection requires only the mitoK$_{ATP}$-ROS signaling platform, whereas the inotropic response requires both platforms. It has been known for several years that the signaling pathway induced by ouabain through cardiac Na^+-K^+-ATPase relies on two intracellular mediators: Ca^{2+} and ROS. Removal of Ca^{2+} from the medium inhibits the ouabain-induced increase in intracellular Ca^{2+} in myocytes but does not affect ouabain-induced ROS production (28), which is mediated by mitoK$_{ATP}$ (41). Thus the two pathways exhibit a certain degree of independence. However, disruption of an up-

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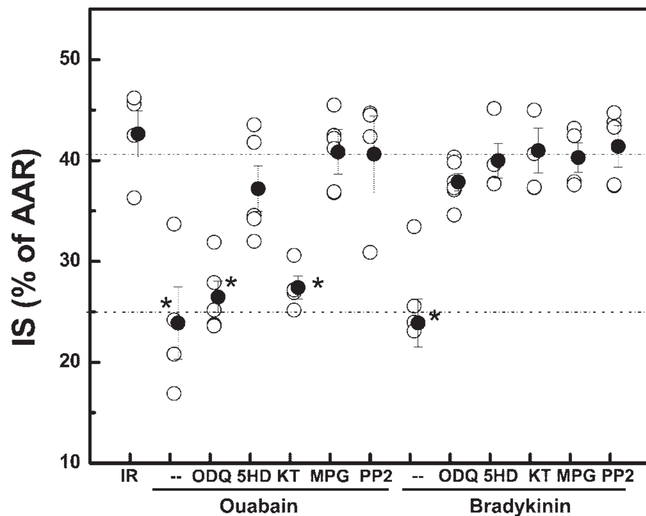

Fig. 7. Differential effects of agents on ouabain vs. bradykinin cardioprotection. IS is presented as % of risk zone in isolated adult rat hearts subjected to 25-min global ischemia and 120-min reperfusion. See MATERIALS AND METHODS and *protocol B* of Fig. 1 for details on perfusion protocols. Individual hearts (○) and group means (●) with SE bars are shown. Hearts treated with 100 nM bradykinin or 50 μM ouabain had significantly smaller IS than untreated IR hearts. In bradykinin-treated hearts, this effect was blocked by all inhibitors tested: 300 μM 5-HD, 1 μM KT-5823, 2 μM ODQ, 1 mM MPG, and 1 μM PP2. In ouabain-treated hearts protection was not blocked by ODQ or KT-5823 but was blocked by 5-HD, MPG, and PP2. $n \geq 4$ in all trials. $^*P < 0.05$ vs. control.

stream component of the cascade, such as the Na^+-K^+-ATPase/c-Src binary receptor (through inhibition of c-Src, for example), prevents ouabain-induced increases in both

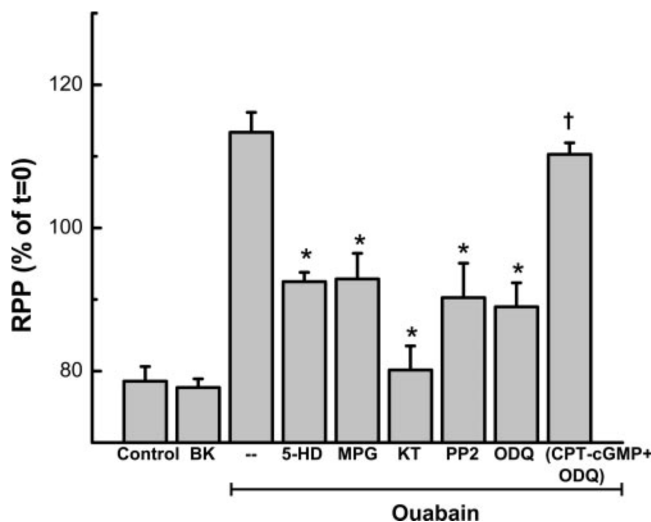

Fig. 8. Differential effects of ODQ and KT-5823 on ouabain-induced inotropy. The inotropic response of the hearts is expressed as the average of RPP during the 15-min drug treatment. Data have been normalized to the initial RPP at $t = 0$. Bradykinin does not have a positive inotropic effect; the positive inotropic response induced by 50 μM ouabain treatment was abolished by 300 μM 5-HD, 2 μM ODQ, 1 mM MPG, 1 μM KT-5823, and 1 μM PP2. The ODQ-inhibited positive inotropy was restored by addition of the PKG activator 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (CPT-cGMP; 10 μM) when added simultaneously with ODQ 5 min before the addition of ouabain, as described in MATERIALS AND METHODS and Fig. 1, *protocol B*. $^*P < 0.05$ compared with ouabain, $^†P < 0.05$ compared with ODQ-inhibited ouabain.

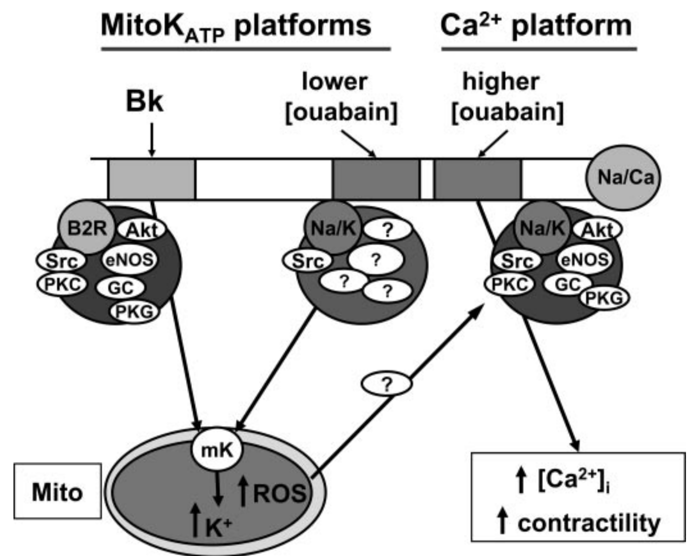

Fig. 9. Proposed model of bradykinin and ouabain signaling in the heart. The signaling platforms associated with the bradykinin receptor (B2R) and the ouabain receptor (Na^+-K^+-ATPase, Na/K) are shown. We propose that bradykinin and ouabain induce formation of 2 different mitoK$_{ATP}$ signaling platforms that cause mitoK$_{ATP}$ (MK) opening, an increase in mitochondrial ROS production, and cardioprotection. The bradykinin mitoK$_{ATP}$ platform includes Src, phosphatidylinositol 3-kinase, Akt, endothelial nitric oxide synthase (eNOS), PKC, guanylate cyclase (GC), and cGMP-dependent protein kinase (PKG). The ouabain mitoK$_{ATP}$ platform also relies on Src activation for its formation, but little is known about the downstream components. In addition, and at higher doses, ouabain induces a Ca^{2+} platform that mediates the positive inotropic response. Interestingly, the ouabain-dependent Ca^{2+} platform requires ROS for activation and appears to contain the same components as the bradykinin-dependent mitoK$_{ATP}$ platform. $[Ca^{2+}]_i$, intracellular Ca2+ concentration.

Ca^{2+} and ROS, and, as shown here, prevents ouabain-induced inotropy and cardioprotection.

It appears that the common element connecting the two pathways is ROS, since mitoK$_{ATP}$-dependent ROS production is required for both cardioprotection and inotropy (15). Although earlier work by Xie and coworkers (40) found no effect of *N*-acetylcysteine on contractility, a recent study from the same group (22) found that ouabain-induced prolongation of the Ca^{2+} transient and increased contractility were blocked by *N*-acetylcysteine, and these effects were mimicked by incubation with 10 μM H_2O_2. ROS are known to inhibit or activate most of the Ca^{2+} channels and transporters of the heart [reviewed by Zima and Blatter (51)]; however, the levels of H_2O_2 achieved by mitoK$_{ATP}$ are unlikely to reach 10 μM. Moreover, a direct role of ROS in inotropy cannot account for the participation of guanylyl cyclase and PKG in the inotropic pathway, which implies a role for phosphorylation, either of a Ca^{2+} transporter or of the contractile mechanism (32). PKCs are activated by H_2O_2 (8) and are an essential component of the Ca^{2+}-regulating platform (30), and it is possible that mitoK$_{ATP}$-dependent H_2O_2 serves the role of activating a PKC in the system. Noland et al. (32) showed that a PKC phosphorylates troponin I to sensitize the contractile system to Ca^{2+}, and Ogbi et al. (34) showed that PMA-activated PKC-ϵ increased contractile amplitude and increased the quiescent period between contractions in neonatal cardiomyocytes. In summary, our results show that the mitoK$_{ATP}$-ROS platform is activated at low concentrations of ouabain that do not increase

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contractility. Although the precise role of increased ROS in this system remains to be established, it may be required to activate a step within the Ca^{2+}-regulating platform, which then moves to increase cytosolic Ca^{2+} or Ca^{2+} sensitivity and thereby initiate the inotropic response.

It is unclear at present how these mechanisms fit into the more established view of ouabain's mechanism of action, in which inhibition of Na-K-ATPase leads to elevation of intracellular Na^+ concentration, which causes reduced Ca^{2+} extrusion and, possibly, increased Ca^{2+} influx via the Na^+/Ca^{2+} exchanger (NCX) (4). Thus Philipson and coworkers (38) found that NCX activity is essential for the action of cardiac glycosides, and Bers and coworkers (1) found that a Na^+ gradient and a functional NCX are essential for glycoside-induced inotropy in cardiac myocytes. Our finding here that ouabain-induced inotropy is blocked by ROS scavengers and inhibitors of Src, guanylyl cyclase, and PKG points to an essential role of cell signaling in the inotropic response to ouabain. These two sets of requirements are not, of course, mutually exclusive; however, it remains for future work to determine how they are interconnected.

In summary, we have shown that ouabain is cardioprotective in the perfused rat heart model. The cardioprotective signaling pathway triggered by ouabain leads to mitoK$_{ATP}$ opening and mitochondrial ROS production, and this pathway differs from that triggered by BK in that guanylyl cyclase and PKG are not involved in ouabain cardioprotection. The mitoK$_{ATP}$-ROS pathway is also necessary, but not sufficient, for the inotropic response, and we hypothesize that a second signaling pathway involving the Ca^{2+}-regulating platform is also necessary for inotropy. These new features of ouabain signaling via the Na^+-K^+-ATPase clearly require additional investigation. Further investigation is also necessary to reveal whether the preconditioning effect of ouabain could explain, at least in part, why it remains more beneficial for the treatment of heart failure patients than the other drugs of the inotropic class (27, 48).

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