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Research Paper

Effect of Piperitenone Oxide on the Skeletal muscle of Toad

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ABSTRACT

Previous studies showed that piperitenone oxide (PO), a major constituent of essential oil of *Mentha x villosa* (EOMV) induces skeletal muscle contraction probably by releasing Ca^{2+} from sarcoplasmic reticulum (s.r.). Here we present evidences that Piperitenone oxide induces muscle contraction in both depolarized (80 mM of K^+) and non-depolarized (4 mM K^+) sartorius muscle of toad. Procaine blocked the contraction induced by PO in non-depolarized muscles. Considering that in saponin permeabilized muscle fibers PO induced contraction, this suggests that the contracture induced by this compound is probably due to the activation of the calcium channel (Ryanodine receptor) present in the membrane of sarcoplasmic reticulum.

Keywords: Piperitenone oxide: Muscle contraction; Sarcoplasmic reticulum; skeletal muscle.

INTRODUCTION

Piperitenone oxide (PO), an oxygenated monoterpene (1-methyl-4-propan-2-ylidene-7-oxabicyclo[4.1.0]heptan-5-one) has been reported to characterize the oils of some chemotypes of *Mentha spicata* (Pine, et al., 1999), M. *longifolia* (Abu-AI-Futuh, et al., 2000; Venskutonis, 1996), M. *villosa* (Matos, et al., 1999) and M. *rotundifolia* (Lorenzo, et al., 2002; Brada, et al., 2006). PO is the major constituent in the essential oil of *Mentha x villosa* Huds. (EOMV), (*Labiatae*), where it occurs in percentages usually higher than 50 % of the total oil weight (Maia, et al., 1992). *Mentha x villosa* is an aromatic, creeping herb, used in folk medicine of northeast of Brazil as stomachic, ansiolytic, and to treat menstrual colic and diarrhea with blood in the stools (Matos, 1994). *Mentha x villosa* has been exploited by local industry of phytotherapics, under the name *Mentha crispa*. Studies looking for the identification of its active principle have been documented activity of the EOMV; and have been referred to the high content of piperitenone oxide, as contrasting to the participation of 23 other minor constituents of this oil (Craveiro, et al., 1990; Hiruma, et al., 1992).

Studies in our laboratory showed that EOMV induces muscle contraction and blocks potassium contracture (Fogaça, et al., 1997). In the present study we

demonstrated that PO induced muscle contraction by releasing calcium from the s.r., probably by the activation of the Ryanodine receptor (RYR). This could explain the previous finding that the muscle contraction induced by EOMV is probably due to the presence of PO in its composition.

MATERIALS AND METHODS

Piperitenone oxide was graciously provided by the Laboratório de Produtos Naturais of the Universidade Federal do Ceará. The isolation was done using the method described by Craveiro et al., 1976. Briefly, freshly chopped plant leaves were placed in a glass flask, connected to one end of a glass vessel with water and the other end, to a water cooled condenser. The water was heated to boiling and the steam percolated through the leaves and collected in the condenser. The oily phase was separated from the water phase. The water phase contained 97% of piperitenone oxide and 3% of other constituents.

All procedures and techniques used in this work were conducted following the rules established by the Helsinki Declarations for the care and laboratorial use of animals. Animal Experimentation Ethics Committee of the Biological Sciences Section at Federal University of Paraná approved all experimental protocols use in this study. Toads (*Buffo paracnemis*) of either sex were anesthetized with sodium pentobarbital (60 mg/kg, intra-coelomically) and then pithed. Sartorius muscles were dissected by the traditional method. The experiments were done in isolated intact muscle or saponin-permeabilized fibers. Intact muscle was mounted vertically in an organ chamber of 20 ml capacity with both ends fastened. One end was tied to the bottom of the chamber, the other end was tied to an optical force transducer (Grass Instrument). Force was recorded on a strip chart recorder (Beckmann Recorder). Ringer solution had the following composition (in mM): NaCl 110, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 3.9, and Tris-(hydroxymethyl)-aminomethane buffer 10, pH 7.4, bubbled with air, at 22°C. The solutions containing high KCl content, NaCl was replaced isotonically by KCl. The contractions induced by PO were normalized to K^+ induced muscle contraction and are expressed as average ± standard error. The PO and procaine were prepared by adding the pure substance directly to the Ringer solution. In other experiments, bundles of sartorius muscle fiber were dissected in relaxing solution, and kept for 30 min. in the same solution containing µg/ml of saponin. This glycoside makes perforations in the sarcolemma but the s.r. and the contractile machinery functions are kept intact (Endo and Lino, 1980, Lofrano-Alves, et al., 2005). The ends of a single muscle fiber were attached by small clamps to an isometric force transducer (Scientific Instruments, GMBH, Heiderberg, Germany) and a stationary arm. Fibers were transferred through a set of solutions contained in small troughs (3ml capacity) built into a block of Plexiglas. The solutions used in all experiments (table 1) were formulated using a computer program which describes the multiple binding equilibria of ions in solution, using binding constants given previously (Godt and Lindley, 1982). The basic relaxing solution had the following composition (in mM, excepted where indicated) Mg^{2+} 1, MgATP 2, NaCP (Na_2phosphocreatine) 15, EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N',-tetraacetic acid) 5, BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) 50, pCa > 8.5 (pCa= -log [Ca^{2+}]), pH 7.0, ionic strength 200 (adjusted with $KMSO_2$), creatine kinase 100 units/ml, at 22°C. This standard solution (solution D)

was modified, as described below. Loading of the sarcoplasmic reticulum with Ca^{2+} was done reducing the pCa to 6.4 (solution L) and the maximal activation of the contractile system (F max.) was obtained in a solution pCa 4.0 (solution A). To be able to record force induced by Ca^{2+} released from the s.r., the concentration of EGTA was reduced to 0.05 and Mg^{2+} to 0.1 mM (solution E). Protocol: By adding caffeine (30 mM) to the solution D the s.r. was depleted of Ca^{2+}; after washed out of caffeine (in solution D) the fiber was transferred to solution L for 120 s and then pre-incubated in a solution E for 60 s. The fiber was transferred to the same solution, containing PO (1.5, 2.0, 3 or 4.0 mg/ml) or caffeine (30 mM). When the force reached the plateau, the fiber was transferred to a solution D, and a new cycle was repeated. At the end of the experiment, F max was obtained. The results were normalized to F max and presented as Means ±SEM.

RESULTS

All six intact preparations exposed to PO (1 to 3 mg/ml), dose dependently developed contraction with an EC50 ≈ 2 mg/ml and the maximal contraction reached 173.83 ± 8.7 % (n = 8) of the contraction induced by 80 mM of K^+ (K^+-contraction). In depolarized muscles (80 mM of K^+), the contraction induced by 3 mg/ml of PO was 141.0 ± 34 % (n=4) of the K^+-contraction.

Table-1: Composition of bathing solutions (sol.) used in saponin skinned skeletal muscle fibers experiments (in mM, except for creatine kinase, pCa and pH).

Component	Diss. sol.	Sol. D	Sol. L	Sol. E	Sol. A
Mg^{2+}	5	1	1.0	0.1	1
MgATP	5	2	2.0	2.0	2.0
NaCP[a]	0	15	15	15	15
EGTA	50	5	5	0.05	5
Imidazole	20	20	0	0	0
BES[b]	0	0	50	50	50
Creatine kinase (units/mL)	0	100	100	100	100
pCa[c]	> 8.5	>8.5	6.4	>8.5	4
pH	7.0	7.0	7.0	7.0	7.0
Ionic strength[d]	210	200	200	200	200
Temperature	22°C	22°C	22°C	22°C	22°C

- [a]NaCP- Na_2phosphocreatine; [b]BES- N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; [c]pCa is the -log of $[Ca^{2+}]_o$; [d]The ionic strength was adjusted by using potassium methanessulfonate.

The figure 1 is a typical chart recording of the contracture induced by PO in depolarized muscle. In six other preparations previously exposed (for 5 min.) to procaine (10 mM), addition of PO (2 mg/ml) for 30 min. did not induce contraction; 1 hour after wash out of both substances, addition of PO induced contraction, which was 131.5 ± 10.41 (n= 6) of K^+-contraction. In six saponin-permeabilized fibers, PO (1.5, 2.0, 3.0 and 4.0 mg/ml) induced contractions which were respectively, 0.52 ± 0.52; 30.92 ± 8.43; 32.97 ± 4.92; 42.78 ± 6.37 % of the F max. The figure 2 is a chart recording of force showing the contraction induced by PO (4 mg/ml), caffeine and by solution pCa 4.0.

DISCUSSION

It is widely accepted that in skeletal muscle fibers the interaction of two receptors, dihidropiridine receptor (DHPR) and ryanodine receptor (RYR) is fundamental for the process of excitation-contraction coupling (Stern, et al., 1997). The depolarization of the sarcolemma is sensed by DHPR, present in tubular transverse membrane, inducing the activation of the RYR, which is located in the terminal cysterna of the s.r. The activation of RYR allows the release of a massive amount of calcium stored in the s.r. (Rios, et al., 1993). The muscle contraction induced by PO could be due to the activation of DHPR. However, muscle contraction was obtained either in depolarized as well as in non depolarized muscle. In depolarized muscle fiber (80 mM of extracellular K^+), DHPR is inactivated and the excitation-contraction coupling is blocked on the step before the releasing of calcium (Rios, et al., 1993). It makes unlikely that muscle contraction induced by PO, could be due to the activation of DHPR. In intact as well as in saponin skinned muscle fiber, caffeine induces muscle contraction by releasing calcium from s.r. (Endo, 2009; Lofrano-Alves, et al., 2005). This effect is blocked by procaine (Endo, 1977; Endo, 2009). Similarly, in intact muscle the contraction promoted by PO was blocked by procaine, which suggests that PO induces calcium release from s.r. Moreover, the contraction induced by PO in saponin-permeabilized muscle fibers where the selective permeability of the sarcolemma is lost and consequently there is no transmembrane potential, suggests that the contraction induced by PO is due to the calcium released from s.r. This is also reinforced because in saponin-permeabilized muscle fiber the only source of calcium necessary for contraction is provided by the sarcoplasmic reticulum. Taken all together, the results obtained is this study demonstrated that: a) muscle contraction induced by PO is due to calcium release from the s.r. probably by the activation of the RYR; b) identical results previously obtained with EOMV (Fogaça, et al., 1997) suggest that PO is the compound responsible for such effects.

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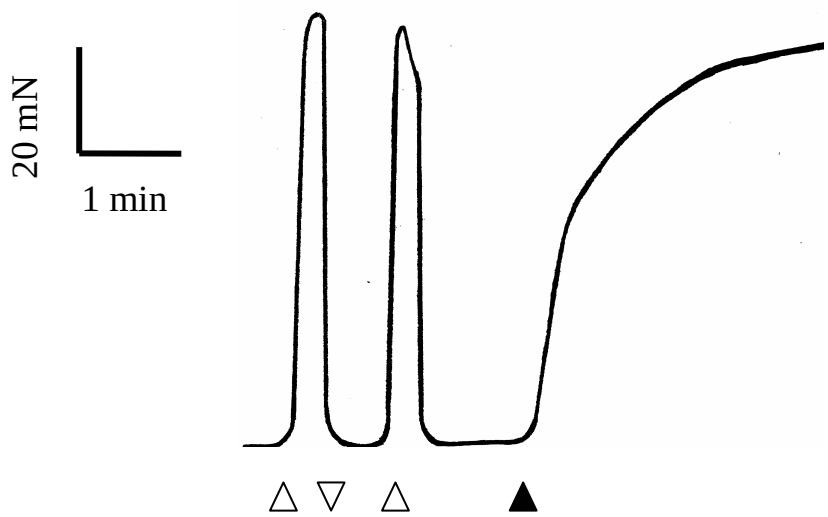

Figure-1: Contraction induced by PO (3.0 mg/ml) in sartorius muscle.

- Triangles: Upward and downward, application and removal, respectively of Ringer solution with 80 mM K^+ (empty triangle). OP was added to 80 mM K^+ containing solutions (full triangle).

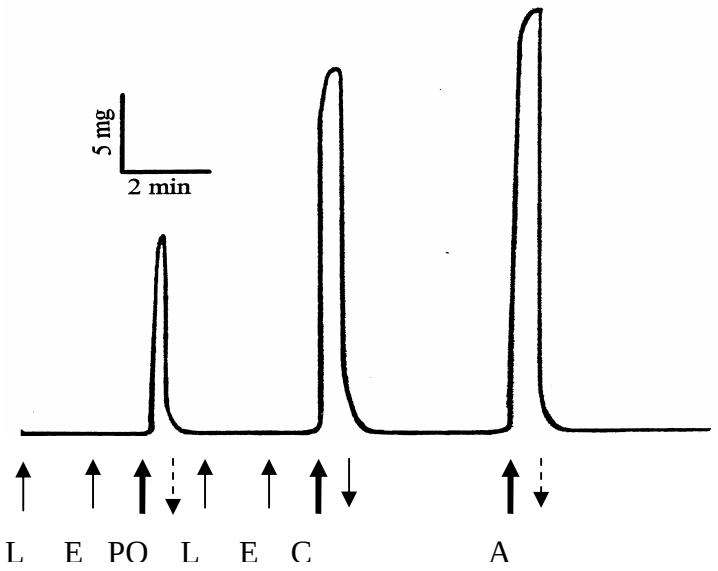

Figure-2: A typical chart recording of force showing the contractions due to calcium released from s.r. induced by caffeine (C; 30 mM) or PO (PO; 4 mg/ml) of a single saponin-permeabilized muscle fiber.

- The contractions were obtained after the s.r. had been loaded in solution pCa 6, 4 (L; solution L). The fiber was previously incubated in solution containing both low EGTA and magnesium (E; solution E) before the exposition to caffeine or PO. When the contraction reached the plateau the relaxation of the fiber was obtained by transferring it to a solution pCa > 8, 5 (solution D, downward arrow). F max was obtained by transferring the fiber to a solution pCa 4.0 (A; solution A). Fiber diameter: 50 μm.