

Ph.D. THESIS

EFFECTS OF THYMOL ON CARDIAC AND SKELETAL MUSCLE

NORBERT SZENTANDRÁSSY, M.D.



Supervisor:

János Magyar M.D., PhD.

**UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
MEDICAL SCHOOL
DEPARTMENT OF PHYSIOLOGY
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INTRODUCTION

Thymol and its analogues (carvacrol, eugenol, guaiacol, vanillin and zingerone) are all monoterpenoid phenol derivatives. Vanillin and guaiacol are soluble in water the others are all soluble in alcohol, chloroform, ether. They are found in natural substances, spice plants, most of them are part of vegetable oils. Thymol is found in thyme, oregano and wolf's bane. Eugenol is the major component of clove oil, and is also found in cinnamon, and thyme. Carvacrol is component of the thyme, oregano and marjoram. Vanillin is found in the vanilla and the zingerone in the root of ginger. Guaiacol occurs naturally in the resin of the beech tree. Thymol, carvacrol and eugenol are widely used as a general antiseptic, in medical practice, agriculture, cosmetics and food industry. They are used in the chemical industry to stabilize and to store solutions and serum samples. The guaiacol is widely used to treat influenza, catarrh and coughing and as dental medicaments. Vanillin is used as food additive for various cakes, chocolates or vanilla ice cream.

Thymol, eugenol and carvacrol are applied in dentistry due to their potent fungicide, bactericide and antioxidant properties. They inhibit the proliferation of bacteria and have disinfectant effect. Many mouthwash contains 1 percentage thymol. Carvacrol and thymol in 1 millimolar concentration inhibit the ATP production of several pathogen bacteria, damage the cell membrane and cause the efflux of intracellular molecules when applied higher than 3 millimol. Thymol is used to prevent the infections in pleurodesis. Similarly to caffeine thymol and carvacrol evoked calcium release from intracellular stores in *Helix pomatia* neurons and in smooth muscle cells. Eugenol and guaiacol exert its effect on both pre- and postsynaptic membrane in mollusc neurons. They inhibit the calcium channels on the presynaptic membrane and decrease the stimulus evoked excitatory postsynaptic potentials in a concentration dependent manner. Both guaiacol and vanillin inhibited the calcium and the A currents in the *Helix* neurons. The activation and inactivation of the A current were accelerated by these drugs. Vanillin caused spontaneous action potentials and lengthened the action potentials in a small extent but did not effect on the delayed potassium currents. Zingerone inhibited the A current of the mollusc neurons in lower concentration than guaiacol and vanillin. Eugenol inhibits the activity of the Na/K-ATPase on jejunal and renal tubular cells in rats. This drug also relaxes the smooth muscle of rabbit aorta and guinea pig ileum by affecting the calcium sensitivity and uptake. Eugenol inhibits the calcium current of guinea pig ventricular myocytes, shortens their action potential and have negative inotropic effect. Zingerone has scavenger activity but its antioxidant effect is much weaker than that of thymol. Zingerone induce inward current in a concentration dependent manner on trigeminal ganglion neurons in rats. The former effect of zingerone showed tachyphylaxis and could be prevented by 10 μ M of the vanilloid antagonist capsazepine. 30 μ M zingerone decreased the amplitude of the transient outward potassium current and delayed rectifier potassium current in rat

ventricular myocytes. The inhibition was 52 % and 35 %, respectively. Thymol inhibited the growth and proliferation of melanoma B16(F10) cells. Due to its antioxidant effect thymol is used to stabilize the fluid halothane. The so-called halothane hepatitis can be due to the presence of thymol in the vaporizer.

OBJECTIVES

Our goal was to examine the effects of thymol and its analogues on the electrophysiological properties of myocardial and skeletal muscle cells derived from different species (human, canine and rat). The effect of thymol on the contraction and calcium handling of myocardium was also studied.

The aim of our work was to obtain information on the followings:

- What is the effect of thymol on the morphology of myocardial action potentials?
- Which myocardial ion currents are affected by thymol and what is the effect of thymol on the kinetics of these currents?
- Is there any difference between the effects of thymol on the L-type calcium current in human and canine cardiomyocytes?
- What is the effect of thymol on the ionic currents of rat skeletal muscle cells?
- Is there any difference between the effects of thymol on the ionic currents in cardiomyocytes and skeletal muscle cells?
- What is the effect of thymol on the cardiac contraction?
- What is the effect of thymol on the intracellular calcium concentration, on the calcium release from intracellular stores and on the activity of the sarcoplasmic reticulum Ca^{2+} -ATPase?
- Thymol or caffeine is better to evoke calcium release from intracellular stores?
- What are the effects of the thymol analogues on the myocardial L-type calcium current?
- Has carvacrol similar effects on the L-type calcium current in human and canine cardiomyocytes?
- What is the relationship between the structure of the analogues and the effects exerted by them?

MATERIALS AND METHODS

1. Cell isolation

Isolated canine ventricular myocytes were obtained from hearts of adult mongrel dogs using the segment perfusion technique. The animals were anaesthetized and their hearts were rapidly removed from the chest. One of the coronary arteries (usually LAD) was cannulated and perfused with calcium-free JMM solution for 5 min in order to remove calcium from the tissues. Dispersion of the cells was performed during a 30 min perfusion with JMM solution containing 50 μM CaCl_2 and 1 mg/ml collagenase type CLS2. Cells were stored in MEM solution at 15 °C before we use them for experiments. Similar procedure was used for human hearts.

Single skeletal muscle fibers were isolated enzymatically from rats. Briefly, rats were anaesthetized and muscles were removed and were treated with collagenase (Type I) for 60-90 min at 37 °C. After dissociation, fibers were allowed to rest for at least 20 min and only those were used that showed no signs of membrane damage.

2. Action potential recording

Action potentials were recorded from Ca^{2+} -tolerant canine ventricular cells superfused with normal Tyrode solution at 37 °C. Transmembrane potentials were recorded using glass microelectrodes filled with 3 M KCl and having tip resistance between 25 and 30 M Ω . These electrodes were connected to the input of an Axoclamp-2B amplifier. The cells were continuously paced through the recording electrode at a steady cycle length of 1 s using 1 ms wide rectangular current pulses with 120 % threshold amplitude. Action potentials were digitized at 100 kHz using Digidata 1200 A/D card and stored for later analysis. Action potentials were visualized on oscilloscope.

3. Voltage clamp technique

Transmembrane currents were recorded in normal Tyrode solution at 37 °C. Suction pipettes, fabricated from borosilicate glass, had tip resistance of 2-3 M Ω after filling with pipette solution composed of KCl 110, KOH 40, HEPES 5, EGTA 10, TEACl 20, K-ATP 3 and GTP 0.25 mM, or alternatively, K-aspartate 100, KCl 45, MgCl_2 1, EGTA 10, HEPES 5, K-ATP 3 mM, when measuring Ca or K currents, respectively. The pH of these solutions were adjusted to 7.4 with KOH. I_{Ca} was blocked by 5 μM nifedipine, and 3 mM 4-aminopyridine was used to suppress I_{to} (both drugs were applied externally). Cells were clamped to -40 or -80 mV holding potential when measuring Ca or transient outward K current, respectively. Ca currents were evoked by 400 ms long depolarizing voltage pulses to a test potential of +5 mV and their amplitude was obtained as a difference between

the peak of the current and the non inactivated component measured at 200 ms. The slow and the fast component of the delayed rectifier potassium current were measured with different voltage square pulses from the -40 mV holding potential. Currents were recorded with an Axopatch-200B amplifier using the whole cell configuration of the patch clamp technique. Ionic currents were normalized to cell capacitance. The series resistance and the pipette capacitance were compensated. Outputs from the clamp amplifier were digitized at 20 kHz using a Digidata 1200 A/D converter under software control (pClamp 6.04). The sampling frequency was between 0.5 and 20 kHz depending on the examined current. Analog signals were filtered with the one third of the sampling frequency according to the Nyquist's theory. Each current was normalized to cell capacitance.

A double vaseline gap chamber was used for measurements on rat skeletal muscle cells. The selected fiber was transferred into a recording chamber filled with Relaxing solution containing K-glutamate 150, MgCl₂ 2, HEPES 10 and EGTA 1 mM. After completing the permeabilization the solutions were exchanged to Internal solution in the open-end pools (containing Cs-glutamate 120, MgCl₂ 5.5, Na₂-ATP 5, Na-phosphocreatine 10, glucose 10, HEPES 5 and EGTA 5 mM) and to External solution in the middle pool (containing TEA-CH₃SO₃ 140, MgCl₂ 2, HEPES 5, tetrodotoxin 0.0003 and 3,4-diaminopyridine 1 mM). For Ca current measurements 5 mM CaCl₂ was added to the External solution and TEA-CH₃SO₃ was reduced appropriately, while the EGTA concentration in the Internal solution was increased to 20 mM with reducing Cs-glutamate. For K current measurements the Internal solution contained 120 mM K-glutamate instead of Cs-glutamate and the External solution was modified to have 140 mM N-methyl-D-glucamine instead of TEA and 4 mM MgCl₂ instead of 2 mM. All solutions were adjusted to pH 7.2 and 300 mOsm. Fibers were voltage-clamped and the holding potential was set to -100 mV. All experiments were performed at 16-18 °C. Ca current was measured using 800 ms long depolarizing voltage pulses exploring the -50 to +60 mV voltage range. The linear capacitive component was subtracted by applying 20 mV hyperpolarizing pulses. K current was measured using 100 or 200 ms long depolarizing voltage steps exploring the -80 to +40 mV voltage range.

4. Measurement of contractility in canine right ventricular trabeculae

Thin right ventricular trabeculae, having diameters less than 1 mm, were obtained from hearts of adult mongrel dogs. The animals were anesthetized and the freshly excised preparations were mounted in a Plexiglass chamber allowing continuous superfusion with Krebs solution. The solution was equilibrated with 95% O₂ plus 5% CO₂ at a temperature of 37 °C. Developed force was recorded under isometric conditions using a capacitive mechano-electronic transducer fixed to a micromanipulator. Each preparation was stretched to the length at which maximum developed force was evoked and allowed to equilibrate for 60 min. The preparations were paced at a constant cycle

length of 1 s. Records were digitized at 1 kHz using Digidata 1200 A/D card and stored for later analysis.

5. Recording of left ventricular pressure and $[Ca^{2+}]_i$ transients in Langendorff-perfused guinea pig heart

Male guinea pigs (weighing 300-500 g) were anesthetized and their heart was rapidly removed and fixed to the cannula of a Langendorff-perfusion device. The heart was perfused with Krebs solution. The coronary perfusion was maintained at 37 °C using a peristaltic pump. Left ventricular pressure was continuously monitored using a Braun 2021-02 arterial pressure transducer that was connected to the left ventricular cavity. Heart rate was maintained at 200 beats/min by left atrial pacing.

To record $[Ca^{2+}]_i$ transients, the heart was loaded with the acetoxymethylester of the fluorescent dye, Fura-2 (5 mM). The dye was excited at both 340 and 380 nm wavelengths. The emitted light was collected at 510 nm using a trifurcated quartz fiber optic bundle connected to a Deltascan device. $[Ca^{2+}]_i$ was calculated from the background-corrected fluorescent ratio (F_{340}/F_{380}) signals. The analogue fluorescence and pressure signals were sampled at 1 kHz. In each case 10 subsequent beats were averaged and stored for later analysis.

6. Recording of single channel currents of canine ryanodine receptor (RyR) incorporated into artificial lipid bilayer

Heavy sarcoplasmic reticulum (SR) vesicles were isolated from ventricular free wall of dogs, and the solubilized RyR was purified. Single channel bilayer measurements were carried out using solubilized RyR incorporated into planar lipid bilayer. Bilayers were formed across a 250 μ m aperture of a nolrene cap using symmetrical buffer solution (containing 250 mM KCl, 100 μ M EGTA, 150 μ M $CaCl_2$ and 20 mM PIPES) at pH 7.2. The chamber into which the small aliquot of the solubilized RyR has been added was designated as the *cis* (cytoplasmic) side, while the other chamber was labelled as *trans* (luminal) side and was kept on ground potential. Current signals, obtained under voltage clamp conditions, were filtered at 1 kHz using an 8 pole low pass Bessel filter and digitized at 3.3 kHz using Axopatch 200 amplifier and pClamp 6.02 software. Channels having conductance higher than 400 pS were considered as RyR in the presence of 250 mM K^+ used as current carrier. Thymol was applied on the *cis* side of the channel in concentrations of either 150 or 300 μ M. At the end of each experiment 2 μ M ryanodine was added to the *cis* side to verify the orientation of the channel. Single channel measurements were carried out at 23 °C, free Ca^{2+} concentrations at both *cis* and *trans* sides were calculated to be 50 μ M using the computer program and stability constants published by Fabiato.

7. Measurement of calcium release from canine heavy SR vesicles

Calcium release from heavy SR vesicles was tested at 37 °C by measuring changes of Ca^{2+} concentration in the extravesicular space using the metallochrome dye arzenazo III (0.25 mM) as an indicator. Measurements were performed in a SPEX Fluoromax single photon counting spectrometer. The vesicles were actively loaded with calcium by successive additions of CaCl_2 followed by inhibition of the calcium pump using 100 nM cyclopiazonic acid. Release of calcium from the SR vesicles was induced by the addition of thymol. Extravesicular Ca^{2+} concentration was calculated as a function of time from the difference in absorbance at 710 and 790 nm. Data were collected at a rate of 1 Hz and stored digitally for later analysis. The rate of initial calcium release was determined from the slope of the thymol-induced change of absorbance.

8. Determination of ATPase activity

Ca^{2+} -ATPase activity of canine cardiac heavy SR vesicles were measured using the coupled enzyme assay in a medium having the composition of KCl 100, TRIS-HCl 20, MgCl_2 5, CaCl_2 0.7, ATP 5, EGTA 0.5, phosphoenolpyruvate 0.42, NADH 0.2 mM, Ca^{2+} ionophor A23187 1 μM , pyruvate kinase 7.5 IU/ml, lactic dehydrogenase 18 IU/ml, and protein 1-5 $\mu\text{g/ml}$ (pH=7.5). The reaction was started with the addition of ATP, and the rate of hydrolysis was determined from the changes of absorbance measured at 340 nm. The recorded absorbance was linear within a range of 5-10 min. Data were corrected for the basal ATPase activity, measured in the absence of calcium, and the results were expressed as $\mu\text{mol P}_i/\text{mg protein/min}$.

9. Statistics

The arithmetic means and the standard error of mean were calculated from our results. Statistical significance was determined using one-way ANOVA followed by Bonferoni test. Differences were considered significant when the P value was less than 0.05.

RESULTS

1. Effect of thymol on action potential configuration in canine myocytes

Thymol induced cumulative concentration-dependent changes in action potential configuration. At a low concentration of 10 μM , the only observed effect was a reduction in phase 1 repolarization and partial attenuation of the notch. Higher concentrations of thymol (100 μM and above) decreased also action potential duration and caused depression of the plateau potential. The shortening of APD was more pronounced at 50 % than at 90 % level of repolarization. Moderate

reductions in the maximum rate of depolarization were observed in the higher concentrations of thymol. No change in the resting potential was observed even at the highest concentration studied.

2. Effect of thymol on L-type calcium current in canine myocytes

Thymol displayed a concentration-dependent suppressive effect on peak I_{Ca} . The effect of thymol on peak I_{Ca} developed rapidly (within 30 sec). Its effect was stable and was fully reversible. Inhibition of the current was statistically significant from the concentration of 50 μ M (inhibition of 14.3 ± 8.65 %) and above. Fitting results to the Hill equation yielded an EC_{50} of 158 ± 7 μ M and a slope factor of 2.96 ± 0.43 .

No shift in the current-voltage relationship was observed after application of 150 and 250 μ M thymol. Ca -conductance was significantly diminished by 250 μ M thymol at each membrane potential studied, however, when G_{Ca} values of both curves were normalized to the respective G_{Ca} obtained at +30 mV, the G_{Ca} - V_m relationships were almost identical. This latter result suggests that voltage dependence of activation of I_{Ca} is not affected by thymol.

In contrast to the unchanged voltage dependence of activation, inactivation kinetics were seriously altered in the presence of thymol in a reversible manner. Superfusion of the cells with 50, 150 and 250 μ M thymol shifted the midpoint potentials by 5.1 ± 1 , 10.4 ± 1.2 and 17.4 ± 1.6 mV, respectively, from the control value of -21.6 ± 0.7 mV towards more negative potentials without significant changes in the slope factor.

In addition to increase steady-state inactivation, thymol also accelerated the time-dependent decay of I_{Ca} . The decay of I_{Ca} followed biexponential kinetics at +5 mV, characterized as a sum of a fast and a slow component in control. Application of increasing concentrations of thymol significantly reduced the relative amplitude of both components and decreased the time constant of the fast component. The slow component of inactivation was more sensitive to thymol than the fast component, since it was completely blocked by higher thymol concentrations (150 and 250 μ M), allowing only monoexponential fitting of these curves.

Both the fast and slow time constants of recovery from inactivation were increased significantly by 150 μ M thymol in a reversible manner (from 47.2 ± 2.7 to 83 ± 4.8 ms and from 292 ± 33 to 569 ± 41 ms, respectively). Thymol slowed the recovery from inactivation of the L-type calcium channels.

3. Effect of thymol on calcium current in undiseased human ventricular cells

Effects of thymol (250 μ M) in the human cells were qualitatively similar to those obtained in canine myocytes. Thymol suppressed the peak value of I_{Ca} at each test potential studied (from -12.1 ± 1.1 to -3.8 ± 1.1 A/F at +5 mV) without changing the shape of the I-V relationship and the normalized G_{Ca} - V_m curve. Effect on the voltage-dependence of steady-state inactivation was also

similar to those seen in the canine cells: the midpoint of the Boltzmann function was shifted from -20.3 ± 0.52 mV to -32.7 ± 0.17 mV (12.4 ± 0.7 mV leftward shift), while the slope factor remained unaltered. Similar results were obtained in canine cells, 250 μ M thymol reduced the amplitude of both components and decreased the respective time constants. Since the suppressive effect of thymol was weaker in human cells than in canine myocytes, the slow component was not fully abolished, only markedly reduced allowing biexponential fit of the current traces.

4. Effect of thymol on the transient outward potassium current in canine myocytes

The transient outward current, I_{to} , was studied using voltage pulses of 400 ms duration clamped from the holding potential of -80 mV to test potentials ranging between -10 and +60 mV. Each of this test pulses were preceded by a 5 ms long prepulse to -40 mV in order to inactivate the Na current. I_{to} was decreased by thymol in a concentration-dependent manner. This effect developed at relatively low concentrations (suppression of 5.2 ± 2.4 % and 17.4 ± 2.7 % were observed in the presence of 1 and 10 μ M thymol, respectively). The blocking effect of 100 μ M thymol on the amplitude of I_{to} developed within 30 sec and was fully reversible within 1 min of washout. The Hill equation, used to describe the concentration-dependency of the thymol-effect, yielded an EC_{50} of 60.6 ± 11.4 μ M and a Hill coefficient of 1.03 ± 0.11 for I_{to} . The blocking effect of thymol on I_{to} was not voltage-dependent. Similarly, no change in the voltage dependence of the steady-state inactivation of I_{to} was observed (midpoint potentials of -38.4 ± 3 mV and -39.3 ± 3.6 mV were determined in the absence and presence of 100 μ M thymol, respectively). The decay of I_{to} was best fitted as a sum of two exponentials in canine cells, described by a fast (2.3 ± 0.3 ms) and a slow (10.4 ± 1.3 ms) time constant at +50 mV in control. Application of 100 μ M thymol had no significant effect on the fast time constant but decreased the slow time constant to 6.2 ± 0.7 ms.

5. Effect of thymol on the rapid and slow components of the delayed rectifier potassium current

To activate only the fast component of the delayed rectifier potassium current depolarizing voltage pulses of 150 ms duration were used from the holding potential of -40 mV to the test potential of +10 mV. The decaying current tails recorded at -40 mV after the end of the test pulse was assessed as I_{Kr} . Tail current amplitudes were significantly decreased by thymol in a reversible and concentration-dependent manner. Fitting these data to the Hill equation yielded EC_{50} value of 63.4 ± 6.1 μ M and a slope factor of 1.29 ± 0.15 . In another set of experiments I_{Kr} was activated at various voltages ranging from -30 to +30 mV for 150 ms. 100 μ M thymol failed to alter the voltage dependence of activation of the current although the inhibition was evident at all voltages positive to -20 mV.

The slow component of the delayed rectifier, I_{Ks} was activated using long (3 s) depolarizing pulses

clamped to +50 mV, and the fully activated current, measured at the end of depolarization, was considered as a measure of I_{Ks} . Thymol caused concentration-dependent and reversible block of I_{Ks} characterized by an EC_{50} value of $202 \pm 11 \mu M$ and a Hill coefficient of 0.72 ± 0.14 . Similar to results obtained on I_{Kr} , the thymol induced block of I_{Ks} was not voltage-dependent between +10 and +60 mV.

6. Effect of thymol on the inward rectifier potassium current

The steady-state current-voltage characteristics of the membrane were studied using voltage clamp steps of 400 ms duration ranging between -125 and +65 mV and arising from the holding potential of -80 mV. 100 μM thymol decreased the currents measured at the end of the impulses but did not alter the negative I-V relationship of I_{K1} . At the test potential of -125 mV 100 μM thymol reduced I_{K1} density from the control value of -43.3 ± 1.6 A/F to -34.9 ± 1.6 A/F. The blocking effect of thymol on I_{K1} developed rapidly remained stable and was readily reversible.

7. Effects of thymol analogues on L-type calcium current in canine myocytes

The experiment procedure was totally the same as previously described at thymol. The L-type calcium current practically was not altered by 300 μM vanillin and 300 μM guaiacol in canine ventricular myocytes. In the presence of 300 μM zingerone, eugenol and carvacrol the amplitude of the I_{Ca} was decreased by 17, 68 and 71 %, respectively. The blocking effect was reversible. The Hill equation, used to describe the concentration-dependency of the blocking effect of the carvacrol and eugenol, yielded an EC_{50} of 98 ± 11 , and $187 \pm 15 \mu M$, respectively. The value of the Hill coefficient was close to 1.5 for both carvacrol and eugenol in sharp contrast to results obtained previously with thymol.

The voltage dependency of I_{Ca-L} was not altered by either eugenol or carvacrol. Ca-conductance was significantly reduced by carvacrol and eugenol at each membrane potential studied in a concentration-dependent manner. When G_{Ca} values of the curves were normalized to the respective G_{Ca} obtained at +30 mV, the G_{Ca} - V_m relationships were almost identical comparing to those maintained at control. This latter result suggests that voltage dependence of activation of I_{Ca} is not affected by either eugenol or carvacrol. The decay of I_{Ca} followed biexponential kinetics at +5 mV, characterized as a sum of a fast and a slow component in control. 300 μM eugenol and carvacrol significantly decreased the time constant of fast component.

The effect of 100 and 300 μM eugenol and carvacrol on the voltage-dependence of steady-state inactivation was similar to each other although carvacrol had bigger effect. Both drug significantly shifted the midpoint potentials toward more negative potentials in a concentration-dependent and partially reversible manner.

300 μM carvacrol significantly slowed the recovery from inactivation of the L-type calcium channels

in reversible manner but eugenol did not exert that kind of effect.

The effect of carvacrol on all examined kinetical properties of the L-type calcium channels was more pronounced than those of eugenol.

8. Effect of carvacrol on calcium current in undiseased human ventricular cells

100 μM carvacrol suppressed the peak value of I_{Ca} at each test potential studied (at +5 mV the blocking effect was about 50 %) without changing the shape of the I-V relationship and the normalized $G_{\text{Ca}}\text{-}V_{\text{m}}$ curve. Effect on the voltage-dependence of steady-state inactivation was also similar to those seen in the canine cells although the effect was bigger in human cells. The midpoint of the Boltzmann function was shifted from -21.38 ± 0.65 mV to -29.52 ± 0.44 mV, while the slope factor remained unaltered. The effect of carvacrol on the voltage-dependence of steady-state inactivation was reversible. Similar to our results obtained in canine 100 μM carvacrol accelerated the decay of the calcium current in human myocytes too but failed to modify the recovery from inactivation of the L-type calcium channels.

9. Effect of thymol on contractility in canine right ventricular trabeculae

Thymol caused a concentration-dependent negative inotropic action on right ventricular trabeculae isolated from canine hearts. Although the effect of 30 μM thymol was statistically significant, suppression of the contractile force was not complete even at a concentration of 1000 μM . Hill plot of the data yielded an EC_{50} of 297 ± 12 μM and a Hill coefficient of 0.95 ± 0.04 . The shape of the contraction curve was not affected by thymol and accordingly, no significant change was observed in time to peak tension (146 ± 3 ms versus 145 ± 20 ms) or half-relaxation time (67 ± 3 ms versus 70 ± 3 ms) when comparing contractility in control and in the presence of 1000 μM thymol, respectively.

10. Effects of thymol in Langendorff-perfused guinea pig hearts

In Langendorff-perfused isolated guinea pig hearts thymol displayed a concentration-dependent action both on the contractile parameters and $[\text{Ca}^{2+}]_{\text{i}}$ transients. Thymol at a concentration of 150 μM , or higher, significantly decreased the systolic pressure. The diastolic pressure was significantly increased already from 50 μM thymol. These additive changes resulted in a marked reduction of the pulse pressure falling to values close to zero in the presence of 350 μM thymol.

In the presence of higher concentrations of thymol (150 μM , or above) the alterations observed in contractility and cytosolic calcium concentration were largely congruent: systolic $[\text{Ca}^{2+}]_{\text{i}}$ decreased, while diastolic $[\text{Ca}^{2+}]_{\text{i}}$ increased on the effect of thymol, and the amplitude of the $[\text{Ca}^{2+}]_{\text{i}}$ transient was

reduced to zero by 350 μM thymol. In contrast to results obtained with these higher thymol concentrations, lower concentrations (50-100 μM) appeared to induce a calcium-sensitizer effect, since the systolic pressure failed to decrease in spite of the reduced systolic $[\text{Ca}^{2+}]_i$ values, and similarly, diastolic pressure increased without significant increases in diastolic $[\text{Ca}^{2+}]_i$. In summary, the effect of thymol on contractility was qualitatively similar in guinea pig and canine ventricular preparations, although its concentration dependence was steeper in guinea pig than in dog (reduction of force generation was practically complete by 350 μM thymol in the former, whereas it was approx. 50% in the latter).

11. Effects of thymol on calcium handling in canine heavy SR vesicles

Application of thymol resulted in a rapid release of calcium from the vesicles. This effect was statistically significant at a concentration of 100 μM or above, and became more pronounced with increasing the concentration of thymol. The rate of calcium release, determined from the slope of the thymol-induced change in absorbance, was highly concentration-dependent exhibiting a maximal rate of release (V_{max}) of 0.47 ± 0.04 nmol/s, an EC_{50} of 258 ± 21 μM , and a Hill coefficient of 3.02 ± 0.54 , showing strong cooperativity between the binding sites.

The maximum velocity of calcium uptake into the vesicles is proportional to the Ca^{2+} -ATPase activity of the preparations, a value of 0.79 ± 0.07 $\mu\text{mol P}_i/\text{mg protein/min.}$ was obtained in control. The activity of the calcium pump (Ca^{2+} -ATPase) was inhibited by thymol in a concentration-dependent manner. The thymol-induced inhibition was significant statistically from a concentration as low as 50 μM , and was characterized with an EC_{50} of 253 ± 4.7 μM and a Hill coefficient of 1.62 ± 0.05 .

12. Effect of thymol in single canine ryanodine receptors (RyR) incorporated into lipid bilayer

Under control conditions (in the absence of thymol) the specific conductance of RyR was 512 ± 55 pS. Binding of thymol to the RyR failed to alter the specific conductivity of the channel (521 ± 55 and 507 ± 59 pS in 150 and 300 μM thymol, respectively). The number of openings of the channels was increased in the presence of thymol and long lasting open events was induced. The mean open probability was 0.013 ± 0.024 , and mean open time 0.305 ± 0.087 ms under control conditions. Application of thymol (150 and 300 μM) increased the open probabilities to 18 and 21 times of their control values, respectively, (to 0.233 ± 0.103 and 0.272 ± 0.11). However, these open probabilities did not differ significantly from one another, indicating that the effect of thymol on open probability was already close to saturation at the concentrations studied. We reanalyzed the records after excluding the long lasting open events observed in the presence of thymol. These new open probabilities (0.024 ± 0.025 and 0.031 ± 0.028), and mean open times (0.357 ± 0.08 ms and 0.385 ± 0.103 ms) obtained in the presence of 150 μM and 300 μM thymol, respectively, were not

significantly different from the control values, indicating the existence of two, well distinguishable gating modes of the RyR channels in the presence of thymol.

The effect of thymol on the RyR was not voltage-dependent, since it was not influenced by the magnitude of the holding potential, varied between -80 and +80 mV. The thymol-activated RyR channel was able to interact with ryanodine. This result indicates that the ryanodine binding site of RyR is still accessible in the presence of thymol.

13. Effect of thymol on Ca current in rat skeletal muscle fibers

Thymol displayed a concentration-dependent suppressive effect on peak I_{Ca} . Inhibition of the current was statistically significant from the concentration of 100 μ M (reduction of I_{Ca} to 83 ± 7 %) and was almost complete at 600 μ M (I_{Ca} was reduced to 9 ± 3 %). Fitting the results to the Hill equation yielded a k_{50} value of 193 ± 26 μ M and a Hill coefficient of 2.52 ± 0.29 .

Thymol displayed a concentration-dependent biphasic effect on these parameters: both activation and inactivation were retarded at concentrations of 100-200 μ M, but both of them were accelerated significantly by 600 μ M thymol.

Effect of thymol (300 μ M) on activation of I_{Ca} was not voltage-dependent. 300 μ M thymol significantly reduced the conductance of calcium channels but left unchanged shape of G_{Ca} -V curve. The estimated variables describing the voltage dependence of channel activation: the voltage of half-maximal conductance (V' , -12.2 ± 2.4 versus -12.8 ± 1.3 mV) and the slope factor (k , 6.1 ± 1.2 versus 4.6 ± 0.7 mV) were not altered significantly by 300 μ M thymol. On the other hand 300 μ M thymol significantly reduced the maximal conductance (G_{max}) from a control value of 120 ± 2.7 to 74.3 ± 1.5 S/F.

14. Effect of thymol on K current in rat skeletal muscle

Thymol displayed a concentration-dependent suppressive effect on peak I_K . Inhibition of the peak current was statistically significant from the concentration of 100 μ M. The cumulative concentration-dependent effect of thymol was fitted to the Hill equation yielding a 93 ± 11 μ M value of k_{50} and a Hill coefficient of 1.51 ± 0.18 . Time constant for activation was not significantly altered by thymol concentrations up to 300 μ M, where as the monoexponential time constant for inactivation was decreased significantly by 200 and 300 μ M thymol. The action of thymol on skeletal muscle I_K was apparently not voltage-dependent. The effect of thymol was reverted partially upon washout at membrane potentials positive to +20 mV, but practically no recovery was obtained at less positive voltages.

DISCUSSION

1. Effect of thymol on the ionic currents and action potential in canine ventricular myocytes

Thymol displayed a concentration-dependent suppressive effect on all of the examined ionic currents and modified the configuration of the action potential. This suppressive effect, however, was substantially different for K and Ca currents. I_{to} and I_{Kr} were found to be more sensitive to thymol than I_{Ks} and I_{Ca-L} . The Hill coefficient estimated for the thymol-induced inhibition of K currents (I_{to} , I_{Kr} and I_{Ks}) were close to unity, suggesting the involvement of single, independent binding site. In contrast suppressive effect of thymol on I_{Ca} was characterized by a Hill coefficient close to 3 suggesting positive cooperation between the binding sites involved, or alternatively, contribution of more than one mechanisms in the block. Except the acceleration of decay of I_{to} , no changes in kinetic properties of the potassium currents were observed in the presence of thymol.

Marked leftwards shift in the voltage-dependence of steady-state inactivation of I_{Ca} was observed with thymol. This can result much more inactive calcium channels in the presence of 250 μ M thymol (for example 85 % instead of 10 % at -30 mV). Based on this the blocking effect of thymol on cardiac Ca current resembles that of the conventional Ca-entry blocker verapamil. Thymol increased the time constant of the recovery from inactivation of calcium channels, which may result appearance of permanently inactive channels especially at higher heart rates. Thymol caused acceleration of decay of I_{Ca} which might be due to the calcium release from the SR caused by thymol. This latter effect might not substantially modify the interpretation of present results on ion currents due to the presence of 10 mM EGTA in our pipette solution. This was not the case in action potential measurements, performed with conventional sharp microelectrodes containing EGTA-free KCl, therefore, the possible contribution of the thymol-induced Ca release to changes in action potential morphology cannot be ruled out in these experiments. All the changes in the kinetical parameters of I_{Ca} results decreased portion of active calcium channels during heartbeats. Due to this less calcium ion can enter the myocytes from the extracellular space explaining the negative inotropic effect of thymol found in our experiments.

The effect of thymol on action potential configuration of canine myocytes can be well explained by the differences in EC_{50} values estimated for the suppressive effects of thymol on various ion currents. Low concentration (10 μ M) abolished the notch of the action potential without further changes in the action potential morphology, whereas higher concentrations (100 μ M and above) caused shortening of APD and depression of the plateau. The former effect is likely due to inhibition of I_{to} , and the latter due to blockade of I_{Ca} . This thymol-induced shortening of APD suggests that the effect of high thymol

concentrations on APD is dominated by suppression of I_{Ca} in contrast to the inhibitory effect on the repolarizing potassium currents, in spite of the lower EC_{50} values obtained for K channel blockade (except I_{Ks}).

2. Effects of thymol on calcium current in undiseased human ventricular

It is important to emphasize that the effects of thymol on I_{Ca} were qualitatively similar in canine and healthy human ventricular cells, however, they were weaker in the latter. Thymol decreased the peak value of the L-type calcium current and accelerated its decay in both canine and human ventricular myocytes. At a concentration of 250 μ M thymol decreased peak I_{Ca} by 82.6 %, the faster time constant for inactivation by 49.6 %, and shifted the steady-state inactivation curve by -17.4 mV in canine myocytes. The respective values obtained in human cells were 68.6 %, 38.2 %, and -12.4 mV. In spite of these minor differences observed in canine and human myocytes, one may conclude that the mechanism of action of thymol on calcium channels is essentially similar in dog and human.

3. Effect of thymol on Ca and K current in rat skeletal muscle fibers

Similarly to the results obtained in cardiac myocytes Thymol exerts concentration-dependent blocking effect on both Ca and K current in skeletal muscle fibers too. Hill coefficients estimated for the thymol-induced block of K currents in cardiac and skeletal muscle cells were close to unity (values ranging from 0.72 to 1.51), indicating the involvement of a single, independent binding site. In contrast, the inhibitory effect of thymol on I_{Ca} of skeletal and cardiac muscle cells was characterized by Hill coefficients of 2.52 and 2.96, respectively. This suggests strong positive cooperation between the binding sites involved, or alternatively, contribution of more than one mechanism in the block. The k_{50} values of cardiac I_{to} and skeletal muscle I_K currents (60.6 and 93 μ M, respectively) were significantly lower than those obtained for calcium current (158 and 193 μ M).

The effect of thymol on the time course of activation of the channels was variable too. In ventricular myocytes thymol caused acceleration of decay of I_{Ca} in all examined concentration, while in skeletal muscle the effect was biphasic. Thymol increased the value of the inactivation time constant in a concentration range between 100-300 μ M but its value was significantly smaller in the presence of 600 μ M thymol comparing to control.

4. Effect of thymol on contractility in canine right ventricular trabeculae and in Langendorff-perfused guinea pig hearts

Thymol exerts a concentration-dependent cardiodepressant action in mammalian ventricular

myocardium. Thymol increased diastolic pressure and $[Ca^{2+}]_i$, whereas systolic pressure and $[Ca^{2+}]_i$ were decreased by the compound. Thus reduction in pulse pressure, observed in Langendorff-perfused guinea pig hearts in the presence of higher concentrations of thymol, can be well explained with the decreased amplitude of the $[Ca^{2+}]_i$ transient. However, there were differences in the concentration dependence of the thymol-induced negative inotropy in canine and guinea pig preparations. Thymol induced a monotonic depression of contractility from relatively low (30 μ M) concentrations in canine ventricular trabeculae, whereas the developed tension in guinea pig heart was decreased only at a concentration of 150 μ M and above. In addition, this effect was fully saturated in the presence of 350 μ M thymol in guinea pig, while 25 % of contractility was still preserved in canine preparations treated with 1000 μ M thymol. The reason for this interspecies difference remains hidden, since $[Ca^{2+}]_i$ transients were not recorded in working canine heart. The pulse pressure was not impaired by thymol at concentrations lower than 150 μ M in guinea pig hearts, in spite of the fact that $[Ca^{2+}]_i$ transients have already been strongly depressed. This result suggests that low concentrations of thymol may display a calcium sensitizer effect in guinea pig preparations, which might probably be absent in canine cardiac muscle. Further studies, involving simultaneous measurements of $[Ca^{2+}]_i$ and contractility in canine hearts, are required to better elucidate this point.

5. Effects of thymol on calcium handling in canine heavy SR vesicles and in single canine ryanodine receptors (RyR) incorporated into lipid bilayer

Thymol induced a concentration-dependent calcium release from canine cardiac heavy SR vesicles. Single channel studies, performed in canine RyR incorporated into planar lipid bilayer, revealed that thymol binds to, and thus modifies, the gating properties of the calcium release channel in the SR. The appearance of the “long lasting open events” (LLOE) observed in the presence of thymol may account for the thymol-induced calcium release, since the mean open time of the LLOEs was longer than that of the normal openings by three orders of magnitude (approximately 0.3 s *versus* 0.3 ms). It is, however, important to note that the mean open probability of the RyR after exclusion of the LLOEs from the analysis, was not altered by thymol. The alternation of LLOEs with periods of normal channel gating may be explained by assuming the existence of two gating modes of the thymol-modified RyR channel: one having normal gating kinetics and another one resulting in appearance of the LLOEs. Alternatively, development of LLOE may be also the consequence of rapid binding of thymol to and slow unbinding from the channel. Present results, however, do not allow safe distinction between these possibilities.

According to our results, the mechanism of the negative inotropic action of thymol is based on at least two independent actions of the drug (i.e. induction of calcium release from the SR *via* activation of RyR and inhibition of the SR calcium pump), both leading to elevation of diastolic $[Ca^{2+}]_i$ and

calcium depletion of the SR. This explanation is supported by the very close EC_{50} values estimated for negative inotropy (297 μ M), calcium release (258 μ M) and inhibition of the calcium pump (253 μ M). In contrast to the similar EC_{50} values estimated for depression of contractility, calcium release and inhibition of the pump, the corresponding Hill coefficients were markedly different (0.95, 3.0 and 1.62, respectively).

6. Consequencies of the effects of thymol

The results of our experiments can be useful for both the basic and applied research. Caffeine is widely used to release calcium from intracellular stores in experiments exploring the excitation-contraction coupling in skeletal and cardiac cells. The effect of caffeine develops slowly and the molecule is poorly soluble in both water and in the usually used other solvents like alcohol, ether and dimethyl-sulfoxide. Regarding these facts caffeine is not the most perfect drug to elicit calcium release from the intracellular stores. Based on our results thymol is capable to release calcium from the SR in canine ventricular myocytes. This effect of thymol developed faster and diminished faster upon washout, than those of caffeine. The efficient concentration of thymol was 100 fold lower than those of caffeine. Because of all these facts thymol can replace the application of caffeine in those experiments where fast, reversible and reproduceable calcium release is needed. For this case it is worth to have a knowledge of other effects of thymol, because these may alter the result of these experiments. Among these other effects the effects of electrophysiological properties in cardiac and skeletal muscle cells can be mentioned.

Thymol modifies the calcium handling of myocardium and inhibits its contraction. Because of all these effects thymol may cause cardiac arrhythmias in case of incorporation of the appropriate amount of the chemical. Since the compound is a commonly applied constituent of mouthwash, herbal complexes, and dental medication, its overdose resulting in intoxication (accidental swallowing by children or intentional ingestion by adults in case of suicide) may not be ruled out. For instance, several types of mouthwash contain 1 % thymol. Ingestion of 200 ml mouthwash will build up approximately 300 μ M concentration in the whole body fluid, calculating with equal distribution of thymol in extracellular and intracellular body water compartments. This is in the range of the present study, indicating that serious alterations in cardiac and skeletal muscle ion currents can be anticipated in case of thymol intoxication.

Probably it is more important from the therapeutic point of view that thymol is used to stabilize liquid halothane when applying for general anesthesia. Although the concentration of thymol is relatively low (0.01%) in the original halothane-thymol mixture, its concentration may dramatically increase due to accumulation of thymol in the vaporizer in case of improper use. In a perioperative cardiac arrest registry performed in pediatric patients two third of cases of cardiac arrest due to

medication was considered to be a consequence of application of halothane. Indeed, in guinea pig ventricular myocytes halothane was shown to block I_{Ca} and caused a -11 mV leftward shift of the steady-state inactivation curve. Halothane also inhibited I_K but had just little effect on I_{K1} in guinea pig ventricular myocardium while it depressed I_{to} in rat ventricular cells. These effects obtained with liquid halothane (containing also thymol) resembles our present results with thymol, raising the possibility that some of the cardiac effects ascribed previously to halothane may probably be attributed to the concomitant presence of thymol in the superfusate. The previously mentioned so-called halothane hepatitis can be due to the presence of thymol in the vaporizer. Further studies, involving measurements of independently applied thymol and halothane, are required to better elucidate this point.

7. Effects of thymol analogues on L-type calcium current in canine ventricular myocytes

Guaiacol and vanillin hardly affected the I_{Ca-L} in canine ventricular myocytes even in the presence of the highest applied concentration. Zingerone had larger effect, in 300 μ M concentration the amplitude of the I_{Ca} was decreased by 17 %. The decay of the current was not affected by these three analogues. In contrast, eugenol and carvacrol had many effect on the kinetical parameters of I_{Ca} . Both eugenol and carvacrol was able to display total block of the calcium current. Comparing to thymol carvacrol was more effective and eugenol was less effective to block the calcium current. Regarding to these facts it could be surprising that the obtained Hill coefficients for both eugenol and carvacrol was about 1.5 in sharp contrast to those obtained for thymol (2.96 ± 0.43). Neither eugenol nor carvacrol was able to modify the voltage-dependence of the peak of I_{Ca-L} . In contrast, both drugs accelerated the decay of the current and slowed the recovery from inactivation. Based on these results the transition from open to inactive state of the calcium channel was accelerated and the transition from inactive to closed state was slowed down by both eugenol and carvacrol. As found in the presence of thymol both eugenol and carvacrol caused a marked leftward shift of the steady-state inactivation curve in a concentration dependent manner.

8. Effect of carvacrol on calcium current in undiseased human ventricular cells

The effects of carvacrol on I_{Ca-L} were similar in canine and healthy human ventricular cells. Carvacrol failed to modify the voltage-dependence of the peak and the steady-state inactivation of the calcium current in both canine and human myocytes. The amplitude of the current was decreased, its decay was accelerated and its steady-state inactivation curve was shifted toward more negative potentials by carvacrol in human and canine cells too. In contrast to our results obtained with thymol human ventricular myocytes were more sensitive to carvacrol than canine cells (except the time constant of the decay of I_{Ca}). At a concentration of 100 μ M carvacrol decreased peak I_{Ca-L} by 49 %,

the faster time constant for inactivation by 30 %, and shifted the steady-state inactivation curve by -7.2 ± 0.4 mV in canine myocytes. The respective values obtained in human cells were 51 %, 24 %, and -8.1 ± 0.6 mV. In spite of these minor differences observed in canine and human myocytes, one may conclude that the mechanism of action of carvacrol on calcium channels is essentially similar in dog and human.

9. Relationship between the structure of thymol analogues and their effect on the L-type calcium current

The physico-chemical properties of an aromatic molecule depends on the substituents of the benzene ring and on the distribution of the electrons along the molecule. The hydrophobic tail substituent is known to promote the lipid solubility of the compound. In our experiments guaiacol and vanillin, which are water soluble, had no effect on the Ca current. In contrast, eugenol, thymol, carvacrol, and zingerone are practically insoluble in water and they blocked the current in a concentration-dependent manner. These results suggest that terpenoid phenol derivatives, due to their hydrophobic character, may exert their effects on the lipid-protein interface by altering the local environment of the channels. Indeed, the fractional (not integer) values of the Hill coefficients obtained for carvacrol, eugenol, and zingerone, may give further support of this explanation. However, in the case of thymol the Hill coefficient was close to 3, suggesting strong positive cooperation between discrete binding sites involved, or, alternatively, contribution of more than one mechanism in the block. It is possible, therefore, that in addition to the postulated lipid interactions, thymol may bind to and thus modify the channel proteins directly.

Surprisingly we found that the EC_{50} values do not fully correlate with the lipophilic nature of the molecules. Since the longer tail and the other substituents of the benzene ring may decrease the dissociation of proton from the hydroxyl group, the binding potency of the molecule to the channel protein can consequently decrease. Indeed, among the molecules which suppressed Ca current effectively, zingerone has the longest tail and the weakest potency to block the current. The size of tail decreases from eugenol toward thymol and carvacrol, accordingly, the calcium channel blocking potency of the molecules increased. The observed differences in the EC_{50} values observed between carvacrol and thymol can be explained by the different position of the hydroxyl group within the molecule.

In extenso articles that provide the basis of the thesis:

1. J. Magyar, N. Szentandrassy, T. Bányász, L. Fülöp, A. Varró, PP. Nánási: Effects of thymol on calcium and potassium currents in canine and human ventricular cardiomyocytes. *Br J Pharmacol* 2002; 136:330-338. **IF=3.45**
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