Phytomedicine 17 (2010) 980–986

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Juice of *Bryophyllum pinnatum* (Lam.) inhibits oxytocin-induced increase of the intracellular calcium concentration in human myometrial cells

A.P. Simões-Wüst[a,*,1], M. Grãos[b,1], C.B. Duarte[b,c], R. Brenneisen[d], M. Hamburger[e], M. Mennet[f], M.H. Ramos[f,2], M. Schnelle[f], R. Wächter[g], A.M. Worel[h], U. von Mandach[g]

[a] *Research Department, Paracelsus Hospital, Richterswil, Switzerland*
[b] *Biocant – Center for Innovation in Biotechnology, Cantanhede, Portugal*
[c] *Center for Neuroscience and Cell Biology and Department of Zoology, University of Coimbra, Coimbra, Portugal*
[d] *Department of Clinical Research, University of Bern, Bern, Switzerland*
[e] *Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland*
[f] *Clinical Research, Weleda AG, Arlesheim, Switzerland*
[g] *Department of Obstetrics, University Hospital Zurich, Zürich, Switzerland*
[h] *Medical Department, Weleda AG, Arlesheim, Switzerland*

ARTICLE INFO

Keywords:
Bryophyllum pinnatum
Crassulaceae
Oxytocin
Intracellular calcium concentration
Tocolysis

ABSTRACT

The use of preparations from *Bryophyllum pinnatum* in tocolysis is supported by both clinical (retrospective comparative studies) and experimental (using uterus strips) evidence. We studied here the effect of *B. pinnatum* juice on the response of cultured human myometrial cells to stimulation by oxytocin, a hormone known to be involved in the control of uterine contractions by increasing the intracellular free calcium concentration ($[Ca^{2+}]_i$).

In this work, $[Ca^{2+}]_i$ was measured online during stimulation of human myometrial cells (hTERT-C3 and M11) with oxytocin, which had been pre-incubated in the absence or in the presence of *B. pinnatum* juice. Since no functional voltage-gated Ca^{2+} channels could be detected in these myometrial cells, the effect of *B. pinnatum* juice was as well studied in SH-SY5Y neuroblastoma cells, which are known to have such channels and can be depolarised with KCl.

B. pinnatum juice prevented the oxytocin-induced increase in $[Ca^{2+}]_i$ in hTERT-C3 human myometrial cells in a dose-dependent manner, achieving a ca. 80% inhibition at a 2% concentration. Comparable results were obtained with M11 human primary myometrial cells. In hTERT-C3 cells, prevention of the oxytocin-induced increase in $[Ca^{2+}]_i$ was independent of the extracellular Ca^{2+} concentration and of voltage-dependent Ca^{2+}-channels. *B. pinnatum* juice delayed, but did not prevent the depolarization-induced increase in $[Ca^{2+}]_i$ in SH-SY5Y cells.

Taken together, the data suggest a specific and concentration-dependent effect of *B. pinnatum* juice on the oxytocin signalling pathway, which seems to corroborate its use in tocolysis. Such a specific mechanism would explain the rare and minor side-effects in tocolysis with *B. pinnatum* as well as its high therapeutic index.

Introduction

Plants of the genus *Bryophyllum* (family Crassulaceae) occur in tropical Africa, America and Asia, Hawaii, India, China, Australia and Madagascar, and have been traditionally used in these regions in multiple pathological situations (Yadav and Dixit 2003; Lans 2006). In Europe the use of remedies prepared from the species *Bryophyllum pinnatum* [(Lam.), syn. *Kalanchoe pinnata* (Lam.), *Bryophyllum calycinum* (Salisb.)] is limited almost exclusively to anthroposophic medicine (Hamre et al. 2006; Simões-Wüst and Rist 2007), where it was introduced by Rudolf Steiner in 1921 (Daems 1982). Several components have been identified in *B. pinnatum* preparations, which are likely to have biological effects and might therefore have therapeutic potential, namely bufadienolides (McKenzie et al. 1987; Yamagishi et al. 1988; Yamagishi et al. 1989; Supratman et al. 2000; Supratman et al. 2001), flavonoids (Cao et al. 2005; Muzitano et al. 2006a), flavonoid glycosides (Gaind and Gupta

* Corresponding author at: Research Department, Paracelsus Hospital, Bergstrasse 16, CH-8805 Richterswil, Switzerland. Tel.: +41 44 787 24 93; fax: +41 44 787 23 51.
E-mail address: simoes@paracelsus-spital.ch (A.P. Simões-Wüst).
[1] These authors contributed equally to the work and should be considered as joint first authors.
[2] Left Weleda in the meantime.

0944-7113/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.phymed.2010.03.005

1971; Muzitano et al. 2006b), phenols (Gaind and Gupta 1973) and organic acids (Marriage and Wilson 1971). Our previous work on the pharmaceutical characterisation of an aqueous extract directly obtained from juice of *B. pinnatum* as the one used in the present study (Erni 2006), revealed the presence of flavonoids, cinnamic acid derivatives and bufadienolides (ca. 66 μg/100 ml; Rist et al. 2007). The later were present in higher concentrations in similar extract from *Bryophyllum daigremontiana* (ca. 215 μg/100 ml; Rist et al. 2007), in which several structural types have been previously identified (Wagner et al. 1986).

B. pinnatum was first introduced in 1970 at the Community Hospital of Witten-Herdecke, Germany, as a tocolytic for preventing premature labour (Hassauer et al. 1985). A retrospective analysis from an obstetric clinical practice revealed better outcomes and less side-effects in patients treated with *B. pinnatum* (ethanolic tincture, 33%) compared to treatment with fenoterol (Daub 1989). More recently, the tolerability and the tocolytic outcome of patients treated with *B. pinnatum* (aqueous extract, 5%) was compared with those of patients treated with beta-agonists in a retrospective clinical study with closely matched pairs ($n = 67$) (Plangger et al. 2006). Treatment with *B. pinnatum* revealed similar effectiveness but less side-effects than treatment with beta-agonists currently used in clinic as standard therapy for prevention of pre-term delivery (Plangger et al. 2006). *B. pinnatum* (aqueous extract, 100 mg/ml) inhibited the *ex vivo* contractility of human term myometrium strips (biopsies from caesarean section, $n = 14$) under physiological conditions (Gwehenberger et al. 2004).

A tight regulation of the intracellular free calcium concentration ($[Ca^{2+}]_i$) is known to play a crucial role in the myometrial contractant and relaxant signalling pathways (Sanborn et al. 1998). An oxytocin-induced sensitization of the cellular contractile apparatus to Ca^{2+} is thought to play an important role in term and pre-term labour (reviewed in Shmygol et al. 2006; Arthur et al. 2007). In the absence of contractions, the $[Ca^{2+}]_i$ is rather low. Activation of surface receptors and/or depolarization of the plasma membrane increases significantly $[Ca^{2+}]_i$ due to influx of extracellular Ca^{2+} and/or activation of signalling pathways which result in the release of Ca^{2+} from intracellular stores such as the endoplasmatic reticulum (Sanborn et al. 1998; Berridge et al. 2003).

In the present work, the effect of *B. pinnatum* juice on $[Ca^{2+}]_i$ changes occurring in cultured human myometrial cells in response to oxytocin has been investigated.

Materials and methods

Materials

Human myometrial hTERT-C3 immortalized by transfection of a telomerase reverse transcriptase (hTERT-C3 cells) (Condon et al. 2002; Devost and Zingg 2007) were provided by Dr. H.H. Zingg (McGill University, Montreal, Québec, Canada). Human primary human myometrial M11 cells (Devost and Zingg 2007) were obtained from John A. Copland (Mayo Clinic College of Medicine, Jacksonville, FL). SH-SY5Y human neuroblastoma cells were obtained from the European Collection of Cell Cultures – United Kingdom (ECACC, Salisbury, Wiltshire, UK).

Cell culture

hTERT-C3 human myometrial cells (Condon et al. 2002; Devost and Zingg 2007) were cultured in a 1:1 mixture of DMEM and F-12 supplemented with 10% heat-inactivated fetal bovine serum (all from Gibco-Invitrogen, Carlsbad, CA, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Lonza-BioWhittaker, Basel, Switzerland). Primary human myometrial cells M11 (Devost and Zingg 2007) were maintained in DMEM with high glucose, supplemented with 10% fetal bovine serum (all from Gibco-Invitrogen) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Lonza-BioWhittaker). SH-SY5Y human neuroblastoma cells were cultured in a 1:1 mixture of DMEM and F-12 supplemented with 10% heat-inactivated fetal bovine serum (all from Gibco-Invitrogen), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Lonza-BioWhittaker), 1% non-essential amino acids, 7.35 mg/l glutamate and 55 mg/l sodium-pyruvate (all from Sigma–Aldrich, St. Louis, MO, USA). All cell lines were cultured at 37 °C, in an atmosphere of 5% CO_2–95% air and 90% humidity. When cells reached near confluence, they were detached with trypsin (Gibco-Invitrogen) and plated at a 1:2, 1:3 or 1:6 dilutions. Medium was changed every 3 days.

For the $[Ca^{2+}]_i$ measurements, hTERT-C3 cells were plated on solid black 96-well polystyrene microplates (Corning Inc., Schiphol-Rijk, The Netherlands) at a density of 31,250 cells/well (i.e. ca. 98,730 cells/cm^2) 2 days before experiments were performed, to allow cells to fully recover from trypsinisation. M11 and SH-SY5Y cells were plated under similar conditions, except that the cell densities used were 25,000 cells/well (ca. 79,000 cells/cm^2) and 62,000 cells/well (ca. 200,000 cells/cm^2) respectively. In all cases, the cells were confluent on the day of the experiment, as assessed by phase-contrast microscopy, using cells seeded on a clear-bottom 96-well microplate.

Plant material

B. pinnatum plants were provided by Weleda Brazil. A voucher specimen number 292.697 is deposited at the herbarium of Rio de Janeiro's Botanical Garden, Brazil. The plants were harvested in Brazil (19.03.07) by D. Magano, Weleda Brazil, before flowering, in the morning. After macroscopic confirmation of the identity, the fresh plant material was placed in a refrigerated box and immediately sent by airplane to Weleda Arlesheim, Switzerland. The plants were further kept refrigerated and processed within 3 days upon arrival by mechanical pressing in a roller mill to obtain *B. pinnatum* juice; 580 mg juice were obtained per g fresh plant. The procedure used corresponded to the production process for the active ingredient of Weleda Bryophyllum 50% (Weleda AG, Arlesheim). The juice was frozen and sent to the laboratory in Cantanhede, Portugal, where the cell biology experiments were performed. The suspension was thawed, homogenized, sonicated in an ultrasonic bath (Sonorex RK100) for 30 s and then filtered, using a 595.5, 70 mm diameter filter (Whatman/Schleicher & Schuell, Kent, UK). Aliquots of the filtered juice were re-frozen and stored at −20 °C. For each experiment, a freshly thawed aliquot was used.

HPLC profiling of B. pinnatum juice

Lyophilized *B. pinnatum* juice was reconstituted in methanol (corresponding to 2.15 ml *B. pinnatum* juice in 1 ml methanol), filtrated and submitted to HPLC using an Agilent 1100 HPLC-DAD (Agilent, Waldbronn, Germany) system equipped with a Waters SunFire C18 column (150 mm × 3 mm i.d., 3.5-μm particle size, Waters, Baden-Dättwil, Switzerland) and a Waters SunFire C18 guard column (3 mm × 20 mm). The peaks separation was achieved under the following conditions: solvent A = 0.1% formic acid, solvent B = acetonitrile; linear gradient 0–30 min, 5–100% B in A; flow: 500 μl/min; column temperature: 25 °C; detection: 298 nm; DAD spectra: 190–400 nm; sample: 1 ml; injection volume: 10 μl.

$[Ca^{2+}]_i$ measurements using the fluorescent Ca^{2+} indicator Fura-2

hTERT-C3, M11 and SH-SY5Y cells were loaded with the $[Ca^{2+}]_i$ fluorescent indicator Fura-2 (Grynkiewicz et al. 1985)