An Estrogen Metabolite, 2-Methoxyestradiol, Disrupts Cardiac Microtubules and Unmasks Muscarinic Inhibition of Calcium Current

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ABSTRACT

Microtubules provide a chemical signaling function as well as structural support for heart cells. Microtubules modulate autonomic signaling in the heart, and their disruption by colchicine unmasks muscarinic inhibition of Ca (ICa) current. In this study, we compare the actions of the estrogen metabolite, 2-methoxyestradiol (2-ME), with those of colchicine on microtubule stability and chemical signal function in guinea pig-isolated ventricular myocytes. Like colchicine, 2-ME binds to microtubules and disrupts the cytoskeleton of cardiac myocytes. Incubation with 2-ME increased the soluble fraction of tubulin and decreased the polymerized fraction at concentrations ranging from 10 to 100 μM. 2-ME was less potent than colchicine in causing microtubular disruption. Treatment with 2-ME for up to 4 h was accompanied by a progressive increase of ICa amplitude. There was no change in the rates of ICa inactivation. Carbachol, which has no effect on ICa in untreated ventricular myocytes, inhibited this current in the presence of 2-ME. The extent of inhibition increased with incubation time in 2-ME such that carbachol completely removed the increment of ICa by the estrogen metabolite. The results illustrate the important role of microtubules in modulating cardiac autonomic signaling.

Microtubules of the cytoskeleton have a structural function that is paralleled by a chemical signaling function (reviewed in Janmey, 1998). The signaling function of microtubules has been shown in several tissues, including the heart. The tubulin subunits comprising microtubules are stabilized by nonexchangeable GTP bound at the end or cap. Microtubules depolymerize in the presence of colchicine, and the cap is removed. Thus, guanine nucleotide exchange not only regulates tubulin stability but also modulates the activity of guanine nucleotide binding proteins. When depolymerized by agents such as colchicine, exchangeable GTP becomes available for modulating signal transduction. The effects of colchicine were attributed to the release of GTP from disrupted microtubules (Gómez et al., 2000; Malan et al., 2003).

When colchicine has disrupted microtubules, Ca signaling in the heart is increased. This is evident in the increase of the L-type Ca2+ current (ICaL), intracellular Ca2+ transients, and contractions (Gómez et al., 2000, 2004; Kerfant et al., 2001; Malan et al., 2003). Colchicine treatment causes effects like those of the β-adrenoceptor agonist isoproterenol. It was proposed that free tubulin transferred exchangeable GTP to the stimulatory guanine nucleotide binding protein, Gs, to activate adenylyl cyclase (AC) (see Yan et al., 2001). Washout reversed the effects of colchicine (Kerfant et al., 2001).

In addition to mimicking the action of β-adrenergic agonists on ICaL, Ca transients, and contractions, colchicine unmasked muscarinic inhibition of these variables (Malan et al., 2003; Gómez et al., 2004). In general, muscarinic agonists have a negligible effect on ICaL and contractions in the mammalian ventricle (Löffelholz and Pappano, 1985; reviewed in Hartzell, 1988; Harvey and Belevych, 2003). Muscarinic inhibition of ICaL and of contractions requires elevation of cAMP either by stimulation of AC or by inhibition of phosphodiesterase in the ventricular myocytes of mammals. This phenomenon has been termed “accentuated antagonism” (Levy, 1971).

Colchicine binds to tubulin dimers and thereby suppresses microtubule assembly. Treatment of the chorioallantoic membrane of chick embryos with colchicine interfered with angiogenesis (D’Amato et al., 1994). 2-Methoxyestradiol (2-ME; [17β]-2-methoxyestra-1,3,5[10]triene-3,17-diol) replicated this

ABBREVIATIONS: ICa, inhibition of Ca; AC, adenylyl cyclase; 2-ME, 2-methoxyestradiol; CCh, carbachol; ISO, isoproterenol; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; CTR, control.
action of colchicine on blood vessel growth. Furthermore, 2-ME was shown to bind to the same site as colchicine on microtubules and to displace it competitively. 2-Methoxyestradiol is a major metabolite of estradiol and of the oral contraceptive drug ethinylestradiol. Thus, 2-ME, which is found in human blood and urine, is a naturally occurring compound that disrupts microtubules. Moreover, this estrogen metabolite is an endogenous inhibitor of mammary carcinogenesis (Zhu and Conney, 1998).

The present study evaluates the effect of 2-ME on microtubules in mammalian cardiac myocytes. We wished to determine whether, like colchicine, this estrogen metabolite disrupts microtubules and affects Ca signaling in the heart. Experiments were also done to ascertain whether 2-ME also affected \( I_{Ca} \), and the regulation by muscarinic agonist of this current that triggers contraction.

Materials and Methods

**Ventricular Myocytes.** The investigation conformed to the Institute of Laboratory Animal Resources (1996) and was approved by the University of Connecticut Health Center’s Institutional Animal Care and Use Committee. Male guinea pigs (250–450 g) were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and anticoagulated with heparin (1000 IU i.p.). The heart was placed on a Langendorff apparatus and perfused with modified Tyrode’s solution containing the following: 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 0.33 mM NaH\(_2\)PO\(_4\), 10 mM HEPES, and 20 mM glucose (pH adjusted to 7.4 with NaOH). Single ventricular myocytes were isolated with the procedure reported previously (Shen and Pappano, 2001). After disrupting the extracellular matrix with collagenase and protease, the enzymes were washed out by perfusion with 50 ml of Recovery solution containing the following: 130 mM K aspartate, 5 mM K\(_2\)ATP, 5 mM HEPES, and 20 mM glucose (pH was adjusted to 7.4 with KOH). Ventricular cells were dispersed in Recovery solution and kept at 4°C for at least 1 h before experiments.

**Electrophysiology.** A cell suspension droplet was placed in a chamber (500–\( \mu \)l volume) on the stage of an inverted microscope. After 10 min, superfusion began with Tyrode’s solution (2 ml/min) containing 10 mM glucose and 10 mM CsCl (22–24°C). The patch rupture, whole-cell voltage-clamp technique used an EPC 7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Voltage clamp protocols were performed with a low-pass filter at 520 nm. Parameters were first adjusted with a control cell and maintained constant to examine all cells. Relative quantification of microtubules (tubulin) was performed by determining the mean pixel intensity associated with individual myocytes using PhotoShop 6.0 software (Adobe Systems, Mountain View, CA). To obtain pixel intensity values corresponding solely to specific protein immunoreactivity, a background “noise” value selected from an irrelevant and constant area of the image was subtracted from each myocyte value obtained.

**Western Blot.** Myocytes were resuspended in 0.5 to 1 ml of microtubule-stabilizing buffer composed of 50% glyceral, 5% DMSO, 10 mM sodium phosphate, 0.5 mM EGTA, and 0.5 mM MgSO\(_4\). The myocyte suspension was centrifuged at 100,000g at 25°C for 10 min, and the supernatants were saved as the free-tubulin fractions. The pellets were resuspended in 0.5 to 1 ml of microtubule-depolymerizing buffer composed of 0.25 M sucrose, 10 mM sodium phosphate, and 0.5 mM MgSO\(_4\). After 1 h at 4°C, they were centrifuged at 100,000g at 4°C for 15 min, and the supernatants were saved as polymerized tubulin fractions (Ishibashi et al., 1986).

An equal amount of total protein (30 \( \mu \)g) of each free tubulin and polymerized tubulin sample was loaded into each lane and electrophoresed on 10% SDS-polyacrylamide gel. Total protein was determined by microbichininuous acid assay using bovine serum albumin as a standard (Pierce, Rockford, IL). Separated proteins were electrically transferred (50V, 2 h) onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The transfer was blocked with 5% dry milk (fat-free) overnight at 4°C. The blocked transfer was then probed with a 1:2000 dilution of a mouse monoclonal antibody to acetylated tubulin (Sigma-Aldrich). Bound antibodies were visualized with horseradish peroxidase-conjugated anti-mouse IgG (1:3000) and enhanced chemiluminescence technique (ECL; SuperSignal West Femto Maximum Sensitivity; Pierce, Rockford, IL). The intensity was analyzed by exposure to Kodak X-OMAT AR films (Eastman Kodak, Rochester, NY). Densitometric quantification of ECL exposure was performed with PhotoShop.

Data Analysis. Measurements are reported as the mean ± S.E.M. The statistical significance of mean differences was determined by Student’s \( t \) test. A value of \( p \leq 0.05 \) was considered statistically significant. The data in Figs. 1 and 2 were analyzed with analysis of variance followed by post-hoc corrections using Dunnett’s multiple comparison test.

Results

**Microtubule Structure.** Either colchicine or 2-ME, when applied for 4 h, disrupted the microtubule component of the

\[ I_{Ca} \] was recorded at steady state (2–3 min). 2-Methoxyestradiol was present in the CCh-containing solution.

**Immunofluorescence.** Myocytes were incubated with different concentrations of colchicine or 2-ME in Tyrode’s solution at room temperature for 1 to 4 h. Control myocytes were incubated in Tyrode’s solution containing the same concentration of DMSO as those exposed to 2-ME. Myocytes were sedimented onto polylysine-coated microwells of a 12-well microtiter plate for 30 min, permeabilized for 1 min with 0.2% Triton X-100 in phosphate-buffered saline (PBS), and fixed for 30 min with 3.7% formaldehyde. After applying blocking buffer (5% normal goat serum and 0.1% Triton X-100 in PBS) for at least 2 h at room temperature, the myocytes were incubated with a 1:300 dilution of a mouse monoclonal antibody to acetylated tubulin (Sigma-Aldrich, St. Louis, MO) in a blocking buffer overnight at 4°C. Myocytes were then washed with PBS and incubated for 2 h in a 1:200 dilution of a fluorescein-conjugated goat anti-mouse IgG (Sigma-Aldrich) in the dark at room temperature. After excess antibody was washed out with PBS, the myocytes were placed on a slide with a drop of mounting medium (Vectorshield) and covered with a coverslip sealed with nail polish. Myocytes were observed with an Olympus IX70 microscope (Olympus; Precise Instrument Co., Whitman, MA) equipped with a epifluorescence system. Emission was collected through a low-pass filter at 520 nm. Parameters were first adjusted with a control cell and maintained constant to examine all cells. Relative quantification of microtubules (tubulin) was performed by determining the mean pixel intensity associated with individual myocytes using PhotoShop 6.0 software (Adobe Systems, Mountain View, CA). To obtain pixel intensity values corresponding solely to specific protein immunoreactivity, a background “noise” value selected from an irrelevant and constant area of the image was subtracted from each myocyte value obtained.

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that of colchicine. The results of a representative experiment that compared the amounts of free and polymerized tubulin in cardiac myocytes are shown in Fig. 2. In untreated cells, the amount of soluble or free tubulin is less than the amount of polymerized tubulin [Fig. 2 insert, control (CTR)]. Densitometric measurements showed that the amount of polymerized tubulin (82 ± 17.9 arbitrary units) exceeded that of free tubulin (62 ± 11.5 arbitrary units) by 1.3-fold. Colchicine at 1 μM increased the amount of soluble or free tubulin by 50 ± 8.3% relative to control and reduced that of polymerized tubulin to 34 ± 7.3% (Fig. 3). Similar results were obtained in four additional preparations. Like colchicine, 2-ME (30 μM) increased the amount of soluble tubulin and decreased that of polymerized tubulin. Free tubulin increased by 23 ± 6.2%, and polymerized tubulin diminished to 69 ± 3.8%. All of these changes were statistically significant. Thus, the reduction in cytoskeleton immunofluorescence by either colchicine or 2-ME can be attributed to the disruption of microtubules in cardiac myocytes with an attendant increase in soluble tubulin.

Calcium Current. In a previous study, colchicine was shown to increase the L-type Ca current in rat-isolated ventricular myocytes (Gómez et al., 2000). We found that 2-ME, at concentrations that disrupted microtubules, mimicked the effect of colchicine on the L-type Ca2+ current. This effect depended on the incubation time in the presence of the estrogen metabolite. The results in Fig. 3 indicate that the I_{Ca} density increased linearly as a function of incubation time in 2-ME. Data collection began at 1 h after incubation in 2-ME. The Ca current was recorded from cells within 5 min after patch rupture at each hour from different cells. Calcium current at 4 h in 2-ME averaged 15.5 ± 3.39 pA/pF and had increased by ~72% compared to its average value of 9.0 ± 2.55 pA/pF at 1 h. The data were fit well by least-squares linear regression, and the extrapolated value of I_{Ca} was 6.8 pA/pF. The effect of 2-ME is like that reported for colchicine (Gómez et al., 2000; Malan et al., 2003) with the difference that the effect of 2-ME is slower to develop and slower to dissipate.

The inactivation rate of I_{Ca} could be described by the sum of two exponentials. In controls (n = 7), the inactivation time constant was 2.55 pA/pF at 1 h. The data were fit well by least-squares linear regression, and the extrapolated value of I_{Ca} was 6.8 pA/pF. The effect of 2-ME is like that reported for colchicine (Gómez et al., 2000; Malan et al., 2003) with the difference that the effect of 2-ME is slower to develop and slower to dissipate.

Fig. 1. Immunofluorescence analysis of guinea pig ventricular myocyte cytoskeleton. Ordinate: relative luminosity of myocytes; abscissa: experimental condition. Colchicine significantly reduced luminosity at 3 and 10 μM but not at 1 μM. Similar significant effects to reduce luminosity were observed at 30 and 100 μM but not at 10 μM 2-ME. Each column is the summary of 10 measurements from three different heart preparations. The inset shows representative micrographs from an untreated cell (CTR) and from myocytes treated with 10 μM colchicine (Col 10) or 30 μM 2-ME (Met 30). *, p < 0.05; **, p < 0.01.

Fig. 2. Western blot of free and polymerized tubulin in cardiac myocytes. The inset shows a gel (30 μg of protein for each sample) displaying free and polymerized tubulin in the absence of treatment (CTR) and in the presence of 1 μM colchicine (Col) or 30 μM 2-ME. The graph summarizes the data from five experiments with the level of free and polymerized tubulin set at 100%. Either colchicine or 2-ME increased the amounts of free and polymerized tubulin significantly (*, p < 0.05; **, p < 0.01).

Fig. 3. Change of I_{Ca} during incubation with 2-ME. Ordinate: calcium current density in pA/pF; abscissa: time in hours. Measurements were made within 5 min of patch rupture (n = 4–5 cells) and show I_{Ca} increasing linearly with time in the presence of 2-ME. The line through the data is fit by the equation: \[ y = 2.2 x + 6.83 \] with R^2 of 0.99. Recordings in 2-ME are from four ventricular cells from four different hearts at 1, 2, and 3 h; data at 4 h are from five cells in as many hearts.

cytoskeleton (Fig. 1). Preliminary experiments indicated that this time was required to observe a maximal effect of 2-ME (data not shown). Microtubule disruption was evident at 1 μM colchicine; increasing concentrations caused correspondingly greater disruption as indicated by the luminosity measurements. At 10 μM, 2-ME had a small effect on microtubule structure. The disruption in 30 μM 2-ME was comparable with that seen with 3 μM colchicine. No additional effect was observed at 100 μM 2-ME, which was a maximal concentration (Fig. 1). The results, taken from 10 myocytes in three different preparations, indicate that treatment with 2-ME, like colchicine, reduced the intensity of tubulin fluorescence in intact cardiac myocytes. Thus, 2-ME could disrupt the structure of microtubules when applied to myocytes in vitro.

Effects of Colchicine and 2-ME on Myocyte Tubulin. If 2-ME disrupts microtubules, it should change the amounts of free and polymerized tubulin in a manner comparable with
constants were 26 ± 6.1 and 181 ± 29.6 ms for the fast (τ_f) and slow (τ_s) components, respectively. These did not change in 2-ME (n = 8), where τ_f averaged 24 ± 4.5 and τ_s averaged 184 ± 27.3 ms.

Addition of the muscarinic agonist alone has a negligible effect on I_{Ca} in mammalian ventricular myocytes (see Introduction). We observed that CCh became effective as an inhibitor of I_{Ca} in cells treated with 2-ME. An example is shown in Fig. 4. The I_{Ca} was initially large and ran down gradually with time (Fig. 4A). The addition of 10 μM CCh, per se, reduced the Ca current significantly (Fig. 4B), an action reversed by the addition of 100 nM ISO (Fig. 4C). This result indicates that I_{Ca} became sensitive to muscarinic inhibition in the presence of 2-ME. In addition, the effect of ISO consisted of returning I_{Ca} to its original amplitude.

The summary of these experiments is given in Fig. 5, which illustrates another feature of muscarinic inhibition. The Ca current, which increased linearly during incubation with 2-ME (Fig. 3), became progressively more sensitive to suppression by 10 μM CCh (Fig. 5). In effect, CCh removed the increment of I_{Ca} that occurred in the presence of 2-ME.

**Discussion**

Treatment of cardiac myocytes with 2-ME 1) disrupted the microtubular network, 2) increased the amount of soluble tubulin while reducing that of polymerized tubulin, 3) caused a progressive increase of I_{Ca} magnitude, and 4) allowed CCh to inhibit I_{Ca} without addition of ISO to stimulate AC. All of these actions mimic those reported for colchicine (Gómez et al., 2000; Malan et al., 2003; Gómez et al., 2004).

2-Methoxyestradiol, an estrogen metabolite, has acute, nongenomic effects in the cardiovascular system (reviewed in Dubey and Jackson, 2001). Among estrogenic compounds, 2-ME was the most potent to inhibit colchicine binding and to disrupt polymerization in a brain tubulin fraction (D’Amato et al., 1994). Like colchicine, 2-ME inhibits tubulin polymerization in mammalian cardiac myocytes. The disruption was maximal within 4 h of incubation of isolated cells with 2-ME.

Colchicine disrupts microtubules more rapidly (Gómez et al., 2000). Microtubule disruption by 2-ME could modulate cardiac myocyte signaling by either physical or chemical means. For the chemical hypothesis, αβ-tubulin dimers serve as GTP donors. For the physical hypothesis, microtubules may modulate membrane signaling component distribution and function.

**Chemical Hypothesis.** Microtubules are stabilized by non-exchangeable GTP that is bound at the end (Jannmey, 1998). In polymerized tubulin, exchangeable GTP is located between tubulin dimers and is unavailable for exchange. Depolymerization of microtubules by 2-ME, like colchicine, allows GTP to become available. Released GTP seems to activate the stimulatory guanine nucleotide binding protein, G_s. This is based on the observations that colchicine increased cAMP content (Malan et al., 2003) and that the AC inhibitor, 2’,5’-dideoxyadenosine 3’-monophosphate, blocked the effect of colchicine on I_{Ca} (Gómez et al., 2000). It is presumed that the same process for stimulation of AC by GTP occurs when microtubules are disrupted by 2-ME. The increase of I_{Ca} during incubation in 2-ME is consistent with this hypothesis as is the appearance of muscarinic inhibition of this current. If the GTP were donated primarily to the inhibitory guanine nucleotide binding protein, G_i, muscarinic inhibition of I_{Ca} should occur in the absence of a “run-up” of this current.

Muscarinic agonists, per se, do not inhibit the L-type Ca current in mammalian ventricular myocytes; prior activation of adenylyl cyclase is required (see Introduction). [An exception to this has been reported in ferret ventricular myocytes (Bett et al., 2002).] Cardiac isoforms of adenylyl cyclase, types V and VI, are inhibited by α/β-subunits but not by βγ-subunits (Sunahara et al., 1996). The failure of α1-GTP, when released by the muscarinic agonist, to signal inhibition of I_{Ca} could arise from a restricted distribution of α1-GTP or insufficient AC activity. For example, dialysis of ventricular myocytes with the rapid Ca chelator, BAPTA, removes inhibition of AC activity by endogenous Ca and unmasks muscarinic inhibition of I_{Ca} (Shen and Pappano, 2001). The data suggest that 2-ME, like colchicine, releases αβ-tubulin dimers, stimulates adenylyl cyclase, and therefore unmasks muscarinic inhibition of I_{Ca}. When the progressive reduction of I_{Ca} by CCh is subtracted from the control current in 2-ME, I_{Ca} displayed no increase over the 4-h observation period. Thus, the muscarinic agonist removed only that portion of I_{Ca} that has been augmented by the action of 2-ME.
In colchicine-treated myocytes, ISO is unable to stimulate adenylyl cyclase or to increase Ca^{2+} transients and contractions (Gómez et al., 2000, 2004; Kerfant et al., 2001; Malan et al., 2003). We found that ISO antagonized the inhibition of ICa by CCh, a reversal of the usual actions of β-adrenoceptor and muscarinic agonists on ICa regulation in mammalian ventricular cells.

**Physical Hypothesis.** Is there a role for the structural function of microtubules in the action of 2-ME on ICa, and its inhibition by muscarinic agonist? β1 integrins anchor proteins with the cytoskeleton. When embryonic cell-derived cardiac myocytes are made β1 integrin-deficient, CCh was unable to inhibit ICa (Bloch et al., 2001). Muscarinic receptor binding was unchanged from control cells, but Gsi distribution was atypical. Defective coupling of Gsi also occurred in cardiac myocytes treated with cytochalasin D, which stabilizes actin like cytochalasin D, which stabilizes actin and disrupts muscarinic signaling; β-adrenergic stimulation of ICa was not affected. If 2-ME stabilized actin like cytochalasin D, Gsi-dependent signaling should be reduced. The converse was observed. When cardiac myocytes were placed in culture medium, the α1-subunit of L-type Ca channels redistributed to the perinuclear region from their usual location at t-tubules (Leach et al., 2005). Cytochalasin D or cycloheximide prevented the redistribution of Ca channels and t-tubules and preserved Ca release during excitation-contraction coupling. However, colchicine had no effect on the redistribution of the α1-subunit of L-type Ca channels. On the favorable assumption that 2-ME mimics colchicine, then the physical signal functions of both integrins and actin are not changed by microtubule disruption.

Microtubule disruption by colchicine facilitated β-adrenergic receptor, Gsi, and type V/VI adenylyl cyclase interaction in rat ventricular membranes (Head et al., 2006). This physical mechanism may be an alternative to the chemical hypothesis for the consequences of microtubule disruption by colchicine and 2-ME. In experiments performed by Head et al. (2006), colchicine had no effect on basal cAMP or on the stimulant effect of forskolin on cAMP synthesis, but it increased cAMP formation by ISO. In contrast, evidence for the chemical hypothesis included an increase of basal cAMP by colchicine and a lack of stimulation by ISO (Malan et al., 2003). In addition, basal ICa increased in colchicine-treated cells (Gómez et al., 2000, 2004; Malan et al., 2003), and ISO produced no further increase of ICa. Basal intracellular Ca^{2+} transients increased in colchicine as did cell shortening (Gómez et al., 2000; Kerfant et al., 2001; Malan et al., 2003); again, the ISO had no effect on these processes. Forskolin increased ICa when ISO was unable to do so, and muscarinic inhibition of ICa was unmasked in either colchicine or 2-ME (Gómez et al., 2000, 2004; Kerfant et al., 2001; Malan et al., 2003; this report). All of these actions are contrary to those reported for the physical hypothesis (Head et al., 2006). We believe that the evidence favors the chemical hypothesis; however, resolution of this issue requires additional experiments.

Estrogen, via a genomic action, reduced the expression of the L-type Ca channel gene (reviewed in Babiker et al., 2002). 17β-Estradiol, via a nongenomic effect, opposed the stimulation of adenylyl cyclase and the increase of ICa by ISO in the rat ventricle (Li et al., 2000). Thus, the increase of ICa is not readily explained by either a genomic or nongenomic estrogen-like action of 2-ME.

Physiological concentrations of 2-ME are in the picomolar range (Lakhani et al., 2006). 2-Methoxyestradiol has antiangiogenic and antitumor actions (Fotsis et al., 1994; Tinley et al., 2003; Stafford et al., 2005). Clinically observed concentrations of 2-ME range from 1 to 30 ng/ml (3.3–100 nM) in patients who receive the drug for cancer treatment. There is local synthesis of estradiol and its metabolites in cardiac myocytes and fibroblasts (Dubey et al., 2004) and in human coronary smooth muscle (Zacharia et al., 2001). Estrogens exert an antiproliferative effect in aortic smooth muscle cells. This action, which is independent of either estrogen receptor α or β, is attributed to the local synthesis of hydroxyestradiol and methoxyestradiol by the blood vessel (Barchiesi et al., 2002). In a clinical trial, plasma levels of 2-ME increased with dose up to 400 to 600 mg/day and did not increase further at doses >600 mg/day (James et al., 2007). This result indicates that lipid-soluble 2-ME accumulates in tissues. However, it is not known whether the cardiac concentrations of locally synthesized, lipid-soluble 2-ME in vivo approach those used in vitro experiments to disrupt microtubules.

In summary, a mammalian metabolite, 2-ME, mimics the actions of a plant product, colchicine, on cytoskeletal structure and regulation of Ca channel function in guinea pig ventricular myocytes. Like colchicine, 2-ME augments ICa and unmasks muscarinic inhibition of this current in mammalian ventricular myocytes. This is attributed to activation of Gsi by GTP released from αβ-tubulin dimers.

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**References**


James J, Murry DJ, Treston AM, Storniolo AM, Sledge GW, Sider C, and Miller KD (2007) Phase I safety, pharmacokinetic and pharmacodynamic studies of 2-meth oxyestradiol and muscarinic inhibition in Heart 511


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