The phosphoinositide 3-kinase inhibitor LY294002 enhances cardiac myocyte contractility via a direct inhibition of $I_{k,slow}$ currents

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Abstract

Objective: Phosphoinositide 3-kinase (PI3K) is a key component in regulating myocardial growth, survival and contractility. LY294002 and wortmannin are two PI3K inhibitors used widely to establish the role of PI3K. The goal of this study was to examine the effects of acute application of LY294002 and wortmannin on cardiac myocyte contractility and underlying machanisms. Methods: Patch-clamp, indo-1 epifluorescence and video-edge detection techniques were used to measure outward K+ currents, action potentials (AP), Ca2+ transients and shortening of myocytes isolated from mouse left ventricular free wall. Results: In field-stimulated myocytes, LY294002 (10 μmol/l) increased Ca2+ transient amplitude by 23%, and cell shortening amplitude by 60% in the absence or presence of wortmannin, while wortmannin alone had no effect. LY294002 (but not wortmannin) prolonged AP duration by specifically inhibiting slowly inactivating K+ currents (i.e., the 4-aminopyrydine-sensitive $I_{k,slow1}$ and the tetraethylammonium-sensitive $I_{k,slow2}$), leading to an increase in sarcoplasmic reticular Ca2+ levels. It appeared that the AP prolongation was responsible for elevated contractility since AP-clamp of myocytes with prolonged APs (recorded in LY294002-treated myocytes) induced a 29% increase in cell shortening compared with control APs, while LY294002 application did not increase contractility in voltage-clamp studies using either step or AP depolarizations. Conclusions: The putative PI3K inhibitor LY294002 increases Ca2+ release and myocyte contractility via direct inhibition of cardiac $I_{k,slow}$ and AP prolongation, thus limiting the usefulness of this agent in the analyses of the role of PI3K in heart function.

1. Introduction

Phosphoinositide 3-kinases (PI3Ks) are a family of evolutionarily conserved lipid kinases that mediate the formation of D-3 phosphoinositides which are key components of many signaling pathways regulating a broad range of fundamental cellular processes including cell growth, differentiation, survival and migration in response to extracellular stimuli [1,2]. The availability of PI3K inhibitors such as wortmannin and LY294002 has been crucial in advancing our understanding of the biological functions of PI3K enzymes [3,4]. In the heart, for example, these inhibitors have been used to establish the role of PI3Ks in mediating inotropic [5,6], hypertrophic [7,8] and antiapoptotic responses [9,10] of cardiac myocytes to different stimuli. Wortmannin and LY294002 are structurally distinct antagonists of all three classes of PI3Ks. Wortmannin was originally isolated from *Penicillium wortmannii* and irreversibly inactivates PI3Ks by covalent modification of the catalytic subunit [3,4,11,12]. On the other hand, LY294002 is a synthetic compound derived from the broad-spectrum kinase inhibitor quercetin [13] and reversibly inhibits PI3K by competing with ATP for the active site of catalytic subunit p110 [4,13,14].

Recent studies in transgenic mice established that deletion of PI3Kα enhances cardiac contractile function, while dominant-negative inhibition of PI3Kα impairs myocardial growth [15]. Consistent with these mouse studies, acute application of LY294002 increased cell
shortening amplitude [15]. In order to elucidate the mechanism(s) of PI3K-mediated inotropic effects, we examined the actions of wortmannin and LY294002 application on K⁺ current, action potential, Ca²⁺ transient and cell shortening in isolated mouse myocytes. We found that LY294002, but not wortmannin, increased myocyte shortening which was associated with blockade of \( I_{\text{s,slow}} \) currents and action potential prolongation.

2. Methods

2.1. Myocyte isolation

Single myocytes were isolated from the left ventricle free wall of C57BL6 male mice (12 weeks old, Charles River) using Type II collagenase (0.4 mg/ml, Boehringer-Mannheim) and Type XIV protease (0.025 mg/ml, Sigma) as previously described [16]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Electrophysiology

Action potentials (AP) and outward K⁺ currents were recorded with the whole-cell patch clamp technique under current-clamp and voltage-clamp mode, respectively, using an Axopatch 200B amplifier and pClamp 6 software (Axon Instrument). The pipette resistance ranged between 1.2 and 1.8 MΩ when filled with a solution containing (mmol/l) 120 potassium aspartate, 20 KCl, 5 NaCl, 1 MgCl₂, 5 MgATP, 10 HEPES and 10 EGTA, pH 7.2. Cell capacitance and series resistance were electronically compensated by 80–90%. Myocytes were held at −80 mV, and K⁺ currents were elicited by 5-s depolarizations to +40 or +60 mV. The fast Na⁺ current was inactivated by applying a prepulse to −40 mV for 50 ms. Membrane currents were sampled at two different rates (10 kHz for the first 500 ms and 2 kHz for the rest 4.5 s) and filtered at 2 kHz. The bath solution for AP recordings contained (mmol/l) 140 NaCl, 4 KCl, 1 MgCl₂, 1.2 CaCl₂, 10 HEPES and 10 d-glucose (pH 7.4). For K⁺ current recordings, the L-type Ca²⁺ channel was blocked by adding 0.3 mmol/l CdCl₂ to the bath solution. In some experiments, NaCl was replaced by 135 mmol/l tetrae-

Fig. 1. Effects of LY294002 and wortmannin on cell shortening under field stimulation conditions. (A) Representative recordings of steady-state cell shortening from three different myocytes stimulated at 1 Hz under different conditions: (a) before and 5 min after exposure to 10 μmol/l LY294002 (LY); (b) before and 15 min after application of 200 nmol/l wortmannin (WM); (c) before, 10 min after application of 100 nmol/l wortmannin and 5 min after subsequent addition of 10 μmol/l LY294002 in the presence of wortmannin. (B) Mean percentage cell shortening (CS), maximal velocity of shortening (+ dL/dt) and rate of relaxation (−dL/dt) pooled from corresponding experiments shown on (A). (a) \( n = 10 \); (b) \( n = 6 \); (c) \( n = 10 \). *\( P < 0.01 \) vs. Control; **\( P < 0.01 \) vs. Control and wortmannin groups.
thalammonium (TEA)-Cl. Experiments were performed at 35.5 ± 0.5 °C.

2.3. Cell shortening measurements

Unloaded myocyte shortening was measured with a video edge detector coupled to a high frequency (240 Hz) charge-coupled camera (Crescent Electronics, UT, USA). Myocytes were voltage-clamped through patch pipettes or field-stimulated at 1 Hz via square pulses (5 ms × 6 V) delivered by a S44 Grass stimulator. The cell motion was sampled at 1 kHz (Felix software, Photon Technologies). The pipette solution for cell shortening (CS) measurement contained (mmol/l) 125 potassium aspartate, 20 KCl, 5 Na2-phosphocreatine, 0.5 MgCl2, 5 MgATP, 0.4 Na2GTP, 10 HEPES and 0.05 EGTA, pH 7.2. The amplitude of CS was measured at steady state. LY294002 or wortmannin was washed in after the steady-state CS was recorded under control conditions. The effects of drugs on CS were measured when the bath solution was completely exchanged for drug-containing solutions and the CS reached the new steady state.

2.4. Intracellular Ca2+ measurements

Ca2+ transients were measured on myocytes loaded with indo 1-AM (Molecular Probe) before and after addition of LY294002. The intracellular indo 1 was excited at 365 nm. Fluorescence emitted at 405 and 495 nm and collected by two matched photomultiplier tubes was filtered at 50 Hz and sampled at 1 kHz (Felix software). The ratio of the intensity of fluorescence emitted at 405 nm over that at 495 nm (R405/495) was calculated off-line after subtraction of background fluorescence and used as an index of intracellular Ca2+ concentrations in this study.

2.5. Chemicals

Wortmannin (Sigma) and LY294002 (Calbiochem) stocks were prepared in DMSO and then diluted to final concentrations in bath solutions before use.

2.6. Data analysis and statistics

The amplitude of cell shortening was expressed as percentage shortening relative to the resting diastolic length. The maximal velocity of shortening (+dL/dt) and the maximal rate of relaxation (−dL/dt) were measured from time derivatives of the cell shortening signal. The AP and current recordings were analyzed using pClamp software (Clampfit 8.2, Axon, CA, USA). The decay phase of outward K+ currents was fitted to a sum of two or three exponentials with Chebyshev method (Clampfit 8.2, Axon, CA, USA). The fits to the data are considered adequate if the correlation coefficient was greater than 0.98.

Statistically significant differences between two means was assessed using pair or unpaired Student’s t-tests. A P value < 0.05 was considered to indicate significance. Group data are expressed as the mean ± S.E.M.

3. Results

3.1. Differential effects of LY294002 and wortmannin on myocyte contractility and action potentials

To examine the role of PI3K in regulating basal myocyte contractility, we applied the PI3K inhibitors LY294002 and wortmannin to freshly isolated, field-stimulated myocytes.

![Fig. 2. Effects of LY294002 on intracellular Ca2+ transients. (A) Representative recordings of Ca2+ transients in response to field stimulations at 1 Hz obtained from a myocyte before and after bath application of 10 μmol/l of LY294002. R405/495: ratio of indo 1 fluorescence emitted at 405 nm over that at 495 nm. (B) Mean amplitude of Ca2+ transients obtained from six cells in the absence (Control) and presence of 10 μmol/l of LY294002. The amplitude of Ca2+ transients (ΔR405/495) was measured as the difference between diastolic and maximal systolic fluorescence R405/495. *P < 0.001.](https://academic.oup.com/cardiovascres/article-abstract/62/3/509/319579)
Fig. 1A(a) and B(a) shows that application of 10 μmol/l LY294002 increased ($P<0.05$) the amplitude of steady-state cell shortening from $7.0 \pm 0.5\%$ to $11.3 \pm 0.7\%$ ($n=10$) while also enhancing the maximal velocities of shortening and relaxation when myocytes were stimulated at 1 Hz. The effects of higher doses of LY294002 could not be reliably studied due to the frequent appearance of spontaneous contractions, presumably due to excessive SR Ca$^{2+}$ loading. By contrast, cell shortening was unaltered following the application of PI3K inhibitor wortmannin, at concentrations that are more than 50-fold greater than the IC$_{50}$ for inhibition of PI3K [13] (Fig. 1A(b) and B(b)). However, the addition of 10 μmol/l LY294002 in the presence of 100 nmol/l wortmannin enhanced myocyte shortening to the same extent ($P<0.05$) as LY29004 alone (Fig. 1A(c) and B(c)), suggesting that LY294002 increases myocyte contractility by mechanisms independent of PI3K inhibition.

To determine whether the increased myocyte contractility induced by LY294002 was associated with elevated intracellular Ca$^{2+}$, Ca$^{2+}$ transients were measured with indo 1. As shown in Fig. 2, addition of 10 μmol/l LY294002 increased ($P<0.001$) the amplitude of Ca$^{2+}$ transients by

![Image of Fig. 2](https://example.com/image2.png)

Fig. 3. Effects of LY294002 and wortmannin on action potential (AP). (A) Representative APs recorded on current clamp mode (1 Hz). (a) APs recorded before and 5 min after bath application of 10 μmol/l LY294002 (LY). (b) APs recorded in the absence of drugs, 15 min after application of 100 nmol/l wortmannin (WM), and 5 min after subsequent addition of 10 μmol/l LY294002 in the presence of wortmannin. (B) Mean ratio of APD$_{50}$, APD$_{75}$ and APD$_{90}$ obtained in the presence of LY294002 or wortmannin over those obtained on the same cells before application of drugs (Control). $n=7$ for wortmannin group, $n=5$ for LY294002 5 μmol/l group and $n=8$ for LY294002 10 μmol/l group, *$P<0.05$ vs. Control.
23% (n = 6 cells), suggesting that LY294002 enhances myocyte contractility by increasing Ca\(^{2+}\) release.

Since action potential profile and membrane potential can regulate cardiac contractility via modulation of Ca\(^{2+}\) cycling, we examined the effects of LY294002 on action potentials. Fig. 3A shows that LY294002 markedly prolongs the late phase of action potential repolarization while having no effect on the resting potential, the amplitude of action potential or the early repolarization. Indeed, Fig. 3B establishes that 5 and 10 \(\mu\)mol/l LY294002 prolonged \((P<0.05)\) the time to 90% repolarization (i.e., APD\(_{90}\)) but not the time for 50% repolarization (i.e., APD\(_{50}\)). These effects of LY294002 on AP were not mediated by PI3K inhibition, since 100 nmol/l wortmannin did not alter AP profile even 15 min after drug application (Fig. 3 and Table 1). On the other hand, application of LY294002 in the presence of 100 nmol/l wortmannin caused similar changes in AP profile as observed with LY294002 alone (Fig. 3A).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>RP (mV)</th>
<th>APA (mV)</th>
<th>APD(_{50}) (ms)</th>
<th>APD(_{75}) (ms)</th>
<th>APD(_{90}) (ms)</th>
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<tr>
<td>Control</td>
<td>7</td>
<td>-78.4 ± 0.6</td>
<td>142.1 ± 3.5</td>
<td>5.6 ± 0.6</td>
<td>13.1 ± 1.5</td>
<td>32.2 ± 3.9</td>
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<tr>
<td>WM 100 nmol/l</td>
<td>7</td>
<td>-78.3 ± 0.6</td>
<td>141.7 ± 3.9</td>
<td>5.4 ± 0.6</td>
<td>12.9 ± 1.7</td>
<td>31.9 ± 3.4</td>
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<tr>
<td>Control</td>
<td>5</td>
<td>-75.8 ± 0.9</td>
<td>140.1 ± 4.3</td>
<td>4.3 ± 0.5</td>
<td>8.7 ± 1.4</td>
<td>26.1 ± 3.6</td>
</tr>
<tr>
<td>LY 5 (\mu)mol/l</td>
<td>5</td>
<td>-75.1 ± 0.9</td>
<td>140.3 ± 4.7</td>
<td>4.3 ± 0.5</td>
<td>9.7 ± 1.8</td>
<td>42.6 ± 3.3*</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>-78.2 ± 0.7</td>
<td>140.7 ± 2.6</td>
<td>5.2 ± 0.6</td>
<td>11.9 ± 1.6</td>
<td>32.9 ± 3.2</td>
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<tr>
<td>LY 10 (\mu)mol/l</td>
<td>8</td>
<td>-78.4 ± 0.3</td>
<td>140.7 ± 2.9</td>
<td>5.3 ± 0.7</td>
<td>17.5 ± 4.1</td>
<td>67.6 ± 4.5*</td>
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</table>


* \(P<0.01\) vs. Control.

Fig. 4. Effects of LY294002 on cell shortening under voltage clamp conditions. (A) Representative recordings of cell shortening elicited by 10th step pulse from \(-80\) to 0 mV applied at 1 Hz (top) before, 5 min after application of 25 \(\mu\)mol/l LY294002 (LY) and after washout of the drug. (B) Mean amplitude of cell shortening (CS) normalized with respect to that obtained 5 min after whole-cell formation and before application of LY294002 (Control). \(n = 6\). * \(P<0.05\) vs. Control. (C) Mean amplitude of cell shortening recorded at 10 min after forming whole cell configuration (WCC) normalized with respect to that obtained at 5 min after whole-cell access and taken as control. \(n = 5\). * \(P<0.05\).
The results above suggest that changes in AP profile by LY294002 are responsible for the changes in myocyte contractility. To investigate this possibility, we examined the effects of LY294002 on myocyte shortening under voltage-clamp conditions in order to prevent membrane potential changes. Fig. 4A shows representative cell shortening traces recorded in response to 200-ms depolarizations from −80 to 0 mV at 1 Hz before, 5 min after application of 25 µmol/l LY294002 as well as after washout of the drug. In contrast to field-stimulated myocytes, under voltage-clamp conditions, LY294002 application caused a small reduction in cell shortening. This small reduction resulted, at least in part, from a time-dependent rundown of cell contractility since similar reductions in cell shortening were observed in the absence of LY294002 at times corresponding to those when LY294002 was applied (i.e., 10 min after forming whole-cell configuration, Fig. 4B and C). This reduced contractility of voltage-clamped cells might also arise partially from a small measurable (16%) inhibition of L-type Ca\(^{2+}\) currents produced by 25 µmol/l LY294002 application (data not shown).

To further demonstrate that APD prolongation induced by LY294002 is responsible for the enhanced myocyte contractility observed, we assessed myocyte shortening in response to stimulation with typical steady-state APs recorded from myocytes in the absence (Control AP) or in the presence of 5 µmol/l LY294002 (LY-AP, Fig. 5). LY294002 at 5 µmol/l increased (P<0.05) CS in field-stimulated myocytes (7.0 ± 0.5% vs. 8.9 ± 0.4%, n=5). When myocytes were stimulated with a train of LY APs, cell shortening was increased (P<0.05) by 29% compared to the cell shortening measured in the same myocytes stimulated with Control-APs. The degree of increase in contractility is similar to that observed in field-stimulated myocytes, suggesting that the effects of LY294002 on AP duration are sufficient to explain the increased steady-state myocyte contractility observed with LY294002 application. Changes in action potential profile can alter Ca\(^{2+}\) transients and contractility by modulating triggered release of Ca\(^{2+}\) from the SR, by modifying the SR Ca\(^{2+}\) content or both. To distinguish between these possible mechanisms, we measured myocyte shortening evoked by both a Control-AP and a LY-AP under the conditions when equal SR Ca\(^{2+}\) level was kept fixed by using a train of 10 depolarizing pulses of 100 ms to 0 mV (at 1 Hz). Fig. 5C and D shows that, when the SR Ca\(^{2+}\) was kept constant, the amplitude of cell shortenings elicited by a Control-AP (left) vs. LY-AP (right) was not different, establishing that LY294002 enhances...
contractility and Ca$^{2+}$ transients by varying SR Ca$^{2+}$ load as a result of AP prolongation.

To further establish that APD prolongation is critical for the increased contractility observed following LY294002 addition, we examined the effects of LY294002 on myocyte shortening in response to stimulations with both Control AP and LY-AP (Fig. 6). Similar to the results shown in Fig. 5A and B, stimulation with a train of prolonged LY-APs increased ($P<0.01$) cell shortening by 23% in a group of five cells compared to the cell shortening measured in the same myocytes stimulated with a train of Control APs. However, bath application of 25 μmol/l LY294002 did not significantly alter cell shortening when myocytes were stimulated with Control or LY-APs. These findings further support our conclusion that LY294002 increases myocyte contractility primarily via prolongation of APD, although effects of LY294002 on other Ca$^{2+}$ handling proteins or myofilament could still concurrently contribute to the increased myocyte contractility observed.

3.2. Effects of LY294002 on outward K$^+$ currents

Since K$^+$ currents are key determinants of cardiac AP profile, the effects of LY294002 on voltage-dependent K$^+$ currents were examined. Fig. 7A shows representative outward K$^+$ current recordings elicited by 5-s depolarizations to +60 mV in the absence and presence of LY294002 as well as the current that was inhibited by LY294002 (i.e., the LY294002-sensitive current) with the inset displaying currents during the first 120 ms following depolarization. Bath application of 25 μmol/l LY294002 inhibited a large portion of the inactivating outward K$^+$ currents, leaving a very rapidly inactivating component and a sustained component. On average, the peak current, measured as the difference between the maximal outward current and the sustained current remaining at the end of 5-s pulses, was reduced ($P<0.05$, $n=10$) from 67.4 ± 7.8 to 45.4 ± 6.9 pA/pF. The sustained component at the end of 5-s pulses was also reduced ($P<0.05$, $n=10$) from 6.75 ± 0.93 to

![Fig. 6. Effects of LY294002 on cell shortening under AP-clamped conditions. (A) Representative recordings of steady-state cell shortening evoked by Control AP and LY-AP on the same cell in the absence (-LY294002) and presence (+LY294002) of 25 μmol/l LY294002. (B) Mean amplitude of percentage steady-state cell shortening (CS) evoked by Control APs and LY-APs in the absence (-LY294002) and presence (+LY294002) of LY294002. $n=5$. *$P<0.01.$](https://academic.oup.com/cardiovascres/article-abstract/62/3/509/319579/16579)
6.07 ± 0.98 pA/pF (n = 10). The LY294002-sensitive current density was 48.8 ± 6.7 pA/pF and peaked significantly later than the current recorded prior to LY294002 application (24.9 ± 1.6 ms vs. 4.0 ± 0.2 ms). By contrast, superfusion of myocytes with 100 nmol/l wortmannin did not affect outward K+ currents. Addition of 25 μmol/l LY294002 in the presence of wortmannin inhibited K+ currents to the same extent as LY294002 alone (data not shown), suggesting that reductions of K+ currents by LY294002 were unrelated to inhibition of PI3Ks.

The outward K+ current in mouse ventricular free wall is composed of multiple overlapping components: a rapidly inactivating transient outward K+ current (I_{to,f}), two slowly inactivating K+ currents (I_{k,slow1} and I_{k,slow2}) and a sustained non-inactivating component (I_{ss}) [17,18]. Two approaches were utilized to determine the K+ current components that are blocked by LY294002. First, we analyzed the decay phase of control currents and LY294002-sensitive currents (Fig. 7B). The decay phase of currents during 5-s pulses in the absence of LY294002 was well fit with a tri-exponential function with time constants of 180 ± 140 ms (τ_1), 310 ± 24 ms (τ_2) and 79 ± 12 ms (τ_3) which match closely to those reported previously for I_{k,slow2} (τ_1), I_{k,slow1} (τ_2) and I_{to,f} (τ_3), respectively [17–19]. The decay of current that is blocked by LY294002 (i.e., LY294002-sensitive current) could be fit with a bi-exponential equation (τ_1 = 1500 ± 98 ms and τ_2 = 211 ± 8 ms). The slow time constant τ_1 for the LY294002-sensitive currents was not different from the slowest time constant τ_1 of control currents, while the faster time constant τ_2 for the LY294002-sensitive currents had a magnitude closely resembling to the intermediate time constant τ_2 for the control currents (Table 2), suggesting that LY294002 may block I_{k,slow1} and I_{k,slow2}. This suggestion is consistent with the observation that the amplitude of the two components of LY294002-sensitive currents was also very similar to the corresponding amplitudes of I_{k,slow1} and I_{k,slow2} identified in the control currents (Table 2).

The above analysis relies on the accurate dissection of multiple overlapping currents, which is generally only valid if LY294002 does not affect the kinetics properties of the currents being blocked. To investigate further whether LY294002 blocks I_{k,slow1} and I_{k,slow2} currents, we used a combination of 50 μmol/l 4-AP and 135 mmol/l TEA which have been shown (at these concentrations) to block primarily I_{k,slow1} and I_{k,slow2}, respectively [17]. Typical current traces recorded during 5-s depolarizations to +60 mV in the absence and presence of both 4-AP and TEA as well as the corresponding 4-AP + TEA-sensitive current are illustrated on Fig. 7C (inset shows the same recordings for the first 120 ms). The combination of 4-AP and TEA reduced the amplitude of peak current from 56.1 ± 6.2 to 23.2 ± 4.2 pA/pF while also inhibiting I_{ss} from 6.3 ± 0.6 to 3.9 ± 0.5
Table 2
Current decay time constants and current densities obtained in the absence and presence of different agents

<table>
<thead>
<tr>
<th>Currents</th>
<th>n</th>
<th>$\tau_1$ (ms)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>$A_1$ (pA/pF)</th>
<th>$A_2$ (pA/pF)</th>
<th>$A_3$ (pA/pF)</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>1802.0 ± 139.6</td>
<td>310.6 ± 23.7</td>
<td>78.5 ± 11.5</td>
<td>12.2 ± 0.8</td>
<td>28.8 ± 4.5</td>
<td>21.9 ± 3.8</td>
<td>6.0 ± 0.9</td>
</tr>
<tr>
<td>LY294002-sensitive</td>
<td>10</td>
<td>1500.1 ± 94.7</td>
<td>210.8 ± 8.4*</td>
<td>14.5 ± 1.1*</td>
<td>31.8 ± 5.7</td>
<td>0.5 ± 0.3**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>1922.8 ± 237.5</td>
<td>314.2 ± 22.8</td>
<td>57.5 ± 2.7</td>
<td>9.9 ± 1.4</td>
<td>17.3 ± 2.4</td>
<td>18.6 ± 3.5</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>4AP + TEA-sensitive</td>
<td>8</td>
<td>2220.2 ± 408.2</td>
<td>339.1 ± 59.0</td>
<td>69.8 ± 7.5</td>
<td>9.3 ± 0.8</td>
<td>18.3 ± 4.1</td>
<td>11.9 ± 1.7**</td>
<td>1.4 ± 0.6**</td>
</tr>
</tbody>
</table>

$\tau_1$, $\tau_2$ and $\tau_3$ represent slow, intermediate and fast time constant, respectively; $A_1$, $A_2$ and $A_3$ represent the amplitude of the slow, intermediate and fast components, respectively; C represents the amplitude of the sustained component remaining at the end of 5-s depolarizations.

**P < 0.05.
**P < 0.01 compared with Control.

As is evident, 4-AP plus TEA inhibited a large portion of slowly inactivating outward current, leaving a rapidly inactivating component that is similar to the rapid current remaining in the presence of 25 μmol/l LY294002. These currents blocked by 4-AP plus TEA are virtually identical to the current blocked by LY294002, suggesting that LY294002 blocks both $I_{k,slow1}$ and $I_{k,slow2}$. Unlike LY294002, the decay of 4-AP + TEA-sensitive currents was fit with a tri-exponential function (Fig. 7D and Table 2), consistent with the previous finding that the mouse $I_{k,slow}$ was partially inhibited by 4-AP and TEA at these concentrations [17]. These findings suggest that LY294002 selectively inhibits $I_{k,slow1}$ and $I_{k,slow2}$ currents in mouse myocytes. Consistent with this conclusion, preliminary data in two cells showed that while the combination of 4-AP and TEA reduced the amplitude of peak current by 60% (from 45.3 ± 16.7 to 16.7 ± 0.2 pA/pF), addition of 25 μmol/l LY294002 in the presence of 4-AP and TEA caused only 6% further reduction in current amplitude (from 16.7 ± 0.2 to 14.2 ± 0.2 pA/pF).

3.3. Concentration dependence of $I_{k,slow}$ inhibition by LY294002

To evaluate the potency of inhibition of $I_{k,slow}$ by LY294002, we examined the dose–response effects of LY294002 on the current. Fig. 8A presents the outward K+ currents recorded from a myocyte exposed to different concentrations of LY294002. As is evident, increasing concentrations of LY294002 progressively and selectively blocked the slowly inactivating $I_{k,slow1}$ and $I_{k,slow2}$ currents. The percentage of block is plotted against the concentrations of LY294002 in Fig. 8B. The amplitude of $I_{k,slow}$ was determined as the difference between the current level at 80 ms of depolarization and $I_{ss}$ at the end of 5-s pulses. These results demonstrate that LY294002 inhibits $I_{k,slow1}$ and $I_{k,slow2}$ currents with an IC50 of 3.7 μmol/l and a power value of 1.8 (using the logistic function).

4. Discussion

LY294002 and wortmannin are two widely used PI3K inhibitors. In purified enzyme assays, the IC50 of PI3K inhibition was found to be 2–3 nmol/l for wortmannin [11,12] and 1.4 μmol/l for LY294002 [13]. Evidence for an involvement of PI3K in a given biological system including cardiac myocytes has typically been obtained by acutely treating cells with 10–100 nmol/l wortmannin and 5–50 μmol/l LY294002 [1,7,8]. In this study, we found that, at these concentrations, LY294002 exerted very different effects.
from wortmannin on cardiac myocyte K⁺ current, AP profile and contractility. Cell shortening of field-stimulated cardiomyocytes was increased by LY294002 but not by wortmannin, suggesting that increased myocyte contractility by LY294002 does not result from PI3K inhibition. Indeed, the positive inotropic effects of LY294002 could be linked to AP prolongation as a result of K⁺ current inhibition induced by LY294002. This conclusion is supported by the observation that LY294002 did not increase contractility in voltage-clamp studies using either step or AP depolarizations, while stimulation of myocytes with the prolonged APs recorded in LY294002-treated myocytes evoked greater contractions compared with Control APs in the absence or presence of LY294002. Similar differential effects of LY294002 and wortmannin have previously been reported in basophilic leukaemia cells [20] and airway smooth muscle cells [21], although the mechanism for these differences was unclear. Nonspecific effects of LY294002 and wortmannin on L-type Ca²⁺ channels in chromaffin cells have also been suggested by Warashina [22] in an earlier study.

The inability of wortmannin to affect basal myocyte contraction in our studies agrees with previous results in human myocardial strips [6]. However, the lack of effect of wortmannin was not expected given our previous results showing that a selective deletion of gamma isoform of PI3K increased myocardial contractile function [15]. The lack of effect of wortmannin on myocyte contractility would be expected if the basal level of PI3K activity is low in nonstimulated myocytes as reported previously [6,23], or alternatively, simultaneous inhibition of other PI3Ks (i.e., PI3Kα and PI3Kβ) that are also expressed in myocardium may offset the positive inotropic effects of PI3Kγ inhibition. Future studies will be required to fully address the cellular mechanisms accounting for the inability of wortmannin to increase contractility in isolated mouse myocytes.

4.1. Selective inhibition of I_k,slow by LY294002

Outward K⁺ currents in mouse ventricular free wall are composed of four overlapping and kinetically distinct components, I_k,slow, I_k,slow1, I_k,slow2 and I_at, precise details vary between different regions of the heart [17,18]. Studies on genetically engineered mice have demonstrated that Kv1.5 encodes for the faster I_k,slow1 [24,25] while Kv2.1 encodes for the slower I_k,slow2 [19]. Pharmacologically, I_k,slow1 is sensitive to micromolar concentrations of 4-AP, while I_k,slow2 is sensitive to millimolar levels of TEA. In our study, outward K⁺ currents could be fit with a function containing three exponential components with time constants of 80, 310 and 1800 ms, similar to those reported previously for I_k,slow1 and I_k,slow2, respectively [17,18]. LY294002 preferentially inhibited both I_k,slow1 and I_k,slow2 with a potency (IC₅₀ = 3.7 μmol/l) much greater than 4-AP blockade of I_k,slow1 (IC₅₀ = 32 μmol/l [25]) or TEA blockade of I_k,slow2 (IC₅₀ = 638 μmol/l [18]). Interestingly, the potency of Kv channel inhibition by LY294002 is comparable to that for inhibition of PI3K-dependent superoxide generation in neutrophils (IC₅₀ = 6.4 μmol/l) [27], although much lower concentrations biochemically inhibit purified PI3K enzymatic activities (IC₅₀ = 1.4 μmol/l) [13]. The blockade of outward K⁺ currents by LY294002 was not shared by wortmannin, suggesting that LY294002 inhibits I_k,slow via a PI3K-independent mechanism, possibly by direct channel blockade. Consistent with this suggestion, LY294002 has been shown recently to directly block Kv2.1 currents in heterologous system [26].

4.2. Functional implications of I_k,slow inhibition in mouse ventricle

The present study revealed that LY294002 prolonged APD₉₀. This effect was attributable to the inhibition of I_k,slow since our AP recordings were obtained in the presence of high EGTA levels (10 mmol/l) in order to minimize inward Na/Ca exchanger currents which can affect late repolarization. Prolongation of APD₉₀ but not APD₅₀ by LY294002 is consistent with the relatively slow kinetic properties of the LY294002-sensitive current which peaked around 25 ms after the onset of depolarization. The effects of LY294002 on AP profile are also consistent with previous pharmacological studies. Specifically, dominant-negative inhibition of Kv2.1 channels (I_k,slow2) caused APD₉₀ prolongation but not APD₅₀ [19]. Similarly, inhibition of Kv1.x-based current using a truncated Kv1.1 protein increased APD₉₀ but not APD₅₀ in apical myocytes which express Kv1.5 at high levels [28]. On the other hand, replacement of the Kv1.5 gene with the Kv1.1 gene (which is not expressed) in SWAP mice did not affect AP profile, although this was traced to compensatory increases in I_k,slow2 [25].

Prolongation of early repolarization of AP, in rat and mouse, following reduced I_k,slow, strongly enhances the Ca²⁺ transients and myocyte contractility by increasing the trigger L-type Ca²⁺ current as well as SR Ca²⁺ loading [16,30]. Little is known, however, about the role of I_k,slow in the modulation of cardiac contractility. Our findings clearly demonstrate that prolongation of late AP repolarization following I_k,slow inhibition by LY294002 led to an increased myocyte contractility as a result of increases in SR Ca²⁺ loading. Consistent with this conclusion, inhibition of I_k,slow1 by low levels of 4-AP (50 μmol/l) increase left ventricular systolic pressure in mouse [31]. Changes in the late-phase repolarization via I_k,slow inhibition is expected to reduce Ca²⁺ extrusion via the Na/Ca exchanger [32] while having little impact on trigger L-type Ca²⁺ current. The resulting increase in SR Ca²⁺ content by LY294002 will increase SR Ca²⁺ release by enhancing the open probability of SR Ca²⁺ release channels and by increasing the amount of releasable Ca²⁺ in the absence of changes in trigger I_Ca,t [33,34].

Cardiac myocyte contractility is determined by numbers of factors such as AP profile that controls trans-sarcolemmal...
Ca^{2+} influx and efflux, the properties of sarcoplasmic and SR Ca^{2+} handling proteins, the efficiency of excitation–contraction coupling and the properties of myofilaments. Our finding that LY294002 markedly enhanced the Ca^{2+} transients and contractility of field-stimulated myocytes but did not increase cell shortening under voltage clamp conditions strongly support the conclusion that LY294002 exerts its positive inotropic effects primarily via alteration of AP profile. Consistent with this conclusion, stimulation of myocytes with prolonged APs recorded in the presence of LY294002 elevated contractility to a similar extent as that observed in field-stimulated myocytes treated with LY294002. Nevertheless, it is conceivable that alterations in the activities of other targets affecting Ca^{2+} cycling (like Na/Ca exchangers) or contractile proteins might also contribute to the increase in contractility observed in the presence of LY294002. It has been shown that the outward Na/Ca exchange current (reverse mode) facilitates late AP repolarization in canine ventricular myocytes and that Na/Ca exchange current inhibition causes APD_{90} prolongation [29]. The effects of LY294002 on Na/Ca exchange function were not directly examined in our studies. However, if the enhanced contractility observed in the presence of LY294002 was due to Na/Ca exchanger inhibition, a change in contractility would be expected when LY294002 was added in our voltage-clamp studies using either steps or APs, which was not observed. Future studies will be required to directly assess whether LY294002 blocks Na/Ca exchange currents in mouse myocardium.

In contrast to our studies in mouse myocytes, previous studies using rat ventricular myocytes did not observe increases in cell shortening following LY294002 application under basal conditions [5,35]. The inability of LY294002 to enhance contractility in rat probably originate from known molecular differences in K^+ currents between mouse and rat myocytes [36]. Additional studies are clearly necessary to explore the basis for these differences between rat and mouse myocytes.

In summary, we found that LY294002 which is widely used as a “selective” PI3K inhibitor inhibits I_{k,slow} currents in mouse ventricular myocytes via a direct blockade of I_{k,slow} channels. Selective inhibition of I_{k,slow} currents by LY294002 slows late phase repolarization in mouse ventricular myocytes, thereby enhancing SR Ca^{2+} loading, Ca^{2+} release and contractility. Future studies using LY294002 will need to consider the effects of this agent on K^+ currents and membrane repolarization.

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References


[34] Gyorke I, Gyorke S. Regulation of the cardiac ryanodine receptor channel by luminal Ca2+ involves luminal Ca2+ sensing sites. Biophys J 1998;75:2801–10.
