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Comparison of Cardiac Contractile and Intracellular Ca^{2+} Response between Estrogen and Phytoestrogen α-Zearalanol in Ventricular Myocytes

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While the benefit and risk of estrogen replacement therapy for cardiovascular disease remains controversial, women frequently choose alternatives to estrogen such as phytoestrogen for treatment of menopause even though medical indications for estrogens may exist. Phytoestrogens also possess distinct advantages over mammalian estrogens because their usage in men without feminizing side effects. Nevertheless, the cardiac contractile function of estrogen or phytoestrogen has not been clearly elucidated. The aim of the present study was to compare the effect of 17β estradiol (E_2) and phytoestrogen α-zearalanol (ZAL) on cardiac mechanical function and intracellular Ca^{2+} transients at cellular levels. Isolated ventricular myocytes from adult female rats were stimulated to contract at 0.5 Hz. Contractile properties were evaluated using an IonOptix MyoCam® system including peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR_{90}), and maximal velocity of shortening/relengthening (± dL/dt). Intracellular Ca^{2+} properties were evaluated as fura-2 fluorescent intensity change (ΔFFI) and intracellular Ca^{2+} decay rate. Acute administration of E_2 (10^{-9}–10^{-5} *M*) elicited a concentration-dependent increase in PS and ΔFFI, with maximal augmentation of approx 35% and 25%, respectively. TPS, TR_{90}, ± dL/dt, resting intracellular Ca^{2+} level, and intracellular Ca^{2+} decay were unaffected by E_2. None of the mechanical or intracellular Ca^{2+} indices tested was affected by phytoestrogen ZAL (10^{-9}–10^{-5} *M*). Our results revealed a direct cardiac stimulatory action from E_2 but not from phytoestrogen ZAL on ventricular contraction, likely mediated through enhanced intracellular Ca^{2+} release.

Key Words: Estrogen; phytoestrogen; ventricular myocyte; contraction; intracellular Ca^{2+}.

Introduction

For many years, estrogen replacement therapy has been considered to offer not only relief of climacteric symptoms but also protection against osteoporosis, cardiovascular diseases, and cognitive disorders. However, recent randomized clinical trials failed to confirm cardiovascular benefits of estrogen replacement therapy in women with compromised heart conditions and even indicated possibly enhanced cardiac risk associated with this management, in addition to its known aggressive effect on breast, endometrial, and ovarian cancers *(1,2)*. It appears that current indications for estrogen replacement therapy in otherwise healthy women should largely target on reduction of menopausal symptoms and prevention of osteoporosis. The use of estrogen replacement therapy to lessen cardiac risk is still controversial and may not be recommended as a standard care (*1*). Estrogen or estrogen plus progesterone therapy has been demonstrated to improve left ventricular diastolic and aortic elasticity functions in healthy postmenopausal women *(3,4)*. This is consistent with the observations that an acute, single dose of oral or sublingual estrogen administration significantly improves left ventricular diastolic function in postmenopausal women with diastolic dysfunction *(5,6)*. Nevertheless, other randomized double-blind placebo-controlled clinical trials did not show any affect of transdermal estrogen therapy on left ventricular function in healthy postmenopausal women *(7–9)*.

While estrogen replacement therapy has created substantial controversies, attention has recently been drawn toward the clinical value of phytoestrogens, especially natural isoflavones, because of their overt protective effects against certain chronic diseases such as ischemic heart diseases, ventricular dysfunction, atherosclerosis, osteoporosis, and cancer *(10,11)*. Phytoestrogens or "plant estrogen" are naturally occurring compounds from cereals, vegetables, and medicinal plants and share structural similarity with 17β-estradiol (E_2). Phytoestrogens are considered as "attenuated estrogens" with less estrogenic tumor-promoting capacity *(13)*. More important, cardiovascular benefits of phytoestrogens appear to be equal for both males and females, making

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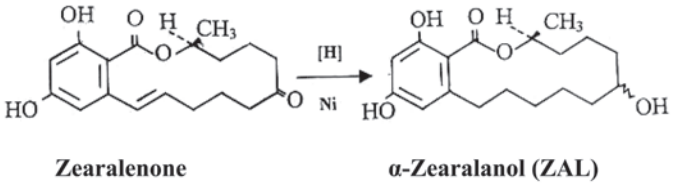

Fig. 1. Chemical structure of ZAL.

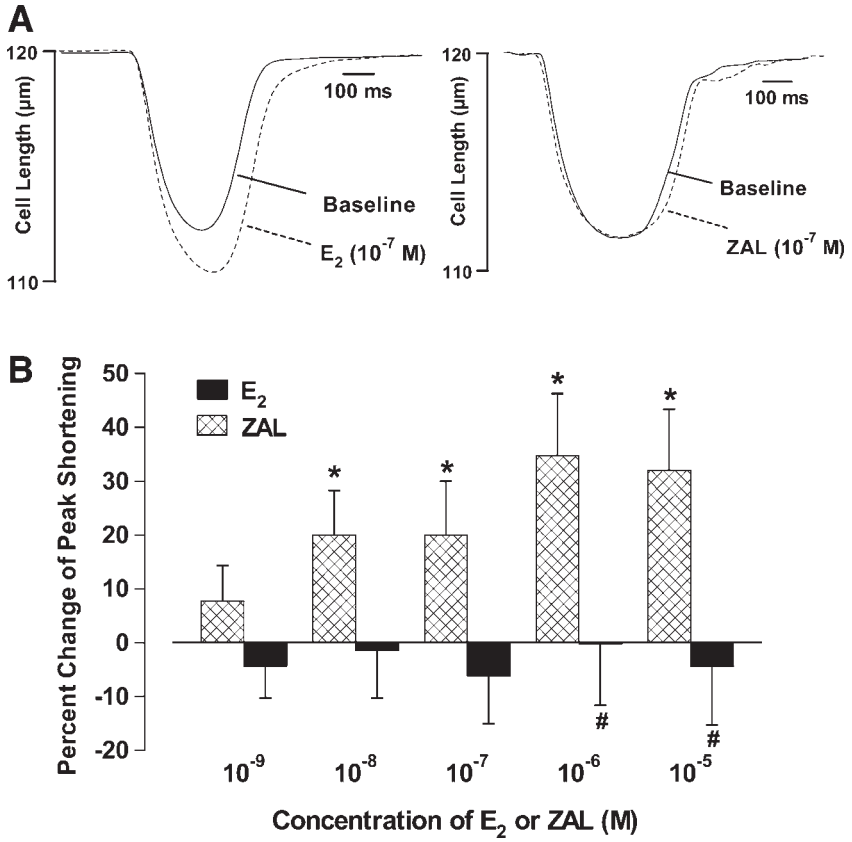

Fig. 2. (**A**) Representative traces depicting the effect of E_2 (10^{-7} *M*) or ZAL (10^{-7} *M*) on cell shortening in ventricular myocytes. (**B**) Concentration-dependent response of E_2 or ZAL (10^{-9}–10^{-5} *M*) on peak cell shortening (PS), indicative of peak ventricular contractility. Data are presented as percentage change from baseline PS, which was 6.48 ± 0.68%. Mean ± SEM, $n = 23$–28 cells per data point. $^{*}p < 0.05$ vs respective control (con) value; $^{\#}p < 0.05$ vs E_2 group at the same dose.

it possible for men to use without worrying about the "feminizing" reproductive effect of estrogen *(10,13)*. However, the cardiac contractile profiles of many phytoestrogens have not been examined or compared to that of estrogen, thus limiting their clinical usage. The aim of this study was to compare cardiac contractile and intracellular Ca^{2+} response of E_2 and a newly identified phytoestrogen α-zearalanol (ZAL) in isolated ventricular myocytes. ZAL is a reduced product of the *Gibberella zeae* metabolite zearalenone (Fig. 1), a member of β-resorcylates commonly used in animal husbandry to facilitate growth *(12)*. ZAL is rapidly metabolized in the body and may promote protein synthesis or increase the lean meat ratio, in a manner similar to the growth-promoting effect of estrogen, however, without estrogenic tissue proliferation *(12,14)*. ZAL and zearalenone have been used as relatively safe and efficient food additives to facilitate growth in animal husbandry *(12,15)*. Recently, ZAL has been demonstrated to have estrogenic properties in preserving ovariectomy-induced bone loss and endothelial dysfunction through an estrogen receptor-mediated mechanism *(16,17)*.

Results

Effect of E_2 and ZAL on Myocyte Shortening (PS)

The average resting cell length of ventricular myocytes used in this study was 108.2 ± 2.7 μm ($n = 51$). Acute exposure (5 min) of E_2 or ZAL did not significantly affect resting cell length over the range of concentrations tested (data not shown). Representative traces depicting the effect of E_2 (10^{-7} *M*) or ZAL (10^{-7} *M*) on myocyte peak shortening (PS) under physiological extracellular Ca^{2+} environment (1.0 m*M*) are shown in Fig. 2A. At the end of a 5 min exposure to this concentration of E_2 or ZAL, PS was increased by approx 20% in response to E_2, whereas negligible change

Table 1
Effect of E_2 and ZAL on Duration and Maximal Velocity of Myocyte Shortening and Relengthening in Adult Rat Ventricular Myocytes[a]

	TPS (ms)	TR_{90} (ms)	+dL/dt (μm/s)	−dL/dt (μm/s)
Control	159 ± 10	246 ± 21	121 ± 12	−96 ± 14
E_2 10^{-9} *M*	166 ± 11	246 ± 20	123 ± 12	−80 ± 11
E_2 10^{-8} *M*	173 ± 9	274 ± 26	117 ± 13	−83 ± 13
E_2 10^{-7} *M*	176 ± 11	276 ± 25	122 ± 16	−78 ± 15
E_2 10^{-6} *M*	188 ± 11	248 ± 16	126 ± 14	−89 ± 13
E_2 10^{-5} *M*	185 ± 10	242 ± 17	128 ± 15	−85 ± 15
Control	187 ± 18	357 ± 30	74 ± 9	−56 ± 8
ZAL 10^{-9} *M*	201 ± 14	347 ± 29	65 ± 8	−50 ± 9
ZAL 10^{-8} *M*	197 ± 17	384 ± 33	62 ± 7	−46 ± 6
ZAL 10^{-7} *M*	200 ± 20	393 ± 34	62 ± 9	−46 ± 7
ZAL 10^{-6} *M*	202 ± 23	384 ± 35	69 ± 11	−59 ± 13
ZAL 10^{-5} *M*	198 ± 20	384 ± 33	67 ± 12	−54 ± 11

[a]Time-to-peak shortening (TPS), time-to-90% relengthening (TR_{90}), maximal velocities of shortening and relengthening (± dL/dt). Data represent mean ± SEM, n = 23–28 cells per data point.

was noted for PS in response to ZAL. E_2 (10^{-9}–10^{-5} *M*) but not ZAL elicited a concentration-dependent increment in PS, with a maximal response of approx 35% for E_2. The threshold of E_2-induced augmentation was between 10^{-9} *M* and 10^{-8} *M* (Fig. 2B). E_2-induced positive myocyte contractile response was maximal within 3 min of exposure and was reversible upon washout (data not shown). Neither E_2 nor ZAL significantly affected the maximal velocities of shortening/relengthening (± dL/dt), duration of shortening (TPS), and duration of relengthening (TR_{90}) (Table 1). There was no significant interaction between the drug (E_2 or ZAL) and doses on all mechanical indices examined in Table 1. Finally, the E_2 (10^{-8} *M*)-induced elevation in PS was prevented by the E_2 receptor antagonist ICI 182,780 (10^{-8} *M*). PS was 7.29 ± 0.48%, 8.48 ± 0.50%, and 6.30 ± 0.59%, in control, E_2 and E_2+ICI 182,780 groups, respectively (n = 19 cells per group, significance was reached between the E_2 group and any other groups). ICI 182,780 (10^{-8} *M*) did not affect basal myocyte cell shortening nor the PS response of ZAL (data not shown), consistent with our previous report *(18)*.

Effect of E_2 and ZAL on Intracellular Ca^{2+} Transients

To determine whether E_2 or ZAL-induced response in cell shortening was due to altered availability of intracellular Ca^{2+}, the effect of E_2 or ZAL on intracellular Ca^{2+} transient fura-2 fluorescent intensity change (ΔFFI) was examined. Representative traces of intracellular Ca^{2+} transients shown in Fig. 3A depict that 10^{-7} *M* E_2 increased electrically stimulated rise of FFI (ΔFFI) by approx 18%, whereas ZAL displayed little effect. Consistent with their response on peak shortening (PS), E_2 (10^{-9}–10^{-5} *M*) elicited a concentration-dependent elevation of ΔFFI, with a maximal response of approx 25% (Fig. 3B). The threshold of inhibition was between 10^{-9} *M* and 10^{-8} *M* for E_2 (Fig. 3B), similar to that in cell shortening response. ZAL (10^{-9}–10^{-5} *M*) did not elicit any significant effect on ΔFFI. The response of intracellular Ca^{2+} transients suggests that an increase in intracellular free Ca^{2+} is likely to be responsible, at least in part, for E_2-induced positive myocyte contractile response. Neither the resting intracellular Ca^{2+} level nor the intracellular Ca^{2+} decay rate was affected by E_2 or ZAL (Table 2). Both resting intracellular Ca^{2+} level and ΔFFI tended to be different in control myocytes between E_2 and ZAL groups, although no statistical significance was reached. There was no significant interaction between the drug (E_2 or ZAL) and doses on all mechanical indices examined in Table 2.

Discussion

Our current study reported that acute administration of estrogen (17β estradiol, E_2) significantly enhanced peak shortening and intracellular Ca^{2+} rise at supraphysiological and pharmacological concentrations, without affecting maximal velocity of shortening/relengthening, duration of shortening/relengthening, resting intracellular Ca^{2+} levels, and intracellular Ca^{2+} decay rate in ventricular myocytes. Unlike E_2, the newly identified phytoestrogen ZAL failed to elicit any effect on cell mechanics and intracellular Ca^{2+} transients. Our study indicated E_2-induced cardiac contractile effect is likely mediated through the E_2 receptor although evidence regarding any role of the E_2 receptor for ZAL in cardiac myocytes is not conclusive at this time. ZAL is a known phytoestrogen with estrogenic activities through the estrogen receptor but with the likelihood of much lower affinity *(17)*. Our observation of the disparate response between E_2 and ZAL may indicate an overt difference in cardiac contractile profiles between estrogen and certain phytoestrogens.

Our current finding that acute E_2 exposure promotes cell shortening and intracellular Ca^{2+} response in isolated ventricular myocytes is somewhat supported by previous reports. It was shown that short-term treatment of physiological levels of estrogen preserves ventricular contractility against cardiac stunning in adult female rabbits *(20)*. Estrogen replacement therapy has been reported to cause modest but significant increase in cardiac output, ejection fraction, and left ventricular mass without affecting heart rate, suggesting a direct myocardial improvement from estrogen replacement therapy *(21)*. In addition, our earlier study showed reduced cardiac contractility in ventricular myocytes from ovariectomized rats compared to those from sham-operated rats *(18)*. Although the precise mechanism(s) of action behind estrogen-induced cardiac contractile response is not fully clear at this time, the similarity in the pattern of response between

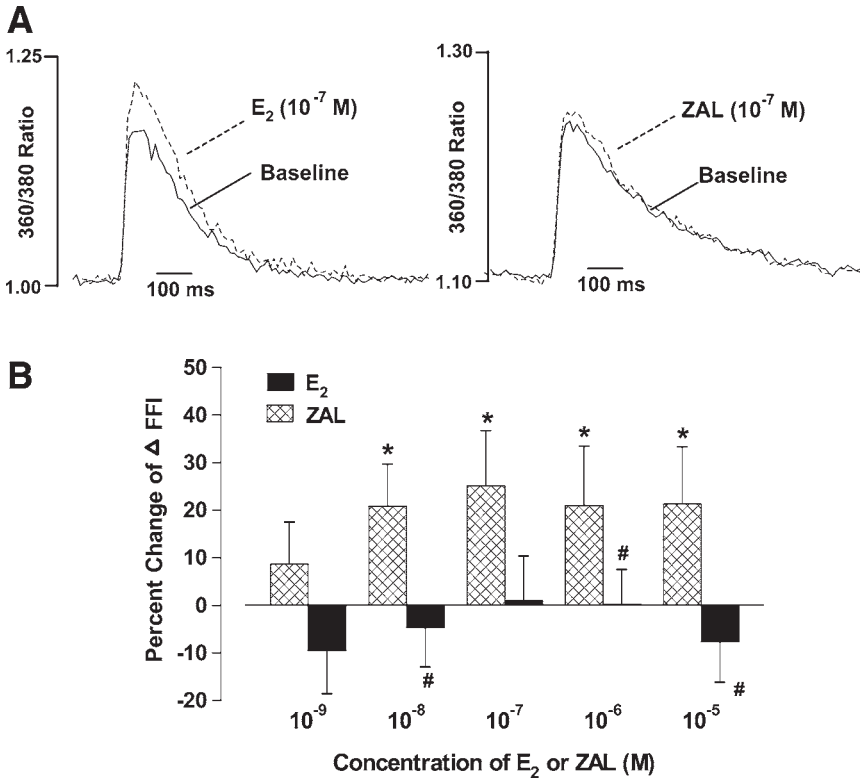

Fig. 3. (**A**) Representative traces depicting effect of E_2 (10^{-7} *M*) or ZAL (10^{-7} *M*) on electrically stimulated rise in fura-2 fluorescent intensity (ΔFFI) of intracellular Ca^{2+} transients, indicative of SR Ca^{2+} release upon electrical stimulation, in ventricular myocytes. (**B**) Concentration-dependent response of E_2 or ZAL (10^{-9}–10^{-5} *M*) on ΔFFI. Data are presented as percentage change from respective basal ΔFFI value. Mean ± SEM, n = 14–16 cells per data point. $^{*}p < 0.05$ vs respective control (con) value; $^{\#}p < 0.05$ vs E_2 group at the same dose.

Table 2
Effect of E_2 and ZAL on Baseline Intracellular Ca^{2+} Level and Intracellular Ca^{2+} Decay Rate in Adult Rat Ventricular Myocytes[a]

	Baseline (360/380 ratio)	Ca^{2+} decay rate (ms)
Control	0.97 ± 0.03	865 ± 144
E_2 10^{-9} *M*	0.97 ± 0.02	938 ± 151
E_2 10^{-8} *M*	0.95 ± 0.03	943 ± 158
E_2 10^{-7} *M*	0.95 ± 0.03	937 ± 153
E_2 10^{-6} *M*	0.97 ± 0.03	909 ± 155
E_2 10^{-5} *M*	0.97 ± 0.03	930 ± 160
Control	1.07 ± 0.05	641 ± 132
ZAL 10^{-9} *M*	1.06 ± 0.04	715 ± 97
ZAL 10^{-8} *M*	1.06 ± 0.04	727 ± 121
ZAL 10^{-7} *M*	0.94 ± 0.13	686 ± 132
ZAL 10^{-6} *M*	1.04 ± 0.04	644 ± 123
ZAL 10^{-5} *M*	1.09 ± 0.04	759 ± 127

[a]Data represent mean ± SEM, n = 14–16 cells per group.

mechanical and intracellular Ca^{2+} effects of E_2 suggests that E_2 may promote cardiac contractile function likely through elevated intracellular Ca^{2+} rise. The direct impact of estrogen and other ovarian hormones such as progesterone on cardiac contractile function has been testified with altered cardiac mechanical function and intracellular Ca^{2+} homeostasis following ovariectomy *(18,22)*. Although a study by Curl and colleagues found facilitated intracellular Ca^{2+} clearing and increased intracellular Ca^{2+} levels in ventricular myocytes from ovariectomized rats when compared with sham or ovariectomized but E_2 re-supplemented groups *(22)*, these findings are somewhat different or are contrary to our findings from previous *(18)* and current studies. Our present study found that E_2 increased intracellular Ca^{2+} release without affecting intracellular Ca^{2+} decay rate. These discrepancies may be related to difference in experimental settings (in vivo vs in vitro), duration of E_2 deprivation or resupplementation, species and age of experimental animals, etc. Other possible scenarios contributing to the direct cardioprotective effects of E_2 may include alteration in glycogen and glucose utilization, as well as improvement in lipid profile *(23)*.

Phytoestrogens have long been known to prevent or delay the development of a number of devastating cardiovascular diseases including coronary heart diseases, atherosclerosis, and hypercholesterolemia *(10,24)*. Although a number of studies have given much credit of such cardiovascular protection to the ability of phytoestrogens to improve blood lipid profile and reduce arterial fatty streaks *(10,23,24)*, the physiological mechanism by which phytoestrogens improve ventricular function has been controversial. Although our study failed to depict any cardiac response for ZAL, the iso-

flavone phytoestrogen genistein has recently been reported to elicit novel cardiac actions including enhanced SR Ca^{2+} load, Na^+/Ca^{2+} exchange, and myofilament Ca^{2+} sensitivity in conjunction with blockade of voltage-dependent Ca^{2+} channels. These actions of genistein should lead to an overall elevation in myocyte contractility *(19)*. Results from our current study do not favor any direct cardiac contractile effect as a major contributing factor for phytoestrogen-elicited beneficial cardiac effects, suggesting that differences in the ability of regulating cardiac contractile function may exist among various kinds of phytoestrogens. Zearalenone, the parent compound of ZAL, is considered as an endogenous hormone present in wheat, cotton, corn, celery, carrots, beets, etc. *(25)*. Toxicological study revealed that the reduced metabolite of zearalenone, α-ZAL, is much safer than E_2 *(15)*. These properties of ZAL, in conjunction with its preservation of bone loss and endothelial dysfunction under ovariectomized states *(16,17)*, would certainly make ZAL a likely candidate in postmenopausal women requiring estrogen replacement therapy. Preliminary evidence from our group indicated that ZAL may effectively prevent high fat-induced fatty plaque formation in experimental atherosclerosis (Dai et al., unpublished data). It is worth mentioning that the effect of ZAL on cardiac contraction may not be representative for other phytoestrogens and therefore caution must be taken when assessing cardiovascular protective effects of other phytoestrogens. For example, genistein, an isoflavonoid phytoestrogen, was shown to acutely enhance cell shortening and intracellular Ca^{2+} transient in guinea-pig ventricular myocytes despite its ability to potently inhibit the L-type Ca^{2+} current. It is believed that such positive cardiac contractile action of genistein is mediated through inhibition of protein tyrosine kinase *(19)*.

During the last decades, hormones from natural sources have played a significant role in disease prevention and treatment, especially in the cardiovascular system. Our study has revealed that cardiac contractile response may be vastly different between estrogen and certain phytoestrogens, which may be used as alternatives for estrogen. The mechanisms of estrogen or phytoestrogens on cardiac contractile components such as SR Ca^{2+} load or release, Na^+/Ca^{2+} exchanger, and myofilament Ca^{2+} sensitivity deserve further scrutinized study. These approaches are essential to understand the cardiac excitation-contraction coupling and other cellular effects phytoestrogen as an alterative for estrogen.

Materials and Methods

Isolation of Ventricular Myocytes

The experimental procedures described in this study were approved by the Institutional Animal Care and Use Committee of University of North Dakota (Grand Forks, ND, USA) and University of Wyoming (Laramie, WY, USA). Single ventricular myocytes were isolated from adult female Sprague–Dawley rats (200–225 g) as described previously *(18)*. Briefly, hearts were rapidly removed and perfused (at 37°C) with oxygenated (5% CO_2–95% O_2) Krebs–Henseleit bicarbonate (KHB) buffer (m*M*: NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, *N*-[2-hydroethyl]-piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) 10, glucose 11.1, pH 7.4). Hearts were subsequently perfused with a nominally Ca^{2+}-free KHB buffer for 2–3 min followed by a 20 min perfusion with Ca^{2+}-free KHB containing 223 U/mL collagenase II (Worthington Biochemical Corporation, Freehold, NJ, USA) and 0.1 mg/mL hyaluronidase (Sigma Chemical, St. Louis, MO, USA). After perfusion, the left ventricle was removed, minced, and further digested with trypsin (Sigma) before being filtered through a nylon mesh (300 μm) and collected by centrifugation. Cells were initially washed with Ca^{2+}-free KHB buffer to remove remnant enzyme and extracellular Ca^{2+} was added incrementally back to 1.25 m*M*.

Myocyte Shortening and Relengthening

Mechanical properties of ventricular myocytes were assessed by an IonOptix Myocam® detection system (IonOptix Incorporation, Milton, MA, USA). Cells were placed in a chamber mounted on the stage of an inverted Olympus IX-70 microscope and superfused (at 25°C) with a buffer containing (in m*M*): 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated at a frequency of 0.5 Hz. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS), indicative of peak ventricular contractility; time-to-90% PS (TPS), indicative of systolic duration, time-to-90% relengthening (TR_{90}), indicative of diastolic duration; and maximal velocities of shortening (+dL/dt) and relengthening (–dL/dt), indicative of maximal velocities of ventricular pressure rise/fall *(18)*.

Intracellular Ca^{2+} Fluorescence Measurement

Myocytes were loaded with fura-2/AM (0.5 μ*M*) for 10 min and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix) as described *(18)*. Myocytes were plated on glass cover slips on an Olympus IX-70 inverted microscope and imaged through a Fluor 40× oil objective. Cells were exposed to light emitted by a 75 W lamp and passed through either a 360 or a 380 nm filter (bandwidths were ± 15 nm), while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm after first illuminating cells at 360 nm for 0.5 s then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol. Qualitative changes in intracellular Ca^{2+} levels were inferred from the ratio of the fluorescence intensity at two wavelengths (360/380) and were used to determine Ca^{2+}-induced Ca^{2+} release (shown as change of fura-2 fluorescent intensity, ΔFFI). ΔFFI was used as an indicative of sarcoplasmic reticulum (SR) Ca^{2+} release upon excitation.

Intracellular Ca^{2+} removal (indicative of intracellular Ca^{2+} clearing/extrusion rate) was evaluated as the rate of fluorescence decay.

Experimental Protocols

Myocytes (either fura-2 loaded or non-loaded) were first allowed to contract at a stimulation frequency of 0.5 Hz over 10 min to ensure steady state (myocytes with rundown >10% were not studied further) before being exposed to either E_2 or ZAL (10^{-9}–10^{-5} *M*) cumulatively. Cell shortening and intracellular Ca^{2+} transients were recorded before and 5 min after each concentration of E_2 or ZAL administration. Cells were then washed with normal contractile buffer for 5 min. The concentration range of E_2 chosen in our study were largely based on the observation that E_2 at physiological (p*M*), supra-physiological (n*M*), and pharmacological (μ*M*), levels may elicit cardiac excitation-contraction, ventricular contractile or other cardiovascular responses *(5–7,18,19)*. Since our earlier study *(18)* and preliminary screening from the current study failed to observe any significant cardiac contractile effect of E_2 or ZAL at concentration < 10^{-9} *M* in rat ventricular myocytes, we started the concentration range from 10^{-9} *M* in the current study. For appropriate comparison of cardiac contractile and intracellular Ca^{2+} response between ZAL and E_2, equal molar concentrations of ZAL and E_2 were examined using the same number of ventricular myocytes (although not the same cell) from the same rat on the same experiment day. In some myocytes, either E_2 or ZAL was examined in the presence of the E_2 receptor antagonist ICI182,780 (10^{-8} *M*).

Data Analysis

Data were presented as Mean ± SEM. Statistical significance ($p < 0.05$) for each variable was estimated by a two-way analysis of variance (ANOVA) with Tukey's test as *post hoc* analysis.

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References

1 Subbiah, M. T. (2002). *Braz. J. Med. Biol. Res.* **35,** 271–276.
2 Welnicka-Jaskiewicz, M. and Jassem, J. (2003). *Cancer Treat. Rev.* **29,** 355–361.
3 Yildirir, A., Yarali, H., Kabakci, G., Aybar, F., Akgul, E., and Bukulmez, O. (2001). *Int. J. Gynaecol. Obstet.* **75,** 273–278.
4 Gorgulu, S., Eren, M., Celik, S., et al. (2003). *Acta Cardiol.* **58,** 1–8.
5 Fisman, E. Z., Tenenbaum, A., Shapira, I., Motro, M., and Pines, A. (1999a). *Maturitas* **33,** 145–152.
6 Fak, A. S., Erenus, M., Tezcan, H., Caymaz, O., Oktay, S., and Oktay, A. (2000). *Fertil. Steril.* **73,** 66–71.
7 Fisman, E. Z., Tenenbaum, A., Shapira, I., Motro, M., and Pines, A. (1999b). *Climacteric* **2,** 174–180.
8 Mercuro, G., Zoncu, S., Paoletti, A. M., Melis, G. B., Vacca, A. M., and Cherchi, A. (1999). *Am. J. Cardiol.* **83,** 1677–1679.
9 Vogelvang, T. E., Mijatovic, V., Kamp, O., et al. (2002). *Am. J. Obstet. Gynecol.* **186,** 729–736.
10. Clarkson, T. B. and Anthony, M. S. (1998). *Baillieres Clin. Endocrinol. Metab.* **12,** 589–604.
11 Deodato, B., Altavilla, D., Squadrito, G., et al. (1999). *Br. J. Pharmacol.* **128,** 1683–1690.
12 Kleinova, M., Zollner, P., Kahlbacher, H., Hochsteiner, W., and Lindner, W. (2002). *J. Agric. Food Chem.* **50,** 4769–4776.
13 Knight, D. C. and Eden, J. A. (1995). *Maturitas* **22,** 167–175.
14. Fu, F. Y. (1991). *Acta Agricul. Universitatis Pekinensis* **17,** 117–118.
15. Draughon, F. and Churchville, D. C. (1985). *Churchville Phytopath.* **75,** 553–556.
16 Draper, C. R., Edel, M. J., Dick, I. M., Randall, A. G., Martin, G. B., and Prince, R. L. (1997). *J. Nutr.* **127,** 1795–1799.
17 Altavilla, D., Saitta, A., Galeano, M., et al. (2001). *Lab. Invest.* **81,** 125–132.
18 Ren, J., Hintz, K. K., Roughead, Z. K., et al. (2003). *Am. J. Physiol. Heart Circ. Physiol.* **284,** H1800–H1807.
19 Liew, R., Macleod, K. T., and Collins, P. (2003). *FASEB J.* **17,** 1307–1309.
20 Gorodeski, G. I., Levy, M. N., Yang, T., Goldfarb, J., and Utian, W. H. (1999). *J. Soc. Gynecol. Investig.* **6,** 80–87.
21 Sites, C. K., Tischler, M. D., Blackman, J. A., et al. (2003). *Fertil. Steril.* **71,** 137–143.
22 Curl, C. L., Wendt, I. R., Canny, B. J., and Kotsanas, G. (2003). *Clin. Exp. Pharmacol. Physiol.* **30,** 489–494.
23 Fraser, H., Davidge, S. T., and Clanachan, A. S. (1999). *J. Mol. Cell. Cardiol.* **31,** 1539–1549.
24 Brandi, M. L. (1997). *Calcif. Tissue Int.* **61,** S5–S8.
25. Zhao, D. G. and Meng, F. J. (1997). *Acta Botanica Sinica.* **39,** 570–573.