Effects of Phloretin and Phloridzin on Ca\textsuperscript{2+} Handling, the Action Potential, and Ion Currents in Rat Ventricular Myocytes

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ABSTRACT

The effects of the phytoestrogens phloretin and phloridzin on Ca\textsuperscript{2+} handling, cell shortening, the action potential, and Ca\textsuperscript{2+} and K\textsuperscript{+} currents in freshly isolated cardiac myocytes from rat ventricle were examined. Phloretin increased the amplitude and area and decreased the rate of decline of electrically evoked Ca\textsuperscript{2+} transients in the myocytes. These effects were accompanied by an increase in the Ca\textsuperscript{2+} load of the sarcoplasmic reticulum, as determined by the area of caffeine-evoked Ca\textsuperscript{2+} transients. An increase in the extent of shortening of the myocytes in response to electrically evoked action potentials was also observed in the presence of phloretin. To further examine possible mechanisms contributing to the observed changes in Ca\textsuperscript{2+} handling and contractility, the effects of phloretin on the cardiac action potential and plasma membrane Ca\textsuperscript{2+} and K\textsuperscript{+} currents were examined. Phloretin markedly increased the action potential duration in the myocytes, and it inhibited the Ca\textsuperscript{2+}-independent transient outward K\textsuperscript{+} current (I\textsubscript{to}). The inwardly rectifying K\textsuperscript{+} current, the sustained outward delayed rectifier K\textsuperscript{+} current, and L-type Ca\textsuperscript{2+} currents were not significantly different in the presence and absence of phloretin, nor was there any evidence that the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was affected. The effects of phloretin on Ca\textsuperscript{2+} handling in the myocytes are consistent with its effects on I\textsubscript{to}. Phloridzin did not significantly alter the amplitude or area of electrically evoked Ca\textsuperscript{2+} transients in the myocytes, nor did it have detectable effects on the sarcoplasmic reticulum Ca\textsuperscript{2+} load, cell shortening, or the action potential.

Phloretin and its glycosylated precursor phloridzin are phytoestrogens belonging to the chalcone class of flavonoids (Thiyagarajah et al., 1991). They are derived from apples and apple products, and, along with other phytochemicals found in apples, they are reported to act as antioxidants, to be cardioprotective, to reduce the risk and symptoms of asthma, and to reduce the risk of diabetes (for reviews, see Rossetti et al., 1987; Boyer and Liu, 2004). The latter effect may be mediated by phlorizin, which is a competitive inhibitor of the sodium-dependent glucose transporter (Alvarado and Crane, 1964). Although the cardioprotective benefits of phloretin and phloridzin as well as those of other phytoestrogens have been supported by clinical studies (Clarkson, 2002; de Kleijn et al., 2002), the mechanisms through which this occurs are not understood and could be mediated through, or independent of, interaction with estrogen receptors. The latter nongenomic actions of phytoestrogens, which can occur quite rapidly, are generally less well known than those mediated by estrogen receptor interactions. The potential health benefits suggested by studies of dietary phytoestrogens, and current interest in alternative or naturopathic therapies, may lead to increased consumption of these compounds, and, in particular, to their inclusion in concentrated form in dietary supplements. It is therefore important that their mechanisms of action be understood.

In a previous study (Olson et al., 2006), we found that phloretin and phloridzin reduce the maximal velocity of Ca\textsuperscript{2+} uptake into the cardiac muscle sarcoplasmic reticulum (SR). The effects of these compounds on the SR are due primarily to their inhibitory effects on the sarcoplasmic reticulum/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA). Phloretin is lipophilic (Bechinger and Seelig, 1991), and phloridzin is transported across cell membranes by the sodium-dependent glucose transporter found in many cell types, including cardiac myocytes (Walle and Walle, 2003; Zhou et al., 2003).
These characteristics, and our results showing SERCA inhibition, suggest that phloretin and phloridzin could affect Ca\(^{2+}\) regulation and contraction in intact cardiac myocytes. To test this possibility, we examined the effects of phloretin and phloridzin on electrically evoked Ca\(^{2+}\) transients, the SR Ca\(^{2+}\) load, and cell shortening of intact rat ventricular cardiac myocytes. The results of these experiments further motivated us to examine the effects of phloretin and phloridzin on the action potential and whole-cell K\(^+\) and Ca\(^{2+}\) currents in these cells.

Materials and Methods

Isolation of Rat Cardiac Myocytes. Myocytes were isolated from adult male Sprague-Dawley rats as described previously (Ward and Giles, 1997). In brief, rats (200–225 g body weight) were killed in accordance with protocols approved by the Canadian Council on Animal Care. Hearts were removed, and they were mounted on a Langendorff apparatus and perfused for 5 min through an aortic cannula with Tyrode’s solution containing 1 mM Ca\(^{2+}\) (perfusion rate was 10 ml/min; all solutions were bubbled with 100% O\(_2\) and kept at 37°C). Hearts were then perfused in nominally Ca\(^{2+}\)-free Tyrode’s solution for 5 min followed by a 7-min perfusion in nominally Ca\(^{2+}\)-free Tyrode’s solution with 0.2 mg/ml collagenase (Yakult, Tokyo, Japan) and 0.004 mg/ml protease (type XIV; Sigma-Aldrich, St. Louis, MO). The right ventricular wall was then resected, and it was minced in 10 ml of Tyrode’s solution with 0.5 mg/ml collagenase, 0.1 mg/ml protease, and 3 mg/ml bovine serum albumin (BSA; Sigma-Aldrich) and gently shaken for 20 to 40 min in a water bath at 37°C. Aliquots of suspended cells were removed from the preparation at 5-min intervals, added to tubes with Krafthruß solution, and stored at 4°C until use. Recordings were made from quiescent single rod-shaped cells. Experiments were done at room temperature (20–23°C).

Measurement of Intracellular Ca\(^{2+}\) Transients in Single Rat Cardiac Myocytes. To measure Ca\(^{2+}\) transients in response to electrical field stimulation, freshly isolated myocytes were allowed to adhere for 20 min to the bottom of a recording chamber on the stage of an inverted microscope. The extracellular Krafthruß storage solution was then replaced with Tyrode’s solution containing 1 mM Ca\(^{2+}\), by perfusing the media through the chamber for 5 min at a rate of 0.5 ml/min. Before each experiment, the background fluorescence and light scatter of the cells was determined, and Ca\(^{2+}\) transients were recorded using a SPEX CMX fluorometer (fura-2 experiments; SPEX, Edison, NJ) or an SFX2 microfluorometer (fluo-3 experiments; Solamere Technologies, Salt Lake City, UT). Cells were visualized through a 100× oil immersion objective. The acetoxymethyl ester (AM) form of fura-2 or fluo-3 (2 μg/ml fura-2/AM or 1 μg/ml fluo-3/AM in Tyrode’s solution containing 1 mM Ca\(^{2+}\) and 1 mg/ml BSA) was then added to the chamber and incubated with the myocytes for 10 min. Excess dye was removed by perfusing the chamber for 5 min with Tyrode’s solution (with 1 mM Ca\(^{2+}\)). The cells were then left for 15 min (with continuous superfusion) to allow cleavage of the AM moiety.

For the experiments using fura-2, fluorescence was excited at 340 and 380 nm, and emission was recorded at 510 nm; 340/380 fluorescence ratios were determined every 10 ms. For the experiments with fluo-3, fluorescence was excited at 480 nm, and emission was monitored at 505 nm. Cells were field stimulated at a frequency of 1 Hz through electrodes on either side of the chamber. Cells were first field stimulated for 5 min to allow the SR to develop a constant amplitude of 100 ms, and they were then recorded when the Ca\(^{2+}\) transients produced after 5 min of continuous field stimulation were recorded for 1 min. Phloretin, phloridzin, or ethanol (vehicle control) was then added under continuous field stimulation.

Before analysis, Ca\(^{2+}\) transients were corrected for background fluorescence and light scatter. Individual peak heights for each Ca\(^{2+}\) transient were determined after subtraction of the fluorescence signal during diastole; 10 to 20 individual consecutive transients were averaged to obtain the mean transient amplitude and area under the transient. Changes in transient amplitude or area are expressed as percentage of control.

Assessment of Sarcoplasmic Reticulum Ca\(^{2+}\) Content. The content of the sarcoplasmic reticulum Ca\(^{2+}\) store was assessed through caffeine exposure (Kargacin et al., 2000, and references therein). Stores were first loaded by continuously field stimulating the cells at 1 Hz for 5 min. Field stimulation was then stopped, and within 3 s, 10 mM caffeine was applied using a rapid solution exchanger. The area of the resulting caffeine-evoked Ca\(^{2+}\) transient was then determined. Field stimulation was started again after the caffeine-evoked Ca\(^{2+}\) transient returned to baseline, and then phloretin, phloridzin, or ethanol only was added to the superfusion buffer for 10 min (with continuous field stimulation). Field stimulation was then stopped and a second caffeine pulse was then applied within 3 s, and the area under the resulting transient was compared with the area under the transient evoked by caffeine during the first (control) pulse. Experiments were also conducted in which two control Ca\(^{2+}\) transients were evoked by caffeine in buffer without ethanol, phloretin, or phloridzin to ensure that there were no significant changes in amplitude and/or area without the addition of drug (also see Kargacin et al., 2000).

Measurement of Cell Shortening. The extent of unloaded shortening of ventricular myocytes was determined and quantified by an edge-detection device (Crescent Electronics, Crescent, CO) that tracked changes in cell length. Shortening was elicited in response to action potentials applied to the cell under current-clamp conditions (see below) through a patch pipette. The extent of unloaded shortening was determined by averaging cell lengths measured from a train of five action potentials delivered at a frequency of 1 Hz. Unloaded cell shortening was calculated as the fractional change of cell length relative to diastolic values before, and after, exposure to phloretin, phloridzin, or ethanol (control). The exposure time (10 min) required to obtain the maximal effects was determined empirically.

Electrical Recording. Patch pipettes were pulled (P-97 puller; Sutter Instrument Company, Novato, CA) from borosilicate glass and polished on a microforge (MF-200; WPI, Sarasota, FL). Pipettes, when filled with internal recording solution (see below), had resistances of 2 to 3 MΩ. For electrical recording, aliquots of isolated myocytes in suspension were transferred to a chamber on the stage of a Nikon (TE300) inverted microscope, and the cells were allowed to settle to the bottom of the chamber. The chamber was perfused at a rate of 2 ml/min (bath solution was exchanged approximately every 6 s). When used, phloretin was added to the superfusion buffer, and, after 10 min of superfusion, current recordings were made. The exposure time (10 min) required to obtain the maximal effects of phloretin was determined empirically.

The Ca\(^{2+}\)-independent transient outward K\(^+\) current (I\(_{\text{so}}\)), sustained outward delayed rectifier K\(^+\) current (I\(_{\text{k1}}\)), and inwardly rectifying K\(^+\) current (I\(_{\text{k2}}\)) were recorded using the ruptured patch whole-cell configuration. Whole-cell K\(^+\) currents were evoked from a holding potential of −80 mV by 500-ms test pulses, in 10-mV increments, to voltages between −120 and +50 mV at a rate of 0.2 Hz.

To determine I\(_{\text{so}}\), whole-cell currents were first recorded with the above-mentioned voltage protocol, and they were then recorded when 100-ms prepulses to −40 mV (to voltage-inactive I\(_{\text{k1}}\)) were applied immediately before the 500-ms test pulses. Off-line subtraction of currents recorded using these protocols yielded I\(_{\text{so}}\). Outside-out currents were recorded in the presence of 200 μM Ba\(^{2+}\) (Ba\(^{2+}\) selectively blocks I\(_{\text{k2}}\)). Steady-state I\(_{\text{k1}}\) current values at the end of the 500-ms test pulses were analyzed.

To determine I\(_{\text{so}}\), I\(_{\text{k1}}\), and I\(_{\text{k2}}\), currents were recorded in the presence of 200 μM Ba\(^{2+}\), and they were evoked by 500-ms test pulses at voltages between −120 and +50 mV following 100-ms prepulses to −40 mV.
from a holding potential of −80 mV. The steady-state current at the end of the 500-ms test pulse is $I_{\text{to}}$ and was used in the analysis.

To measure the voltage dependence of $I_{\text{to}}$ inactivation, voltage-clamped cells were held at a holding potential of −80 mV and stepped for 5 s to conditioning voltages between −120 and −10 mV, in 5-mV increments. A test pulse was then applied by stepping to +50 mV for 1 s to activate and record the remaining noninactivated current. Results are expressed relative to the maximal currents elicited during the test pulses.

Reactivation kinetics of $I_{\text{to}}$ was examined using a standard paired pulse protocol. From a holding potential of −110 mV, $I_{\text{to}}$ was activated during 500-ms steps to current. Results are expressed relative to the maximal currents (50 mV for 1st to activate and record the remaining noninactivated current). A test pulse was then applied by stepping to +50 mV for 1 s to activate and record the remaining noninactivated current. Results are expressed relative to the maximal currents elicited during the test pulses.

To measure the voltage dependence of $I_{\text{to}}$ inactivation, voltage-clamped cells were held at a holding potential of −80 mV and stepped for 5 s to conditioning voltages between −120 and −10 mV, in 5-mV increments. A test pulse was then applied by stepping to +50 mV for 1 s to activate and record the remaining noninactivated current. Results are expressed relative to the maximal currents elicited during the test pulses.

Reactivation kinetics of $I_{\text{to}}$ was examined using a standard paired pulse protocol. From a holding potential of −110 mV, $I_{\text{to}}$ was activated during 500-ms steps to +50 mV (yielding current $P_2$). The cells were then returned to the holding potential of −110 mV for set durations ranging from 5 to 600 ms. This was then followed by a second voltage step to +50 mV (yielding current $P_3$). The current ratio, $P_3/P_2$, was then calculated for sets of currents. The fractional recovery of $I_{\text{to}}$ ($P_2/P_1$) as a function of time was determined from the peak currents obtained after the different recovery periods at −110 mV.

L-type Ca$^{2+}$ currents ($I_{\text{Ca,L}}$) were recorded in Cs$^+$ buffer (see below) to block K$^+$ currents using the perforated patch technique. $I_{\text{Ca,L}}$ was elicited by a 1-s ramp from −80 to −40 mV (to voltage inactivate Na$^+$ currents) followed by a 300-ms step to 10 mV, to activate $I_{\text{Ca,L}}$. Phloretin (20 µM) was added, and the voltage protocol was recorded every 2 min for a total of 10 min.

Action potentials were elicited in current-clamp mode through amphotericin B perforated patches. Currents (800 pA; 5-ms duration) were injected at a frequency of 1 Hz, filtered at 1 kHz, and sampled at 5 kHz. Five consecutive individual action potentials were averaged for analysis. Action potential durations were measured as the time required to reach 90% repolarization.

Analysis of Patch-Clamp Experiments. Whole-cell patch-clamp currents were recorded with an Axopatch 1-D amplifier (Molecular Devices, Sunnyvale, CA) using p-CLAMP 8.0 Digidata 1200 data acquisition and analysis software (Molecular Devices). Currents are expressed as current densities (pA/pF), and they were filtered at 1 kHz, sampled at 5 kHz, and corrected for cell capacitance before analysis. Recordings were also corrected for junction potentials (typically 9–11 mV).

Solutions. Tyrode’s solution contained 140 mM NaCl, 5.4 mM KCl, 1 mM Na$_2$HPO$_4$, 5 mM HEPES, 10 mM glucose, and 1 mM MgCl$_2$ (pH adjusted to 7.4 with NaOH). Kraftbrühe solution for cell storage contained 100 mM potassium glutamate, 10 mM potassium aspartate, 25 mM KCl, 20 mM glucose, 10 mM K$_2$HPO$_4$, 5 mM HEPES, 2 mM MgSO$_4$, 20 mM taurine, 5 mM creatine, 0.5 mM EGTA, and 0.1% BSA (pH adjusted to 7.2 with KOH). For recording K$^+$ currents, the internal pipette solution contained 20 mM KCl, 110 mM K$_2$-aspartate, 10 mM EGTA, 10 mM HEPES, 1 mM MgCl$_2$, 5 mM K$_3$ATP, 1 mM CaCl$_2$, and 10 mM NaCl (pH adjusted to 7.2 with KOH). During the recording of electrically or caffeine-evoked Ca$^{2+}$ transients, cell shortening or K$^+$ currents, cells were superfused with Tyrode’s solution containing 1 mM CaCl$_2$. For recording Ca$^{2+}$ currents, the internal pipette solution was composed of 120 mM Cs-aspartate, 30 mM CsCl, 1 mM MgCl$_2$, 5 mM Na$_2$ATP, and 10 mM HEPES (pH adjusted to 7.2 with CsOH). The external solution was 140 mM NaCl, 3 mM CsCl, 1 mM CaCl$_2$, 5.4 mM KCl, 1 mM Na$_2$HPO$_4$, 5 mM HEPES, 10 mM glucose, 1 mM MgCl$_2$, and 0.25 mM lidocaine (pH adjusted to 7.4 with NaOH). Stock solutions of 100 mM phloretin or 500 mM phloridzin were made up in ethanol and added to the superfusion buffer to obtain a final desired concentration of 20 µM (phloretin) or 100 µM (phloridzin). Control experiments were conducted by adding an equal volume of ethanol to the superfusion buffer. Fura-2/AM and fluo-3/AM were purchased from Invitrogen, Carlsbad, CA; all other chemicals were purchased from Sigma-Aldrich. Results are expressed as means ± S.E. Results were taken as significantly different at $p < 0.05$ after analysis using Student’s $t$ test (paired or unpaired as appropriate).

Results

Effects of Phloretin and Phloridzin on Ca$^{2+}$ Transients of Rat Cardiac Myocytes. Figure 1A shows trains of Ca$^{2+}$ transients recorded from freshly isolated cardiac myocytes in the absence and presence of phloretin or phloridzin. A, transients recorded from an isolated myocyte before (black trace) and 10 min after the addition of 20 µM phloretin to the superfusion buffer (red trace). B and C, summary of measurements of the amplitude (B) and area (C) of electrically evoked Ca$^{2+}$ transients in freshly isolated ventricular myocytes in the absence of, and 10 min after, exposure to ethanol, phloretin, or phloridzin. Mean amplitude and area of the transients are expressed relative to mean amplitude and area of transients evoked before the addition of 20 µM phloretin, 100 µM phloridzin, or ethanol only to the superfusion buffer. Myocytes were electrically stimulated at 1 Hz. Amplitude and area in the presence of phloretin were significantly greater than those measured when ethanol only was added to the superfusion buffer ($p < 0.03$; $n = 6$ for all experiments); amplitude and area values in the presence of phloridzin were not significantly different from those measured when ethanol only was added to the buffer.

Fig. 1. Ca$^{2+}$ transients recorded from electrically stimulated freshly isolated cardiac myocytes in the absence and presence of phloretin or phloridzin. A, transients recorded from an isolated myocyte before (black trace) and 10 min after the addition of 20 µM phloretin to the superfusion buffer (red trace). B and C, summary of measurements of the amplitude (B) and area (C) of electrically evoked Ca$^{2+}$ transients in freshly isolated ventricular myocytes in the absence of, and 10 min after, exposure to ethanol, phloretin, or phloridzin.
Ca\textsuperscript{2+} transients recorded from a single isolated rat ventricular myocyte in response to field stimulation at 1 Hz in the absence of phloretin and 10 min after superfusion of the recording chamber with 20 \mu M phloretin. The amplitudes of the Ca\textsuperscript{2+} transients recorded in the presence of phloretin were clearly greater than the amplitudes recorded in its absence; however, the diastolic Ca\textsuperscript{2+} level was not altered by phloretin. Figure 1B summarizes the results of experiments similar to those shown in Fig. 1A. Ca\textsuperscript{2+} transient amplitudes were significantly increased in the presence of phloretin over the amplitudes recorded before the addition of phloretin to the superfusion buffer or when ethanol alone was added to the buffer. The area of the electrically evoked Ca\textsuperscript{2+} transients was also significantly greater in the presence of phloretin (Fig. 1C). Phloridzin (100 \mu M) seemed to decreased the amplitude and area of the Ca\textsuperscript{2+} transients over those measured when the myocytes were superfused with ethanol only; however, this trend was not significant (Fig. 1, B and C). The rate constant for decay of electrically evoked Ca\textsuperscript{2+} transients in the presence of phloretin was significantly increased (from 169 \pm 12 ms in the absence to 200 \pm 10 ms in the presence of phloretin; \( p = 0.0018; n = 8 \)). The rate constants for decay were not significantly different in the presence and absence of phloridzin or ethanol.

The concentration of phloridzin (100 \mu M) used in the experiments described above had a maximal inhibitory effect on the velocity of SR Ca\textsuperscript{2+} uptake in our previous study (Olson et al., 2006). The concentration of phloretin (20 \mu M) used in the experiments in Fig. 1 was chosen because this concentration of phloretin inhibited SR Ca\textsuperscript{2+} uptake to approximately the same extent as 100 \mu M phloridzin. The phloretin concentration, however, is lower than that shown in our previous work to maximally inhibit Ca\textsuperscript{2+} uptake into cardiac SR vesicles. Therefore, we attempted to examine the effects of higher phloretin concentrations on Ca\textsuperscript{2+} transients in intact myocytes; however, when myocytes were superfused with buffer containing concentrations of phloretin greater than ~20 \mu M, the cells hypercontracted once electrical stimulation was started, and they did not relax again. For this reason, we used 20 \mu M phloretin and 100 \mu M phloridzin for the remaining experiments reported here.

The experiments described above were done using fluo-3 to record the Ca\textsuperscript{2+} transients. Because fluo-3 is a nonratiometric indicator, it is possible that motion artifacts or changes in cell size due to cell shortening (see below) could have affected the transients recorded. To rule out this possibility, electrically evoked Ca\textsuperscript{2+} transients were also recorded using the ratiometric Ca\textsuperscript{2+} indicator fura-2. The results (data not shown) obtained with fura-2 were similar to those recorded with fluo-3.

Assessment of the SR Ca\textsuperscript{2+} Load in the Presence of Phloretin and Phloridzin. To assess the Ca\textsuperscript{2+} load within the SR of cardiac myocytes upon exposure to phloretin and phloridzin, we examined the effects of caffeine on isolated myocytes before, and after, the addition of phloretin or phloridzin to the superfusion buffer. The SR Ca\textsuperscript{2+} load of myocytes, as assessed by the area of the Ca\textsuperscript{2+} transients elicited in response to 10 mM caffeine, was significantly increased after 10-min exposure to 20 \mu M phloretin; transient area in the presence of phloretin was 202 \pm 56\% of control (\( p = 0.03; n = 8 \)). In contrast, the SR Ca\textsuperscript{2+} load of the myocytes was not significantly different from control in the presence of 100 \mu M phloridzin or ethanol alone.

As described above, we noted an increase in the time constant for the decline of electrically evoked Ca\textsuperscript{2+} transients in the presence of 20 \mu M phloretin. This is consistent with an inhibition of SR Ca\textsuperscript{2+} uptake and/or Ca\textsuperscript{2+} extrusion through the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger; however, it is difficult to unambiguously interpret these results. The maximal cytosolic [Ca\textsuperscript{2+}] values recorded in the myocytes in response to electrical stimulation were different in the presence and absence of phloretin, and it is known that the maximal [Ca\textsuperscript{2+}] that SERCA is exposed to can affect its subsequent function (Bers and Berlin, 1995). The maximal intracellular [Ca\textsuperscript{2+}] values measured in response to caffeine varied more from cell to cell than those initiated by electrical stimulation. This offered us an opportunity to compare the rate of decline of caffeine-evoked Ca\textsuperscript{2+} transients from different cells when the maximal [Ca\textsuperscript{2+}] levels reached in control transients were similar in magnitude to those reached in other cells in the presence of phloretin. In analyzing 12 such transients in which the maximal Ca\textsuperscript{2+} levels were not significantly different from one another (five transients from control experiments and seven transients recorded in the presence of 20 \mu M phloretin), we did not detect any differences in the rate constants for the decline of the transients (determined from exponential fits to the declining phases of the transients; data not shown). This suggests that neither SERCA-mediated SR Ca\textsuperscript{2+} uptake nor Ca\textsuperscript{2+} extrusion by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was measurably affected in the presence of phloretin in intact myocytes.

Measurement of Cell Shortening and the Cardiac Action Potential in the Presence and Absence of Phloretin and Phloridzin. The effects of phloretin on electrically evoked Ca\textsuperscript{2+} transients in isolated ventricular myocytes would be expected to be associated with an increase in cell shortening in response to contractile stimuli. The results in Fig. 2 show that, after a 10-min exposure to 20 \mu M phloretin, the extent of cell shortening in response to action potentials...
the effects of phloretin on cell shortening and the action potential. Mean percentage of shortening was 3.3 ± 0.4% before the addition of phloretin, 12.0 ± 0.6% after 10 min of superfusion with phloretin, and 3.3 ± 0.2% after 10 min of washout (five experiments). Mean action potential duration was 34 ± 3 ms before the addition of phloretin, 180 ± 10 ms 10 min after superfusion with phloretin was started, and 52 ± 4 ms after 10 min of washout (five experiments).

Whole-Cell Ca\(^{2+}\) and K\(^+\) Currents in the Absence and Presence of Phloretin. The results presented above indicate that the effects of phloretin on Ca\(^{2+}\) handling and cell shortening in ventricular myocytes are accompanied by significant changes in the action potential elicited from these cells. The changes in action potential that were recorded in the presence of phloretin (Fig. 4) are similar to those reported by others (Zhang et al., 1994; Bogdanov et al., 1998; Biliczki et al., 2002; He et al., 2003) in response to the inhibition of cardiac cell K\(^+\) currents. An increase in L-type Ca\(^{2+}\) current would also be expected to increase action potential duration. We therefore examined the effects of phloretin on individual whole-cell Ca\(^{2+}\) and K\(^+\) currents in isolated myocytes.

L-type Ca\(^{2+}\) currents were measured using the voltage protocol described under Materials and Methods. Peak inward currents at 10 mV were recorded at 2-min intervals for 10 min, and they are expressed relative to the current values at time \(t = 0\) following the addition of vehicle (four experiments) or phloretin (four experiments). There were no significant differences in the L-type Ca\(^{2+}\) currents recorded in the presence and absence of phloretin at any of the time points examined (Fig. 5).

There are three principle K\(^+\) currents in cardiac myocytes that are responsible for maintaining resting potential and for mediating repolarization of these cells following an action potential. The \(I_{K1}\) is a constitutively active current that is responsible for stabilizing the resting potential of both atrial and ventricular myocytes (for review, see Lopatin and Nichols, 2001). It contributes to the final phase of repolarization of the myocytes following an action potential. The \(I_{Na}\) is present in many species, including rat and human (Apkon and Nerbonne, 1991; Li et al., 1998), and it is responsible for the early phase of repolarization of the cardiac action potential. The \(I_{K1}\) is a rapidly activating and noninactivating current that also contributes to the repolarization process in
human and rat cardiac myocytes (Apkon and Nerbonne, 1991; Fedida et al., 1993; Wang et al., 1993).

Representative whole-cell current traces recorded before the dissection of individual K\(^+\) currents are shown in (Fig. 6A). Current traces obtained after prepulses to \(-40\) mV to eliminate I\(_{\text{to}}\) are shown in Fig. 6B, and currents recorded in the presence of 200 \(\mu\)M BaCl\(_2\) to eliminate I\(_{\text{K1}}\) are shown in Fig. 6C. The effects of 20 \(\mu\)M phloretin on these currents are shown in Fig. 6, D to F. From comparison of the traces in Fig. 6, A and D, with those in Fig. 6, D and F, it is clear that phloretin has marked effect on the peak whole-cell outward currents but that it does not seem to affect the sustained phase of the currents. The peak whole-cell currents recorded after I\(_{\text{to}}\) was eliminated, however, do not seem to be affected by phloretin (compare Fig. 6, B and E).

Effects of Phloretin on Individual Ventricular K\(^+\) Currents. Figure 7 shows individual whole-cell K\(^+\) currents obtained as described in Materials and Methods. Representative I\(_{\text{to}}\) currents in the absence (Fig. 7A) and presence of 20 \(\mu\)M phloretin (Fig. 7D) indicate that peak I\(_{\text{to}}\) is reduced by 20 \(\mu\)M phloretin; however, I\(_{\text{K1}}\) (Fig. 7, B and E) and I\(_{\text{sus}}\) (Fig. 7, C and F) do not seem to be altered by phloretin. These results were confirmed when the current-voltage relationships of the three currents were examined. Figure 8A shows that I\(_{\text{to}}\) was significantly lower in the presence of phloretin at all potentials above \(-30\) mV. At \(+50\) mV, I\(_{\text{to}}\) was reduced from a mean control value of \(27 \pm 3\) to \(6 \pm 1\) pA/pF (\(p < 0.05\); \(n = 6\)) in the presence of 20 \(\mu\)M phloretin. I\(_{\text{K1}}\) (Fig. 7D) was not altered by 20 \(\mu\)M phloretin; mean I\(_{\text{K1}}\) at \(-120\) mV was \(-10.3 \pm 1.7\) and \(-8.8 \pm 0.7\) pA/pF (\(p = 0.45\); \(n = 6\)) in the absence and presence of phloretin, respectively. I\(_{\text{sus}}\) (Fig. 8C) in the presence of 20 \(\mu\)M phloretin seemed to be slightly greater at potentials more negative than \(-70\) mV and reduced at positive membrane potentials; however, the current values were

![Representative families of current traces illustrating the protocols used to isolate I\(_{\text{to}}\) and I\(_{\text{K1}}\) in isolated rat cardiac myocytes and the effects of phloretin on these currents. A and D, whole-cell currents obtained from a holding potential of \(-80\) mV by applying 500-ms voltage pulses to potentials between \(-120\) and \(+50\) mV in 10-mV increments. A, currents before the addition of 20 \(\mu\)M phloretin to the superfusion buffer, D, currents after phloretin addition. B and E, currents obtained with the same protocol as in A, except that a 100-ms prepulse to \(-40\) mV to inactivate I\(_{\text{to}}\) was included in the protocol before the 500-ms test pulses. B, currents before the addition of phloretin. E, currents after the addition of 20 \(\mu\)M phloretin to the superfusion buffer. C and F, currents obtained with the same protocol as in A except that 200 \(\mu\)M BaCl\(_2\) was included in the superfusion buffer to block I\(_{\text{K1}}\); C, currents before addition of phloretin; F, currents after the addition of 20 \(\mu\)M phloretin. Currents were measured before and after 10-min exposure to phloretin. Note that only every other current trace is plotted for clarity.](image1)

![Effects of phloretin on the three primary K\(^+\) currents in rat cardiac myocytes. A and D, I\(_{\text{to}}\). A, I\(_{\text{to}}\) recorded before the addition of phloretin to the superfusion buffer. D, I\(_{\text{to}}\) recorded after the addition of 20 \(\mu\)M phloretin to the buffer. B and E, I\(_{\text{K1}}\). B, I\(_{\text{K1}}\) recorded before the addition of phloretin to the superfusion buffer. E, I\(_{\text{K1}}\) recorded after the addition of 20 \(\mu\)M phloretin to the buffer. C and F, I\(_{\text{sus}}\). C, I\(_{\text{sus}}\) recorded before the addition of phloretin to the superfusion buffer. F, I\(_{\text{sus}}\) recorded after the addition of 20 \(\mu\)M phloretin to the buffer. Currents were measured before and after 10-min exposure to phloretin; currents were isolated using the protocols described under Materials and Methods.](image2)
phloretin for conditioning potentials between −80 and −60 mV, and it was significantly higher than control for conditioning potentials between −40 and −30 mV. Fitting the inactivation curves with a Boltzmann-type function indicated that the primary effect of phloretin on inactivation was to decrease the sensitivity of inactivation to changes in voltage (Table 1). There were no significant differences in the reactivation curves for $I_{\text{to}}$ (Fig. 9B) measured in the presence and absence of phloretin; comparison of individual curve fits showed that the time constant for recovery from inactivation was 17.3 ± 1.1 ms ($n = 3$) in control experiments and 17.2 ± 1.5 ms ($n = 3$) in the presence of phloretin.

**Discussion**

The main findings of this study are as follows. 1) Phloretin increases the area and amplitude of electrically evoked Ca$^{2+}$ transients, increases cell shortening, and increases the SR Ca$^{2+}$ load in rat ventricular myocytes. Phloretin also increases the duration of action potentials evoked from these cells under current-clamp conditions and specifically inhibits the Ca$^{2+}$-independent transient outward K$^+$ current of the cells. 2) Phloridzin did not have significant effects on the area or amplitude of electrically evoked Ca$^{2+}$ transients, nor did it measurably change the SR Ca$^{2+}$ load, the extent of unloaded shortening, or the action potential of the myocytes.

In a previous study (Olson et al., 2006), we reported that both phloretin, at concentrations of 20 μM or greater, and phloridzin, at concentrations greater that 50 μM, inhibit Ca$^{2+}$ uptake into isolated cardiac SR vesicles. From these results, one might predict that electrically evoked Ca$^{2+}$ transients, the SR Ca$^{2+}$ load and unloaded cell shortening would be decreased in intact cardiac myocytes in the presence of either compound at the concentrations used in our experiments. Our experiments, however, did not reveal any significant effects of phloridzin on intact cardiac myocytes, and the effects of phloretin are generally opposite of what one would predict from the study of its effects on SR function.

A recent study (Walle and Walle, 2003) showed that phloridzin is transported across cell membranes by the sodium-dependent glucose transporter found in many cell types, including cardiac myocytes (Zhou et al., 2003). Our failure to detect an effect of phloridzin on intact myocytes could be a result of the transport mechanism not allowing enough phloridzin to enter the cells to significantly affect any of the parameters that we measured. In this regard, it should be noted that SR Ca$^{2+}$ uptake velocity was only reduced to a maximal extent of approximately 20% by phloridzin in our previous experiments (Olson et al., 2006). Thus, a limited transport of phloridzin into the myocytes could reduce the extent of it effects to an undetectable level. It is also possible that any inhibitory effects on Ca$^{2+}$ handling were balanced by agonistic effects on other mechanisms.

Our results showing that phloretin increases cell shortening, Ca$^{2+}$ transient amplitude, and the SR Ca$^{2+}$ load in intact cardiac myocytes are consistent with a prolongation of the action potential through a mechanism that involves specific inhibition of $I_{\text{to}}$. The effects of phloretin on $I_{\text{to}}$ develop relatively slowly (maximal effect after ~10 min), suggesting that they may be use-dependent or that they may require phloretin to insert into, or cross, the plasma membrane. Although determination of the time constants for the decline
of electrically evoked Ca\(^{2+}\) transients suggests that Ca\(^{2+}\) removal processes might be affected by phloretin. This was not confirmed when caffeine-evoked transients in control conditions were compared with transients recorded in the presence of phloretin when the transients declined from similar starting Ca\(^{2+}\) levels. This suggests that neither the Na\(^{+}/\) Ca\(^{2+}\) exchanger nor SERCA function was measurably affected by phloretin in our experiments with intact myocytes. It should be noted, however, that the effects of phloretin on intact cardiac myocytes in these experiments were examined at a phloretin concentration of 20 \(\mu\)M. At this concentration, phloretin was shown to have only a minimal inhibitory effect on the rate of SR Ca\(^{2+}\) uptake (\(\approx 10\%\) inhibition) in our previous study (Olson et al., 2006); thus, an effect on SR function would probably be masked by the more robust effect on I\(\text{to}\) and the cardiac action potential. We were unable to examine the effects of higher concentrations of phloretin in the present study because, as noted above, at concentrations above 20 \(\mu\)M, the isolated ventricular myocytes hypercontracted and did not relax again when they were electrically stimulated.

To our knowledge, the present study is the first to examine the actions of phloretin on mammalian cardiac muscle K\(^{+}\) channels, and it is the first to show inhibition of I\(\text{to}\). Phloretin, however, is known to affect some other types of K\(^{+}\) channels. It has been shown to increase the current through mouse and human large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels expressed in Xenopus laevis oocytes or in human embryonic kidney 293 cells (Gribkoff et al., 1996). It also opens Ca\(^{2+}\)-activated K\(^{+}\) channels in myelinated nerve fibers (Koh et al., 1994) and in human glioma cells in culture (Ransom and Sontheimer, 2001). In addition to agonistic effects on Ca\(^{2+}\)-activated K\(^{+}\) channels, phloretin has also been reported to block two types of delayed-rectifier K\(^{+}\) channels (I and F channels) in myelinated nerve fibers from X. laevis (Klusemann and Meves, 1992; Koh et al., 1994) and the fast K\(^{+}\) channel in frog nerve fibers (Klusemann and Meves, 1991).

Although phloretin increases inactivation of I\(\text{to}\) relative to control at conditioning voltages between \(-80\) and \(-60\) mV, this cannot completely explain the effects of phloretin on I\(\text{to}\) in the voltage-clamp experiments. When cells are held at a holding potential of \(-80\) mV, one would expect phloretin to reduce I\(\text{to}\) by approximately 20%, based on its effect on voltage-dependent inactivation (Fig. 9). In the voltage-clamp experiments, however, I\(\text{to}\) was reduced by 80% in the presence of 20 \(\mu\)M phloretin (Fig. 8).

Analysis of the inhibition of I\(\text{to}\) in the presence of phloretin revealed that the extent of inhibition was independent of voltage; the mean value of I\(\text{to}\) in the presence 20 \(\mu\)M phloretin was 21.6% of its value in its absence. The solid line on the phloretin trace in Fig. 8 shows the value of I\(\text{to}\) predicted at each test voltage when the control values of I\(\text{to}\) were reduced by the mean percentage of inhibition determined from all test voltages. What makes these findings even more intriguing is that although we observed a near 80% reduction of I\(\text{to}\) this concentration of phloretin had little or no effect on other K\(^{+}\) currents in these cells (Fig. 8), suggesting a relative specificity for I\(\text{to}\). The observed action potential prolongation and positive inotropic effect is also consistent with I\(\text{to}\) inhibition, probably as a consequence of increased Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels due to a delay in repolarization (for review, see Sah et al., 2003).

As noted above, phytosterogens have been shown in clinical studies to reduce the risk of cardiovascular disease (Clarkson, 2002; de Kleijn et al., 2002), although there is little experimental information pertaining to the mechanisms by which this might occur. The results of the present study suggest that there could be risks associated with high levels of phloridzin or phloretin consumption. Rats ingesting
the equivalent of 22 mg of phloretin in a single feeding (either directly as phloretin or as phloridzin) were found to have plasma phloretin levels of \(~70~\mu M\) (approximately 10% of this total was in the form of free phloretin) after 10 h (Crespy et al., 2001). These levels are of the same order of magnitude as that of the concentration shown to affect the contractility and \(Ca^{2+}\) handling in cardiac myocytes in the present study. The phloretin/phloridzin content of apple pulp depends upon variety, but it may be as high as \(~20~mg/kg\) (Escarpa and Gonzalez, 1998). Thus, it is unlikely that the ingestion of phloretin through the normal consumption of apples or apple products could affect cardiac contractility; however, dietary supplements with concentrated amounts could pose a health risk. Further assessment of this possibility seems warranted if such products are contemplated or become available.

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References


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