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Effect of thymol on kinetic properties of Ca and K currents in rat skeletal muscle

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Abstract

Background: Thymol is widely used as a general antiseptic and antioxidant compound in the medical practice and industry, and also as a stabilizer to several therapeutic agents, including halothane. Thus intoxication with thymol may occur in case of ingestion or improper anesthesia. In the present study, therefore, concentration-dependent effects of thymol (30–600 micro-grams) were studied on calcium and potassium currents in enzymatically isolated rat skeletal muscle fibers using the double vaseline gap voltage clamp technique.

Results: Thymol suppressed both Ca and K currents in a concentration-dependent manner, the EC_{50} values were 193 ± 26 and 93 ± 11 μM, with Hill coefficients of 2.52 ± 0.29 and 1.51 ± 0.18, respectively. Thymol had a biphasic effect on Ca current kinetics: time to peak current and the time constant for inactivation increased at lower (100–200 μM) but decreased below their control values at higher (600 μM) concentrations. Inactivation of K current was also significantly accelerated by thymol (200–300 μM). These effects of thymol developed rapidly and were partially reversible. In spite of the marked effects on the time-dependent properties, thymol caused no change in the current-voltage relationship of Ca and K peak currents.

Conclusions: Present results revealed marked suppression of Ca and K currents in skeletal muscle, similar to results obtained previously in cardiac cells. Furthermore, it is possible that part of the suppressive effects of halothane on Ca and K currents, observed experimentally, may be attributed to the concomitant presence of thymol in the superfusate.

Background

Thymol (2-isopropyl-5-methylphenol) is widely used as a general antiseptic in the medical practice, agriculture, cosmetics and food industry [1–3]. Due to its potent fungicide, bactericide and antioxidant properties thymol is applied primarily in dentistry for treatment of oral infections [4–6]. Thymol was also shown to have strong anti-inflammatory action by decreasing the release of inflammatory metabolites like prostanoids, interleukins, and leukotrienes [7,8]. Thymol is added as a stabilizer to several therapeutic agents, including halothane, and the drug was shown to accumulate in the vaporizer during anesthesia. Thymol was reported to suppress Ca and various K currents in mammalian and human cardiac cells [9], but little is known about its effects in mammalian skeletal muscle. A further purpose of this study was to test

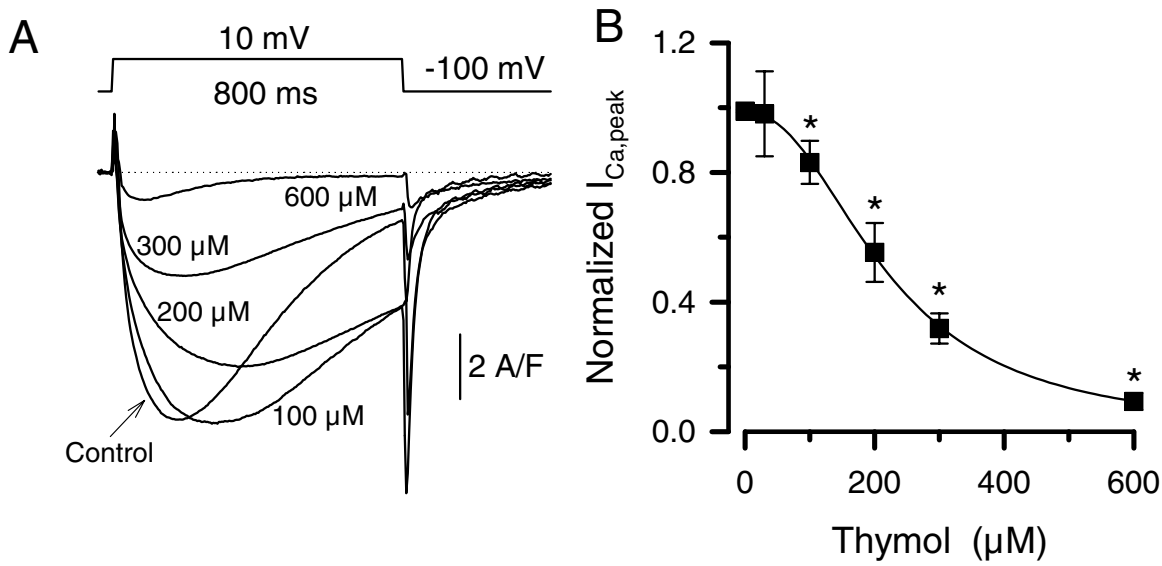

Figure 1

Conentration-dependent effect of thymol on the Ca current. **A:** Superimposed I_{Ca} traces elicited by 800 ms long depolarizations to +10 mV in a muscle fiber before and after cumulative application of 100, 200, 300, and 600 µM thymol. **B:** Average of peak I_{Ca} normalized to control as a function of the thymol concentration. The solid line represents the Hill fit of the averaged curve. Symbols and bars represent mean ± SEM values obtained from 8 preparations, asterisks denote significant ($P < 0.05$) changes from control values.

the effects of the potent antioxidant thymol on the kinetic properties Ca and K currents. Modulation of transmembrane Ca and K currents by antioxidants have been recently reported [10,11].

Results

Effect of thymol on the Ca current

Ca current (I_{Ca}) was activated by sets of 800 ms long depolarizing voltage pulses applied from the holding potential of -100 mV to the test potential of +10 mV, where the peak amplitude of I_{Ca} was close to maximal. In these experiments Na and K currents were blocked by TTX and the combination of external 4-aminopyridine, TEA-CH_3SO_3 plus internal Cs-glutamate, respectively. Thymol displayed a concentration-dependent suppressive effect on peak I_{Ca} in the 8 muscle fibers studied (Figure 1A). Inhibition of the current was statistically significant from the concentration of 100 µM (reduction of I_{Ca} to 83 ± 7%) and was almost complete at 600 µM (I_{Ca} was reduced to 9 ± 3%). Fitting the results to the Hill equation yielded a k_{50} value of 193 ± 26 µM and a Hill coefficient of 2.52 ± 0.29 (Figure 1B).

The superimposed analogue records of Figure 1A clearly show alterations in the current kinetics regarding both activation and inactivation of the current. Due to the known complexity of activation kinetics of I_{Ca} in skeletal muscle [12], the activation of the current was character-

ized with time to peak values (Figure 2A), while the inactivation time constant was determined by fitting the decaying limb of the current to a single exponential function (Figure 2B). Thymol displayed a concentration-dependent biphasic effect on these parameters: both activation and inactivation were retarded at concentrations of 100–200 μM, but both were significantly accelerated by 600 μM thymol.

Current-voltage relations for I_{Ca} were obtained by applying a series of test pulses starting from the holding potential of -100 mV to test potentials increasing from -50 to +60 mV in 10 mV steps in the absence and presence of 300 μM thymol, and peak I_{Ca} was plotted against its respective test potential (Figure 2C). Ca conductance was calculated at each membrane potential using Eqn. 2 and 3 (Figure 2D). Effect of 300 μM thymol on activation of I_{Ca} was not voltage-dependent as indicated by the unchanged shape of the I-V relationship and G_{Ca}-V curves. 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 μM thymol in the 8 muscle fibers studied. On the other hand, 300 μM thymol significantly reduced the maximal conductance (G_{max}) from a control value of 120 ± 2.7 to 74.3 ± 1.5 S/F.

Effect of thymol on K current

K current (I_K) was activated by sets of 100 or 200 ms long depolarizing voltage pulses arising from the holding potential of -100 mV to test potentials up to +40 mV, increasing in 10 mV steps. The cumulative concentration-dependent effect of thymol was evaluated at +20 mV (Figure 3A), and the results were fitted to the Hill equation yielding a 93 ± 11 μM value of k_{50} and a slope factor of 1.51 ± 0.18 in average of the 7 muscle fibers studied (Figure 3B). The analogue I_K records were further analyzed to determine activation and inactivation kinetics. Time constant for activation (determined according to Eqn. 4 in 7 fibers) was not significantly altered by thymol concentrations up to 300 μM, whereas the monoexponential time constant for inactivation was decreased significantly by 200 and 300 μM thymol (Figure 4A). The action on I_K of 300 μM thymol was apparently not voltage-dependent as indicated by the proportional reduction of the current at each test potential between 0 and +40 mV. The effect of thymol was partially reverted upon washout at membrane potentials positive to +20 mV, but little recovery was obtained at less positive voltages (Figure 4B). This was an unexpected result considering the known hydrophobic character of the compound.

Discussion

We have shown in this study that thymol exerts concentration-dependent blocking effect on Ca and K currents in skeletal muscle membranes. This suppressive effect, however, was substantially different in terms of k_{50} values and Hill coefficients. First, the Hill coefficient estimated for the thymol-induced block of K currents was close to unity (1.51), indicating the involvement of a single, independent binding site. In contrast, the inhibitory effect of thymol on I_{Ca} was characterized by a Hill coefficient of 2.52, suggesting a strong positive cooperation between the binding sites involved, or alternatively, contribution of more than one mechanism in the block. The second difference was observed between the sensitivities to thymol. The k_{50} value estimated when blocking I_K (93 μM) was significantly lower than that obtained for I_{Ca} block (193 μM).

Recent experiments have suggested that intracellular as well as sarcolemmal ion channels might be regulated through their thiol redox state. Oxidizing and reducing reagents have indeed been shown to modulate both K [10] and Ca channels [11,14] in a variety of tissues. For K channels antioxidants were found to inhibit transmembrane ion currents [10] similarly as described here for thymol. For Ca channels data are more numerous and more diverse. While Campbell et al. [14] described an activation of L-type Ca current in ferret ventricular myocytes upon oxidation, Fearon et al. [15] found an inhibition in expression systems. Similar inhibition was described in guinea-pig cardiomyocytes, where dithiothreitol was capable of reversing the effect of oxidation [16]. Recently, some antioxidant phenol analogues were shown to suppress L-type Ca current in smooth muscle cells [11]. The observed effects on smooth muscle (little change in kinetics and voltage dependence of activation with a clear reduction in current amplitude) closely resemble those obtained in the present study on skeletal muscle fibers.

Due to the diverse effects of oxidizing and reducing reagents, the effects of thymol can probably be explained only in part with its antioxidant action. As suggested by Fusi et al. [11] direct drug-protein interactions could also take place where thymol may interact with the inactivation machinery of these channels when exerting its inhibitory action. Indeed, thymol failed to modify the voltage dependence of activation of any of the ion currents studied, but caused acceleration of inactivation of the currents. Accordingly, cardiac I_K current, which fails to inactivate upon sustained depolarization, showed only limited sensitivity to thymol [9]. It should be noted, however, that due to the hydrophobic character of the molecule thymol may accumulate in the membrane and could also modify the local lipid environment of the channel protein. These additional unspecific lipid-interactions may account for the fractional values of the Hill coefficients, but not for

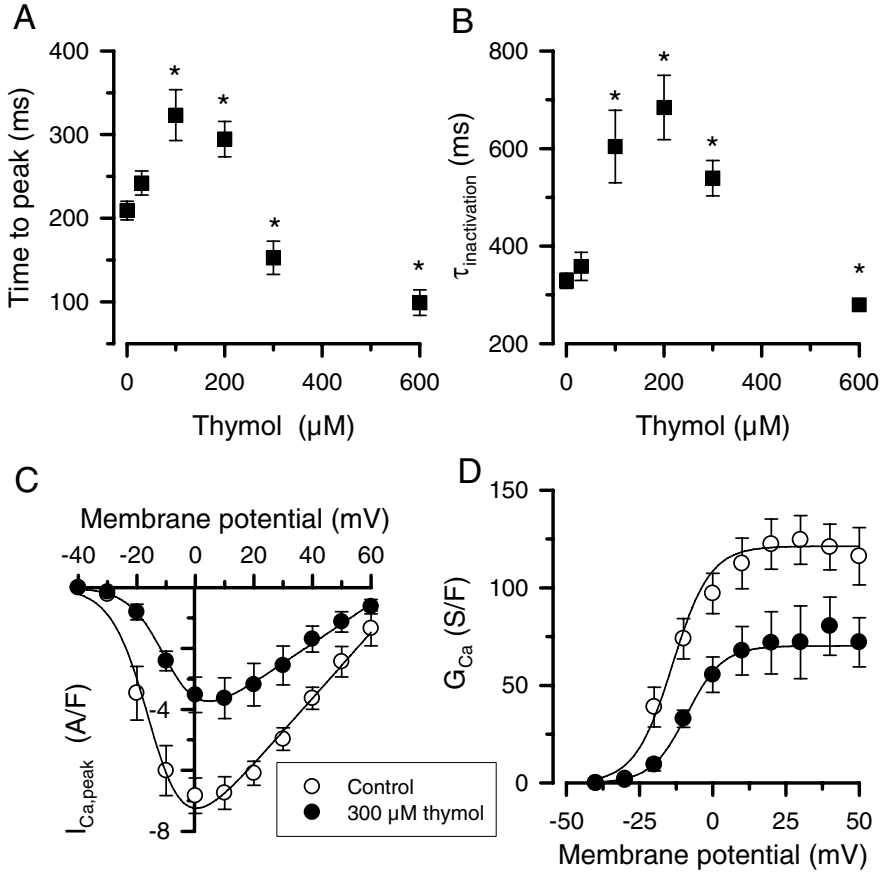

Figure 2

Effect of thymol on the time- and voltage-dependent properties of the Ca current A,B: Concentration-dependent effects of thymol on the time to peak values (**A**) and time constant for inactivation (**B**) of I_{Ca} recorded at +10 mV. **C:** Current-voltage relationship for peak I_{Ca} measured in control and in the presence of 300 μM thymol. Superimposed are the theoretical voltage dependencies obtained by fitting Eqn.2 to the averaged data points. **D:** Ca conductance (G_{Ca}) as a function of the membrane potential. Superimposed are again the theoretical voltage dependencies obtained by fitting data to Eqn. 3.

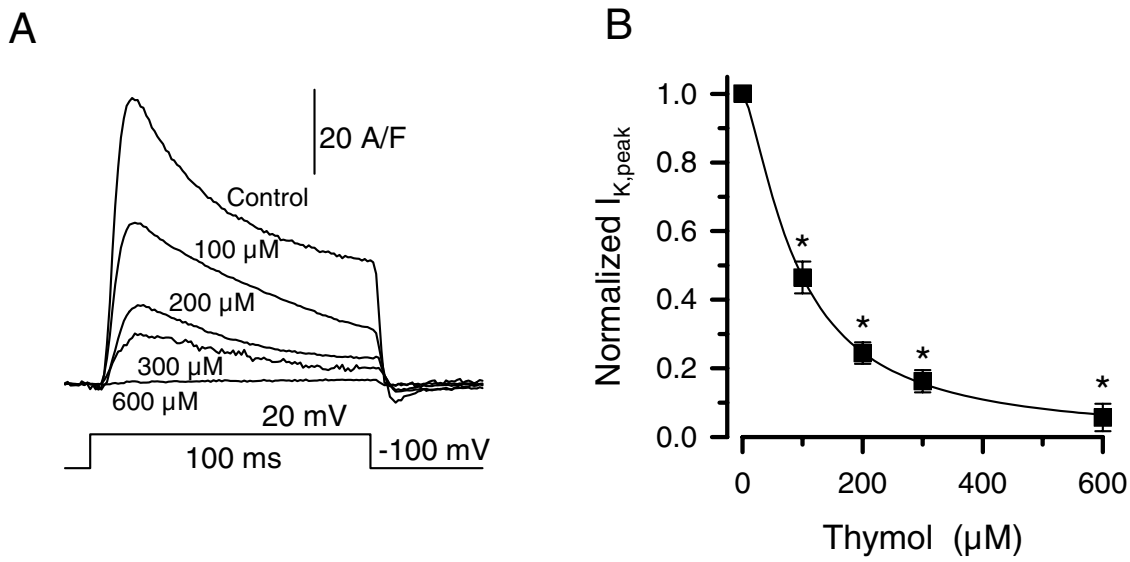

Figure 3
Conentration-dependent effect of thymol on the K current. **A:** Superimposed current records elicited by 100 ms long depolarizations to +20 mV in control and in the presence of increasing concentrations of thymol. **B:** Average of peak I_K normalized to control as a function of the thymol concentration (n = 8). The solid line was obtained by fitting data to the Hill equation.

the differences observed in the thymol-induced block of Ca and K currents.

Since thymol is a commonly applied constituent of mouth wash, 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 mouth wash contain 1% thymol in 200 ml volume. Ingestion of 200 ml 1% thymol solution will build up approximately 300 µM 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 Ca and K currents can be anticipated in case of thymol intoxication.

It has probably more clinical relevance that thymol is used to stabilize liquid halothane when applied for *in vitro* as well as *in vivo* studies, or in case of general anesthesia during surgery. Although the concentration of thymol is relatively low (0.01 %) in the original halothane-thymol mixture [17], its concentration may dramatically increase due to accumulation of thymol in the vaporizer. Indeed, halothane was shown to block Ca and K currents in cardiac [18–20] and skeletal [21] muscle membranes. These effects, obtained with liquid halothane (containing also thymol) in various preparations, strongly resemble the present results and also those of Magyar et al. obtained in canine cardiac cells with thymol [9]. It is possible, therefore, that the suppressive effect of halothane on Ca and K currents are – at least in part – attributable to the concomitant presence of thymol in the superfusate.

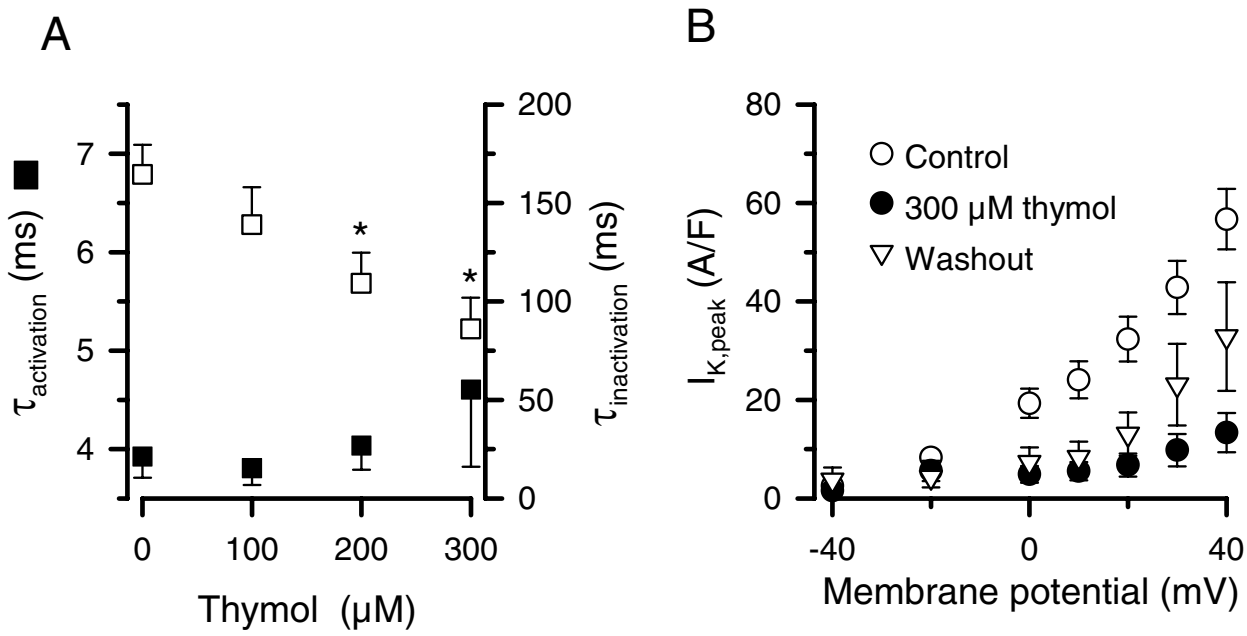

Figure 4
Effect of thymol on the time- and voltage-dependent properties of the K current. **A:** Concentration-dependent effects of thymol on the activation and inactivation time constants of I_K. **B:** Current-voltage relationship obtained for peak I_K in control, in the presence of 300 μM thymol, and after washout.

Methods

Isolation of rat skeletal muscle fibers

Single skeletal muscle fibers were isolated enzymatically from the *extensor digitorum communis* muscles of rats and mounted into a double Vaseline gap chamber as described earlier [22]. Briefly, rats of either sex were anesthetized and killed by cervical dislocation. The muscles were removed and were treated with collagenase (Sigma, 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. The selected fiber was transferred into a recording chamber filled with Relaxing solution containing K-glutamate 150, $MgCl_2$ 2, HEPES 10 and EGTA 1 mM. The fiber segments in the open-end pools were permeabilized using 0.01% saponin. After completing the permeabilization the solutions were exchanged to Internal solution in the open-end pools (containing Cs-glutamate 120, $MgCl_2$ 5.5, Na_2-ATP 5, Na-phosphocreatine 10, glucose 10, HEPES 5 and EGTA 5 mM) and to External solution in the middle pool (containing TEA-CH_3SO_3 140, $MgCl_2$ 2, HEPES 5, tetrodotoxin 0.0003 and 3,4-diaminopyridine 1 mM). For Ca current measurements 5 mM $CaCl_2$ was added to the External solution and TEA-CH_3SO_3 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 $MgCl_2$ instead of 2 mM. All solutions were adjusted to pH 7.2 and 300 mOsm.

Voltage clamp

The experimental set-up and data acquisition have been described in detail in our earlier report [23]. 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 [24]. K current was measured using 100 or 200 ms long depolarizing voltage steps exploring the -80 to +40 mV voltage range.

Concentration dependent effects of thymol on ionic currents were determined using the Hill equation:

$$I = 1 - 1 / (1 + (k_{50} / [thymol])^{n}), \text{ (Eqn. 1)}$$

where k_{50} is the concentration where the effect is half maximal and n is the Hill coefficient.

The peak current *versus* voltage relationship for I_{Ca} was fitted with:

$$I = (V_m - V_{Ca}) * G(V_m), \text{ (Eqn. 2)}$$

where V_m is transmembrane potential, V_{Ca} is the equilibrium potential estimated for Ca, and $G(V_m)$ is the voltage dependence of the conductance given as:

$$G(V_m) = G_{max} / (1 + \exp(- (V_m - V') / k)), \text{ (Eqn. 3)}$$

where G_{max} is the maximal conductance, V' is the potential where the conductance is half of G_{max}, and k is the slope factor. All currents and the maximal conductance were normalized to fiber capacitance to take the size of the individual fibers into account.

The activation kinetics of the K current was fitted with the usual m^4 kinetics:

$$I_K(t) = A * (1 - \exp(- (t - t_0) / T))^{4}, \text{ (Eqn. 4)}$$

where A is the amplitude of the current, T is the time constant of the activation and t_0 is the delay.

Statistics

All values presented are arithmetic means ± SEM. Student's t test for paired data was applied following ANOVA to determine statistical significance. Differences were considered significant when the P value was less than 0.05.

The entire investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and was approved by the local ethical committee.

Authors' contributions

Electrophysiological studies in rat skeletal muscle were performed by PS, NS and LC, while JM and PPN participated in the design of the study. All authors read and approved the final manuscript.

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