

Ouabain triggers preconditioning through activation of the Na^+, K^+ -ATPase signaling cascade in rat hearts

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Abstract

Objective: Because ouabain activates several pathways that are critical to cardioprotective mechanisms such as ischemic preconditioning, we tested if this digitalis compound could protect the heart against ischemia–reperfusion injury through activation of the Na^+, K^+ -ATPase/c-Src receptor complex.

Methods and results: In Langendorff-perfused rat hearts, a short (4 min) administration of ouabain 10 μM followed by an 8-minute washout before 30 min of global ischemia and reperfusion improved cardiac function, decreased lactate dehydrogenase release and reduced infarct size by 40%. Western blot analysis revealed that ouabain activated the cardioprotective phospholipase $\text{C}\gamma 1$ /protein kinase $\text{C}\epsilon$ (PLC- $\gamma 1$ /PKC ϵ) pathway. Pre-treatment of the hearts with the Src kinase family inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) blocked not only ouabain-induced activation of PLC- $\gamma 1$ /PKC ϵ pathway, but also cardiac protection. This protection was also blocked by a PKC ϵ translocation inhibitor peptide (PKC ϵ TIP).

Conclusion: Short exposure to a low concentration of ouabain protects the heart against ischemia/reperfusion injury. This effect of ouabain on the heart is most likely due to the activation of the Na^+, K^+ -ATPase/c-Src receptor complex and subsequent stimulation of key mediators of preconditioning, namely PLC- $\gamma 1$ and PKC ϵ .

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This article is referred to in the Editorial by J. Inverte (pages 446–447) in this issue.

1. Introduction

Ouabain and other cardiac glycosides all share the property of being potent and highly specific inhibitors of the purified Na^+, K^+ -ATPase, the ubiquitous transmembrane

enzyme that transports Na^+ and K^+ across the plasma membrane by hydrolysis of ATP [1–3]. In that context, it had been accepted for years that the physiological and pharmacological actions of these compounds were due to the inhibition of the ATP-driven transport of Na^+ and K^+ ions across the cell membrane. However, in the past few years, it has become apparent that binding of ouabain and other cardiac glycosides to the Na^+, K^+ -ATPase also triggers a series of changes in protein–protein interactions, resulting in the activation of protein kinases within the Na^+, K^+ -ATPase signalplex [4]. This has been shown to take place within the caveolar structures of various tissues and different types of cells where the Na^+, K^+ -ATPase and c-Src interact and form a

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receptor complex [5–8]. Binding of ouabain to this receptor complex activates the Na⁺,K⁺-ATPase-associated Src, resulting in the activation of multiple signaling cascades. For instance, the ouabain-activated Na⁺,K⁺-ATPase/Src complex is able to recruit and activate PLC- γ 1 in LLC-PK1 cells [9]. Significantly, activation of this particular pathway has been demonstrated in cultured neonatal cardiac myocytes [10] and is apparently associated with ouabain-induced inotropy in isolated heart preparations [11].

There is strong evidence that induction of myocardial protection against ischemia/reperfusion injury by a treatment given before the onset of ischemia, known as cardiac preconditioning, is a receptor-mediated process that involves the activation of Src, ERKs, and PKC isozymes [12]. Specifically, activation of PLC/PKC ϵ pathway has been demonstrated during ischemic preconditioning as well as in many G protein-coupled receptor-mediated preconditioning [13–16]. Interestingly, there is evidence that direct activation of PKC ϵ via genetic manipulation is sufficient to protect the heart from ischemia/reperfusion injury [15]. Ouabain at inotropic doses (50–100 μ M) activates Src and increases overall PKC activity in the isolated heart [10,11]. However, it is not known whether lower doses, with little or no effect on contractility, would be capable of activating Src and PKC in this model. Accordingly, as a first step towards an understanding of the physiological and pharmacological impacts of the newly appreciated signaling function of the Na⁺,K⁺-ATPase, we tested whether a low dose of ouabain (10 μ M) can trigger cardiac preconditioning by activation of the Src/PLC- γ 1/ PKC ϵ pathway in the Langendorff-perfused rat heart.

2. Methods

2.1. Isolated perfused rat heart model

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Twelve weeks old male Sprague Dawley rats (350–400 g) were anesthetized with 50 mg pentobarbital sodium injected intraperitoneally. Hearts were rapidly removed and plunged into ice cold (4 °C) Krebs–Henseleit solution. Within 40 s, hearts were perfused in Langendorff mode with an oxygenated Krebs–Henseleit solution containing (in mmol/L) NaCl (118.0), KCl (4.0), CaCl₂ (1.8), KH₂PO₄ (1.3), MgSO₄ (1.2), Ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid (0.3), NaHCO₃ (25), D-glucose [11]. The gas used for oxygenation was a mixture of O₂ (95%) and CO₂ (5%), resulting in a pO₂ of 618 mm Hg, a pCO₂ of 30 mm Hg and a pH of 7.5. The coronary flow was kept constant at a rate initially set to obtain a coronary perfusion pressure of about 100 mm Hg (15 ml min⁻¹, [17]). A drainage cannula was inserted into the apex of the left ventricular cavity to vent the Thebesian flow. Isovolumic left ventricular developed pressure (LVDP,

the difference between systolic and diastolic left ventricle pressures) was measured by inserting a water-filled latex balloon into the left ventricle connected to a P23XL Becton Dickinson pressure transducer, a CP122 AC/DC strain gage amplifier and a Grass Telefactor recording system. The end diastolic pressure (EDP) was adjusted initially at 10 mm Hg. Hearts were paced at 4.5 Hz and the latex balloon was kept inflated throughout the experiment, including during ischemia. The temperatures of the buffer and isolated heart were maintained at 37 °C with a temperature-controlled, circulating water bath. After 10 to 15 min of equilibration, the experiment began, and the left ventricular developed pressure (LVDP) and coronary perfusion pressure were monitored throughout the experiment.

2.2. Choice of ouabain dose

The dose of 10 μ M was chosen based on pharmacological and physiological considerations. We wanted to test a model where the observed effect of ouabain on contractility would be minimal, to keep the interpretation of potential protection as straightforward as possible. According to previous studies [18], the dose of 10 μ M met this requirement.

2.3. Experimental protocol

Six groups of hearts were studied. The ischemia–reperfusion group (IR) was perfused for 20 min, subjected to 30-min zero-flow ischemia, and then reperfused for 120 min. In the ouabain ischemia–reperfusion group (OIR), ouabain 10 μ M was added for 4 min, 12 min before the onset of ischemia. The PP2+OIR and PP2+IR groups were perfused for 20 min with 2 μ M of the c-Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolol[3,4-*d*]pyrimidine (PP2, Calbiochem, San Diego, CA) in the presence or absence of ouabain, respectively. The TIP+OIR and TIP+IR groups were perfused for 20 min with 5 μ M of the protein kinase C epsilon (PKC ϵ) translocation inhibitor peptide EAVSLKPT (TIP, Calbiochem, San Diego, CA) in the presence or absence of ouabain, respectively. After ischemia, all hearts were reperfused with standard Krebs–Henseleit solution. As indicated in Fig. 1, left ventricles for analysis of PLC- γ 1 and PKC ϵ phosphorylation/activities were frozen at time 12 min ($n=3$ /group). LDH release in coronary effluent and contractile function were monitored until time 80 min. At time 170 min, hearts were processed for infarct size measurements ($n=5$ –7/group).

2.4. Lactate dehydrogenase (LDH) activity measurement

Coronary effluent was collected for 30 s at time 20 min (just before the onset of ischemia), and then at 0, 1, 2, 3, 4, 5, 10, 15, 20, and 30 min of reperfusion. LDH activity was determined colorimetrically using a standard assay (TOX 7 kit, Sigma, Saint-Louis, MO, USA), according to the manufacturer recommendation.

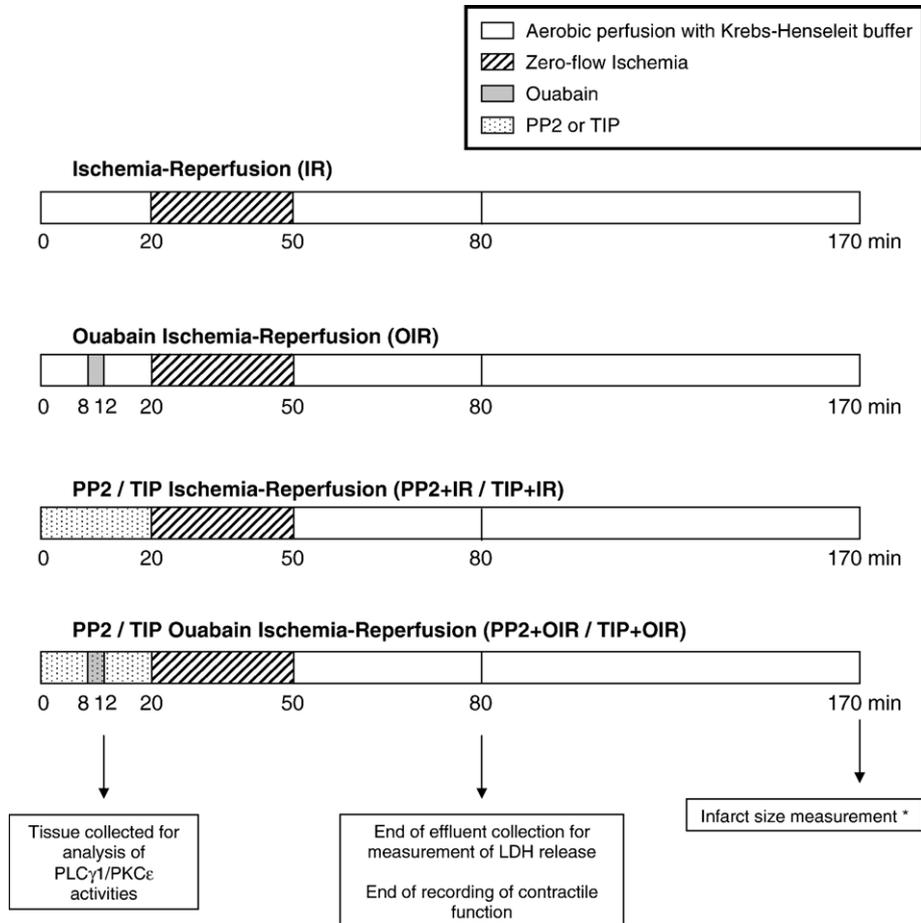


Fig. 1. Experimental protocols. Timing of interventions is shown in relation to ischemia. Ouabain was perfused at 10 μ M. The c-Src kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine, 2 μ M) and the (PKC ϵ) translocation inhibitor peptide (TIP, 5 μ M) were given before, during and after ouabain. After ischemia, all hearts were reperfused with standard Krebs–Henseleit solution. * Except for TIP groups.

2.5. Tissue preparation, SDS-PAGE, immunoprecipitation and immunoblotting

For each experimental group, 3 hearts were quick-frozen in liquid nitrogen after 12 min of perfusion according to their respective protocols (Fig. 1). One hundred mg of powdered left ventricle was placed into an ice-cold buffer containing 30 mM histidine, 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 nM okadaic acid, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and then homogenized in a 30 ml homogenizer by repeating 5 times a series of 8 up-and-down strokes separated by 30 s intervals. For immunoprecipitation experiments, homogenates were diluted 1 to 3 in an ice-cold solution containing 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 50 mM Tris–HCl (pH 7.4) [11]. Lysates were centrifuged at 16,000 \times g for 15 min, and supernatants (2 mg) were immunoprecipitated using an anti-PKC ϵ rabbit polyclonal or an anti-PLC-1 monoclonal-antibody (both from Santa Cruz, CA). The

immunoprecipitates were dissolved in 2 \times Laemmli sample buffer, boiled for 5 min, separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-phospho-PKC ϵ (pan, Cell Signaling Technology, Beverly, MA) or anti-phospho-PLC- γ 1 antibody (Santa Cruz). To assay PKC ϵ translocation from cytosolic to particulate fraction, the same homogenates were centrifuged at 100,000 \times g for 1 h at 4 $^{\circ}$ C. The supernatant designated as the cytosolic fraction was removed and saved. The pellet was homogenized in the solution containing 1% Triton and the particulate fraction was prepared as we previously described [11].

2.6. Histomorphometry

After 120 min of reperfusion, hearts were frozen (-20 $^{\circ}$ C, 2 h), sliced into 3 mm thick transverse sections and incubated in triphenyltetrazolium chloride solution (TTC, 1% in phosphate buffer, pH 7.4) at 37 $^{\circ}$ C for 20 min, then further incubated for 20 min in formalin. TTC stains the viable tissue in a bright-red color, which allows the discrimination between viable (red) and nonviable (pale yellow) tissue.

Slices were electronically scanned and subsequently analyzed using Image J (version 1.32j) software (National Institutes of Health, USA <http://rsb.info.nih.gov/ij/>). The amount of necrosis was then estimated with a macro modified from reference [19] to detect poor uptake of TTC. Infarct size was expressed as a percentage of the risk zone (equivalent to total cardiac muscle mass), as reported previously [20].

2.7. Statistical analysis

Differences between untreated and pharmacologically treated groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. When 2 groups were compared, unpaired bilateral Student's *t* test was used. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Cardiac function

We first determined the effect of ouabain exposure on the recovery of cardiac function. Preliminary experiments on

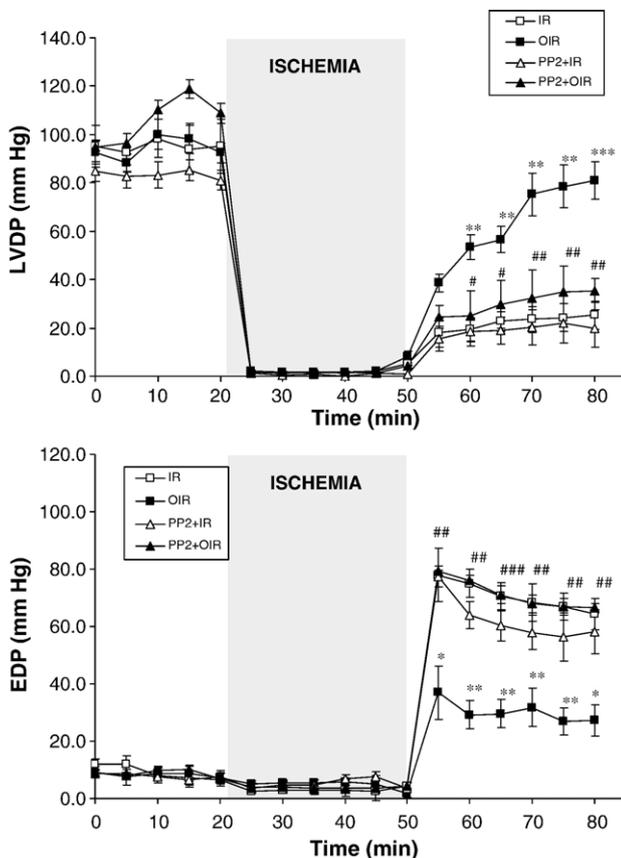


Fig. 2. Effect of ouabain and PP2 on myocardial function. Left ventricular developed pressure (LVDP, upper panel) and end diastolic pressure (EDP, lower panel) were measured in Langendorff-perfused hearts submitted to no treatment (IR, □), ouabain (OIR, ■), PP2 (PP2+IR, △) or PP2+ouabain (PP2+OIR, ▲). Values are means±SEM of 5–7 independent experiments for each group. ** $P < 0.01$ and *** $P < 0.001$ vs. IR. ## $P < 0.01$ and ### $P < 0.001$ vs. OIR.

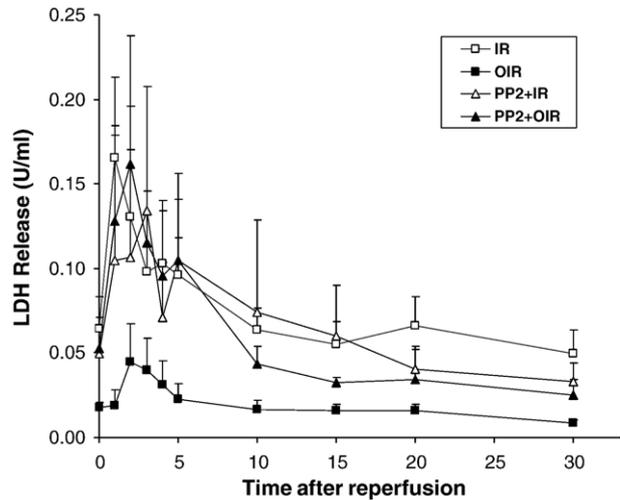


Fig. 3. Effect of ouabain and PP2 on LDH release. LDH release over the 30 min of reperfusion was IR: ischemia/reperfusion (□), 32.3 ± 8.4 U, OIR: ouabain+ischemia/reperfusion (■): 8.0 ± 2.6 U ($P < 0.05$ vs. IR), PP2+IR: PP2+ischemia/reperfusion (△), 24.0 ± 8.6 U and PP2+OIR: PP2+ouabain+ischemia/reperfusion (▲) 15.1 ± 1.8 U ($P < 0.05$ vs. OIR).

continuously perfused Langendorff rat hearts showed that after a 10–15 min equilibration period, cardiac performance was stable with a decrease $< 10\%$ over the next 80-min perfusion period (data not shown). Baseline parameters were identical in all groups (Figs. 2 and 6). Four min infusion of $10 \mu\text{M}$ ouabain caused a small and transient increase in LVDP ($< 10\%$, time=8 min, Fig. 2), followed by a subsequent recovery to baseline value during washout (time=12 to 20 min, Fig. 2). This is in accordance with the fact that ouabain dissociates from the Na^+, K^+ -ATPase $\alpha 1$ isoform rapidly when the drug is removed from the perfusate. As expected, ischemia resulted in cardiac arrest in all groups. Although cardiac performance resumed within seconds of reperfusion, it was significantly compromised in all groups as shown by the significant decrease in LVDP (time=55 min, upper panel of Fig. 2) and the increase in EDP (time=55 min, lower panel of Fig. 2). However, a significant protection by ouabain pre-treatment was clearly detected at 10 min of reperfusion and after (time=60 to 80 min), and the recovery in the ouabain-treated group was faster and greater. At the end of the 30 min reperfusion, the LVDP recovery was $21.3 \pm 3.0\%$ of the pre-ischemic value in control hearts and $75.7 \pm 9.8\%$ in ouabain-treated hearts ($P < 0.001$, Fig. 2). To determine if the ouabain effects on cardiac performance were mediated by the activation of Src, PP2 was administered before, during and after ouabain perfusion. This treatment significantly reduced ouabain-induced improved recovery of LVDP and EDP (Fig. 2).

3.2. Enzyme release (LDH)

Coronary effluent was collected as detailed in the Methods section. The total LDH release over the 30 min of reperfusion (calculated from areas under the curves presented in Fig. 3) was 32.3 ± 8.4 U in the untreated group.

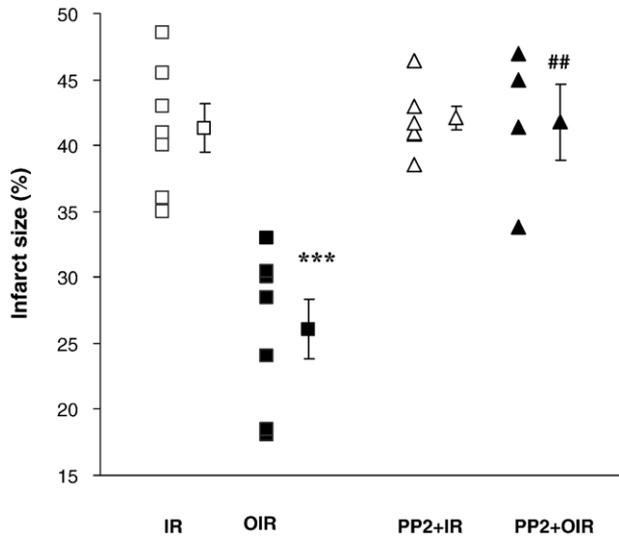


Fig. 4. Effect of ouabain and PP2 on infarct size. Infarct size expressed as a percentage of the risk zone was measured after 120 min of reperfusion. IR: ischemia/reperfusion (□); OIR: ouabain + ischemia/reperfusion (■); PP2 + IR: PP2 + ischemia/reperfusion (Δ); PP2 + OIR: PP2 + ouabain + ischemia/reperfusion (▲). Shown are individual experimental data and means ± SEM of 5–7 independent experiments for each group. ****P* < 0.001 vs IR, ##*P* < 0.001 vs OIR.

Ouabain treatment reduced LDH release to 8.0 ± 2.6 U (*P* < 0.05). PP2 had no significant effect by itself (24.0 ± 8.6 U) but completely blocked the protective effect of ouabain on LDH release (15.1 ± 1.8 U, *P* < 0.05 vs. OIR).

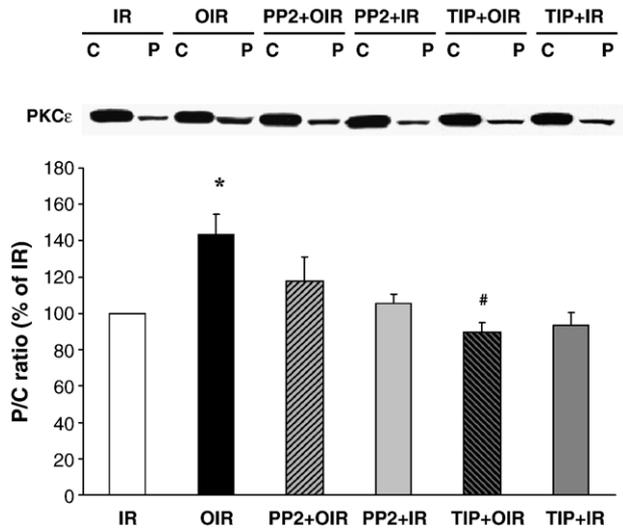


Fig. 5. Effect of ouabain and PP2 and TIP on PKCε activation. Hearts were perfused for 12 min according to the protocols described in Fig. 1. Cytosolic (C) and particulate (P) fractions obtained from tissue lysates were then assayed and compared for their contents in PKCε. IR: ischemia/reperfusion; OIR: ouabain + ischemia/reperfusion; PP2 + IR: PP2 + ischemia/reperfusion; PP2 + OIR: PP2 + ouabain + ischemia/reperfusion. TIP: PKCε-specific translocation inhibitor peptide. TIP + IR: TIP + ischemia/reperfusion; TIP + OIR: TIP + ouabain + ischemia/reperfusion. Upper panel: representative western blot. Lower panel: means ± SEM of 3 separate experiments. **P* < 0.01 vs IR. #*P* < 0.01 vs OIR.

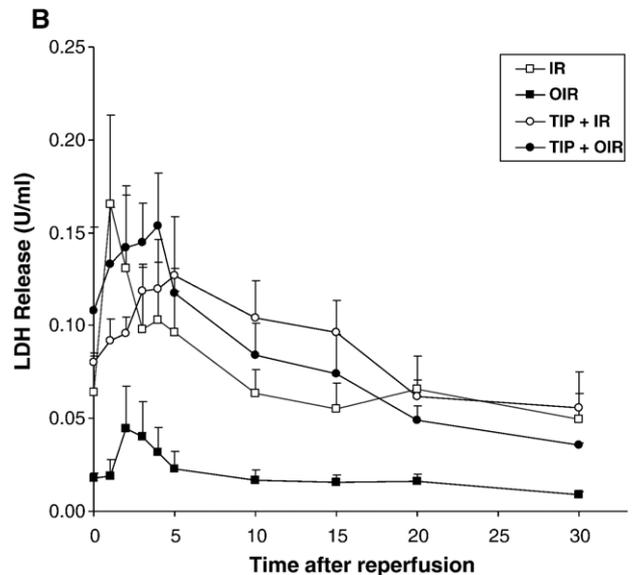
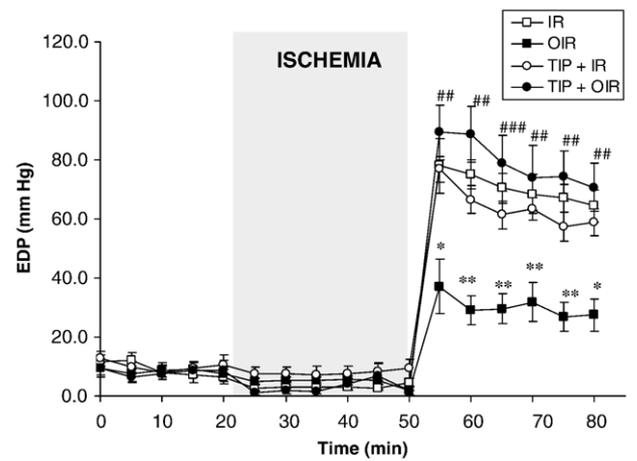
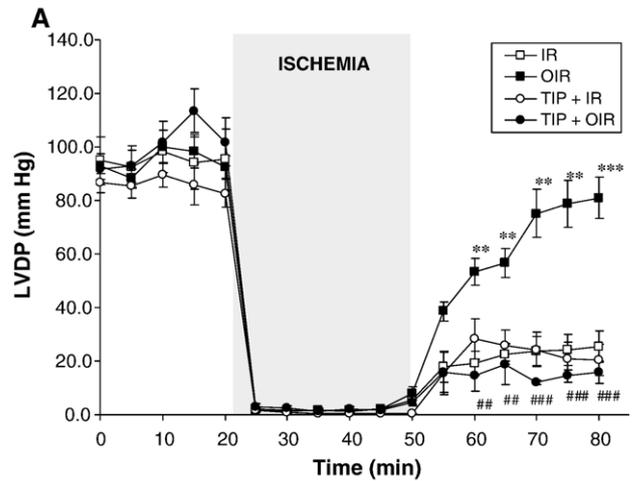


Fig. 6. Effect of PKCε inhibition on ouabain-induced preconditioning. A. Left ventricular developed pressure (LVDP, upper panel) and end diastolic pressure (EDP, lower panel) were measured in Langendorff-perfused hearts submitted to no treatment (IR, □), ouabain (OIR, ■), TIP (○) or TIP + ouabain (TIP + OIR, ●). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs. IR. ##*P* < 0.05, ###*P* < 0.01, and ####*P* < 0.001 vs. OIR. B. LDH release over the 30 min of reperfusion was IR: 32.3 ± 8.4 U, OIR: 8.0 ± 2.6 U (*P* < 0.05 vs. IR), TIP + IR: 39.0 ± 7.7 U and TIP + OIR: 34.7 ± 6.4 U (*P* < 0.05 vs. OIR). Values are means ± SEM of 5–7 independent experiments for each group.

3.3. Tissue necrosis

TTC staining in 5–7 samples demonstrated that 30 min of global ischemia followed by 2 h of reperfusion in the untreated heart brought about the infarction to $43.5 \pm 1.3\%$ of the area at risk. As shown in Fig. 4, ouabain induced a protective effect. In those hearts, infarct size ($26.1 \pm 2.3\%$ of the area at risk) was reduced by 40% with respect to that of untreated hearts ($P < 0.001$). PP2 treatment did not modify the infarct size by itself but blocked ouabain effect.

3.4. Role of PKC ϵ activation in ouabain-induced protection

We have shown that sustained treatment with ouabain at inotropic doses (50–100 μM , 10–25 min without washout) activates c-Src and PKCs in the isolated heart [11] and more specifically c-Src/PKC ϵ in cultured cardiac myocytes [10]. Recently, we also reported that ouabain stimulated Src/PLC- γ pathway in cultured LLC-PK1 cells [9]. These previous observations led to the hypothesis that preconditioning treatment (low dose and transient) applied in the present study may stimulate the Src/PLC- γ /PKC ϵ pathway in the isolated heart, resulting in cardiac protection. To test our working hypothesis, we first measured the effect of ouabain on PKC ϵ translocation from the cytosolic to the particulate fraction as described in Methods. As shown in Fig. 5, ouabain induced a significant increase in PKC ϵ translocation. PP2 reversed ouabain effect (Fig. 5). Concomitantly, both PLC- γ 1 and PKC ϵ phosphorylation were activated by ouabain. These effects were reversed in the presence of PP2 (not shown). To directly address the role of PKC ϵ activation in ouabain-induced protection, we used the PKC ϵ specific translocation inhibitor peptide TIP as previously described [21]. TIP did not have a significant effect by itself but totally blocked ouabain-induced PKC ϵ translocation (Fig. 5). Likewise, TIP did not significantly modify the amount of LDH released over the 30 min of reperfusion (39.0 ± 7.7 U for TIP+IR, $P > 0.05$ vs. 32.3 ± 8.4 U for IR, Fig. 6B), but totally prevented the ouabain-induced decrease (34.7 ± 6.4 U for TIP+OIR, $P < 0.05$ vs. OIR, Fig. 6B). Finally, in terms of recovery of cardiac function, no effect of TIP itself was observed. However, TIP treatment prevented ouabain-induced preconditioning as shown by a limited recovery of LVDP and EDP, similar to the untreated group (Fig. 6A). Although infarct size was not measured, these data strongly suggest that ouabain-induced activation the Src/PLC- γ /PKC ϵ pathway in the isolated heart preparation is critical to ouabain-induced preconditioning.

4. Discussion

Using the rat Langendorff-perfused isolated heart model, the present study shows that ouabain induces myocardial protection against ischemia/reperfusion injury. This is documented by an improved recovery of cardiac function, a reduction in cardiac enzyme release and a reduction of tissue necrosis. The protection occurred at the low dose of 10 μM ,

required c-Src activation and involved the activation of the cardioprotective PLC- γ 1/PKC ϵ . This suggests the signaling function of the Na/K-ATPase/c-Src receptor complex as the cellular mechanism of this effect.

4.1. Ouabain preconditioning vs. ischemic preconditioning (IP)

IP was the first described and remains the best-characterized form of preconditioning. It is defined as a protective mechanism whereby hearts exposed to brief, sublethal ischemic insults are more resistant against subsequent prolonged ischemia, reducing myocellular death and postischemic cardiac dysfunction [22]. Being the first attempt to demonstrate a preconditioning effect of the digitalis ouabain, the present study was designed to allow comparison with previous studies on ischemic injury and/or IP. Accordingly, the protocol described in Fig. 1 was established in a widely used model of rat isolated heart, exposed to 30 min of ischemia and 30 min of reperfusion. The recovery of cardiac mechanical function usually reported ranges from 4% to 40% [23–27] of the initial value in the absence of preconditioning. We observed a recovery of $21.3 \pm 3\%$ of the LVDP, suggesting that this protocol allowed us to characterize a protection against a fairly standard and previously reported extend of injury. In comparable protocols, the reported recovery of mechanical function when the heart is preconditioned ischemically prior to the sustained ischemia typically reaches 60 to 90% [25–27]. Therefore, the recovery of $75.7 \pm 9.8\%$ afforded by ouabain 10 μM is quite superimposable to the reported effect of IP. Like the protection afforded by IP, the effects of ouabain on EDP and myocellular death were significant. The latter was revealed by the significant decrease of LDH release over the course of the reperfusion and the reduction by about 40% of the infarct size compared with the untreated ischemic group.

4.2. Initiation of the ouabain preconditioning effect: pumping vs. signaling

Applying a new concept (signaling through Na⁺,K⁺-ATPase) to an old drug (ouabain) led us to this unexpected yet highly physiologically relevant finding. Indeed, the rationale for this study was clearly based on striking similarities observed between cardiac preconditioning- and ouabain-signaling cascades, both newly characterized and still under intensive investigation. However, based on the classical role of the cardiac enzyme and previous reports on digitalis/Na⁺,K⁺-ATPase in ischemia/reperfusion injury, exposure to a Na⁺,K⁺-ATPase inhibitor, even at a very low concentration, may not have been expected to induce myocardial protection. Indeed, since the pioneer work of Beller et al. [28], alteration of myocardial Na⁺,K⁺-ATPase activity and remodeling have been repeatedly reported as one key adverse consequence of ischemic injury [26,29,30]. Although the exact mechanism is still under investigation [30–32], this

alteration is critical in the intracellular Na^+ accumulation and Ca^{2+} overload during ischemia and reperfusion, both of which are closely related to the outcome of myocardial damage. In apparent contradiction with the paradigm that we chose to investigate are the facts that 1) IP preserves myocardial Na^+ , K^+ -ATPase activity after ischemia/reperfusion injury [26,33] and 2) high concentrations of ouabain given after IP blocked the protective effect of IP [34]. Three important differences in the present study may explain the apparently contradictory outcome. First, we used a low concentration of ouabain (20 times lower than the above-mentioned study, Ref. [34]). Second, the exposure was transient (4 min) and third, ouabain was given and washed out (consistent with the rapid dissociation rate of ouabain when bound to the rodent $\alpha 1$ isoform of Na^+ , K^+ -ATPase) before the onset of ischemia.

By the same token, it seems important to emphasize that the mechanism investigated here is different from the one involved in the well-known positive inotropic action of digitalis. The inotropic effect has long been known to involve an inhibition of the sarcolemmal Na^+ , K^+ -ATPase leading to a significant increase in intracellular Na^+ and Ca^{++} , and in the force of contraction [35]. Although the more recently described signaling cascade is essential to the ouabain's effect on Ca^{++} in rat cardiac myocytes [36], the signaling cascade itself can be initiated without changes in intracellular Ca^{++} concentration and relies on a pathway linked to ROS production [37]. In ouabain-insensitive species like rat (about 1000 times less sensitive than human), ouabain at the dose of 10 μM induces a limited inhibition of the cardiac enzyme activity, of about 20% [38]. This resulted in less than 10% changes in LVDP (inotropic effect) in the present study, in agreement with previous reports [18]. In other words, the ouabain exposure that was given here (low dose and transient) was targeting the initiation of the signaling cascade rather than the ion pumping activity of the enzyme at the time of reperfusion. Therefore, this preconditioning effect of ouabain is not in contradiction with a preservation of Na^+ , K^+ -ATPase activity at the time of reperfusion.

Taken together, these findings suggest that while ouabain-induced inotropy requires continued occupation of the receptor, its effect on preconditioning is solely dependent on the activation of the signaling cascade. In other words, once activated, this signaling cascade will lead to the activation of PLC- $\gamma 1$ /PKC ϵ in the absence of continued receptor occupancy. Admittedly, important issues such as the role played by downstream intracellular messengers (ROS and Ca^{++}) or differences in the mechanisms triggered by continuous vs transient Na^+ , K^+ -ATPase receptor occupancy need to be further addressed in future studies.

4.3. Downstream effectors of the ouabain preconditioning effect

We have recently demonstrated that Na^+ , K^+ -ATPase and c-Src interact and form a receptor complex [8]. Binding of ouabain to this receptor complex activates Src, leading to the recruitment and tyrosine phosphorylation of PLC- $\gamma 1$ in pig

kidney cells [9]. Here we demonstrated that ouabain could also activate the Src/PLC- $\gamma 1$ cascade in the isolated heart preparation. In addition, we observed that ouabain increased phosphorylation of PKC ϵ in a Src-dependent manner. Because phosphorylation is required for PKC ϵ translocation, it is conceivable that the concerted effects of ouabain on PKC ϵ phosphorylation and on DAG generation via the activated PLC- $\gamma 1$ shall lead to a full activation of PKC ϵ in the isolated heart. This conclusion is consistent with the PKC ϵ translocation data (Figs. 5 and 6) and with our prior observation that inotropic doses of ouabain were sufficient to stimulate both c-Src and PKCs in the isolated hearts [11]. Others have shown that the formation of c-Src/PKC ϵ signaling module is an important step in IP [39]. Activation of PKC ϵ facilitates the formation of this complex leading to c-Src activation, which places PKC ϵ upstream from c-Src at least in rabbit species [40]. Evidence of a c-Src-dependant activation of PKC ϵ , more in line with the present report, has also been reported [41]. The current vision seems therefore to include the involvement of a c-Src-dependent and a c-Src-independent mechanism of PKC ϵ activation, probably taking place in different subcellular compartments (membrane vs cytoplasmic) in IP. The present data suggest that the ouabain-induced cardioprotective effect is mediated primarily by the formation of a Na^+ , K^+ -ATPase/c-Src/PLC- $\gamma 1$ tertiary complex preceding PKC ϵ activation. In addition, it should be mentioned that opening of mitochondrial K_{ATP} channel and increases in ROS production may also be important ouabain-induced cardioprotective mediators linked to the PLC- $\gamma 1$ /PKC ϵ pathway [42,43]. Clearly, these issues need to be addressed in future studies.

Continuing research is providing an increasing number of agents capable of preconditioning the heart. Unfortunately, their translation into clinical therapy involves obvious practical challenges that increase the likelihood of significant unanticipated risks [44,45]. Our data suggesting an effect of digitalis on resistance to ischemia/reperfusion injury raises the possibility that this well-studied drug could be used as a preconditioning agent. Clinicians have accumulated a large body of experience and expertise in the use of digitalis under numerous physiological conditions and dosing regimens [46–48]. Nevertheless, the administration of digitalis for preconditioning in human patients is complicated by such obvious concerns as the differences in sensitivity to the drug exhibited by rodents and primates, as well as the distinction between the acute exposures used in this work and the typically chronic exposures in patients. Clearly, further study is needed to determine if an extrapolation to humans is warranted, but the present findings raise the possibility of including this traditional drug in a regimen for reducing ischemia/reperfusion injury.

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