Universidade de Aveiro Departamento de Biologia
2016

Ana Rodrigues Barros

Efeitos do tributilestanho (TBT) ao nível vascular

Tributyltin (TBT) effects at a vascular level

DECLARAÇÃO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Ana Catarina Almeida Sousa, investigadora em pós-doutoramento do Centro de Investigação em Ciências da Saúde, Faculdade de Ciências da Saúde da Universidade da Beira Interior e do Prof. Doutor Carlos Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho à minha família e amigos!

O júri

Presidente	Prof. Doutora Sónia Alexandra Leite Velho Mendo Barroso Professora Auxiliar com Agregação da Universidade de Aveiro
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Palavras-chave

Compostos orgânicos de estanho, TBT, aorta de rato, A7r5, patch clamp, banho de órgãos

Resumo

Os compostos orgânicos de estanho são uma importante classe de compostos organometálicos produzidos pelo homem, e o tributilestanho (TBT) é um dos químicos mais estudados dentro desta classe. O TBT atua como um potente disruptor endócrino, sendo também considerado como um composto obesogénico, imunotóxico e neurotóxico. A exposição humana a este e outros compostos orgânicos de estanho deve-se à sua vasta utilização em aplicações comerciais como estabilizadores de plásticos, agentes catalíticos e biocidas industriais. A sua utilização como agentes catalíticos na produção de silicones faz com que estes compostos estejam presentes em vários produtos à base de silicone, incluindo os usados em aplicações biomédicas como implantes mamários e válvulas cardíacas, o que pode constituir uma fonte potencial de exposição para os humanos. De acordo com a Organização Mundial de Saúde, as doenças cardiovasculares estão a aumentar e são consideradas como a principal causa de morte a nível mundial. Tendo isto em conta, as agências reguladoras recomendam o estudo da toxicidade dos compostos orgânicos de estanho. Considerando o número limitado de estudos sobre os efeitos cardiovasculares dos compostos orgânicos de estanho, o presente trabalho pretende elucidar os efeitos do TBT ao nível vascular.

Os efeitos do TBT na contractilidade de artérias de rato (aorta) foram estudados pela técnica do banho de órgãos e a medição dos canais de cálcio tipo L foi realizada em A7r5 (linha celular de músculo liso vascular derivada de aorta embrionária de rato) através da técnica do *patch clamp* na configuração *whole cell*. Os resultados obtidos demonstram que o TBT parece induzir relaxamento nas artérias sem endotélio contraídas previamente com noradrenalina e com cloreto de potássio, mas esse efeito não é significativamente diferente do controlo de solvente usado. Nas experiências de eletrofisiologia a inibição das correntes de cálcio através dos canais de cálcio tipo L nas células A7r5 também não mostrou ser significativamente diferente do controlo, o que parece demonstrar que o modo de ação pelo qual o TBT induz efeito nas células vasculares é mais complexo e envolve outras vias.

Keywords Organotin compounds, TBT, rat aorta, A7r5, patch clamp, organ bath

Abstract

Organotins are an important class man made organometallic compounds and tributyltin (TBT) is one of the most studied chemicals within this class. TBT is a potent endocrine disruptor being also considered as an obesogenic, immunotoxic and neurotoxic compound. Humans were exposed to this and other organotin compounds as a consequence of their widespread commercial applications including plastic stabilizers, catalytic agents and industrial biocides. Their utilization as catalytic agents in the production of silicones leads to the presence of these chemicals in silicone based products including those used in biomedical applications such as breast implants and cardiac valves which may constitute a potential source of exposure. According to the World Health Organization cardiovascular diseases are sharply increasing and constitute the prime cause of death globally. Taking this into account, regulatory agencies, recommend the study of organotin compounds toxicity. Considering the limited number of studies on the cardiovascular effects of organotins, the present thesis aims to elucidate the effects of TBT at the vascular level.

The study of TBT effect on the contractility of rat artery (aorta) was performed by the organ bath technique and the L-type calcium channels in A7r5 (cell line derived from the smooth muscle of embryonic rat aorta) was measured by whole-cell configuration of the patch clamp technique.

The obtained results demonstrated that TBT seems to relax the rat aorta without endothelium contracted by noradrenaline and potassium chloride but this effect is not significantly different from the respectively ethanol control. The electrophysiological experiments demonstrated that the inhibition of the calcium current by the L-type calcium channels in the A7r5 vascular smooth muscle cells was also not significantly different, which suggests that probably the mode of action of TBT is more complex and involves other pathways.

“Trabalhamos no escuro, fazemos o que podemos, damos o que temos. A nossa dúvida é a nossa paixão e a nossa paixão é o nosso trabalho. O resto é a loucura da arte.”

Henry James, *The Middle Years*

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List of Abbreviations

A7r5: Cell line derived from the smooth muscle of embryonic rat aorta

Ach: Acetylcholine

AFS Convention: International Convention on Control of Harmful Antifouling Systems on Ships

ATP: Adenosine Triphosphate

ATPase: Adenosine Triphosphatase

BSA: Bovine Serum Albumin

BTs: Butyltins

CaM: Calmodulin

cAMP: Cyclic Adenosine Monophosphate

cGMP: Cyclic Guanosine Monophosphate

CICR: Ca^{2+}-Induced Ca^{2+} Release

CNS: Central Nervous System

DBT: Dibutyltin

DG: Diacylglycerol

DMT: Dimethyltin

DOT: Dioctyltin

DPT: Diphenyltin

ER: Endoplasmic Reticulum

FBS: Fetal Bovine Serum

HVA: High-Voltage Activated Ca^{2+} Channels

I_{CaL}: L-Type Ca^{2+} Current

IICR: IP_3-Induced Ca^{2+} Release

IMO: International Maritime Organization

IP_3: Inositol-1,4,5-Triphosphate

IVA: Intermediate-Voltage Activated Ca^{2+} Channels

KCl: Potassium Chloride

K_{ow}: Octanol/Water Partition Coefficient

LTCC: L-Type Calcium Channels

LVA: Low-Voltage Activated Ca^{2+} Channels

MAPK: Mitogen-Activated Protein Kinase

MBT: Monobutyltin

MLC_{17}: Myosin Essential Light Chains

MLC_{20}: Myosin Regulatory Light Chains

MLCK: Myosin Light Chain Kinase

MLCP: Myosin Light Chain Phosphatase

MMT: Monomethyltin

MOT: Monooctyltin

MPT: Monophenyltin

MSCs: Multipotent Mesenchymal Stem Cells

MYPT1: Myosin Phosphatase Target Protein-1

NA: Noradrenaline

NCX: Na^{+}/Ca^{2+} Exchanger

NO: Nitric Oxide

NSCC: Nonselective Cation Channels

OcTs: Octyltins

OTs: Organotin compounds

pGC: Particulate Guanylyl Cyclase

PKA: Protein Kinase A or Cyclic AMP-dependent Protein Kinase

PKC: Protein Kinase C

PKG: Protein Kinase G or Cyclic GMP-dependent Protein Kinase

PLC: Phospholipase C

PMCA: Plasma Membrane Ca^{2+} ATPases

PPARɣ: Peroxisome Proliferator Activated Receptor Gamma

PTs: Phenyltins

PVC: Polyvinyl Chloride

REACH: Registration, Evaluation, Authorization and Restriction of Chemicals

ROCC: Receptor-Operated Ca^{2+} Channels

ROS: Reactive Oxygen Species

RXR: Retinoid X Receptor

SACC: Stretch –Activated Ca^{2+} Channels

SCHER: Scientific Committee on Health and Environmental Risks

SERCA: Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase

sGC: Soluble Guanylyl Cyclase

SOCC: Store Operated Ca^{2+} Channels

SR: Sarcoplasmic Reticulum

TBT: Tributyltin

TET: Triethyltin

TMT: Trimethyltin

TOT: Trioctyltin

TPT: Triphenyltin

VOCC: Voltage Operated Ca^{2+} Channels

VSMCs: Vascular Smooth Muscle Cells

WHO: World Health Organization

Chapter 1

General Introduction

1.1 Organotin compounds (OTs) and tributyltin (TBT)

1.1.1 Chemical and physical properties of OTs

Organotin compounds (OTs) are characterized by a tin (Sn) atom covalently bound to one or more organic substituents (Hoch, 2001). The general formula of organotins is RSnX in which R is an organic group (such as alkyl groups like methyl, ethyl, propyl, butyl, or aryl groups like phenyl) and X is an anionic group (such as chloride, fluoride, oxide, hydroxyl, carboxylate and thiolate) (Hoch, 2001). OTs are organometals essentially produced by humans except methyltins, which can also be produced naturally by bacteria through biomethylation (Guard et al., 1981). OTs are grouped into mono-, di-, tri-, and tetrasubstituted compounds according to the number of organic groups (Hoch, 2001). The physical and chemical properties of these compounds vary considerably with the number and nature of the R groups and type of ligand. Their toxicity is also strongly influenced by these factors. Basically inorganic tin is non-toxic and trisubstituted compounds are the most toxic (Fent, 2006; Hoch, 2001). The solubility of organotins in water is very low and depends on the temperature, salinity, pH and concentration of suspended particles. These compounds have a high affinity for organic solvents and lipids showing a high octanol/water partition coefficient[1] K_{ow} (WHO, 1990).

Table 1. Examples of selected organotin compounds with their respective formula. Adapted from Sousa ACA et al. (2013).

Class	Compound	Abbreviation	Formula
Butyltins (BTs)	Monobutyltin	MBT	C_4H_9Sn
	Dibutyltin	DBT	$C_8H_{18}Sn$
	Tributyltin	TBT	$C_{12}H_{27}Sn$
Phenyltins (PTs)	Monophenyltin	MPT	C_6H_7Sn
	Diphenyltin	DPT	$C_{12}H_{10}Sn$
	Triphenyltin	TPT	$C_{18}H_{15}Sn$

[1] **The octanol/water partition coefficient** (K_{ow}) describes the partitioning of an organic chemical between octanol and water. Octanol is believed to best imitate the fatty structures in plant and animal tissues (Kenaga and Goring, 1980).

	Monooctyltin	MOT	$C_8H_{20}Sn$
Octyltins (OcTs)	Dioctyltin	DOT	$C_{16}H_{34}Sn$
	Trioctyltin	TOT	$C_{24}H_{51}Sn$

1.1.2 Production and applications

The industrial manufacture of organotins started during the 1940's when the plastic industry began to expand, in particular the one responsible for the polyvinyl chloride (PVC) production (Sousa ACA et al., 2013). In fact, PVC industry produces about 76% of the total annual organotin manufacture, mainly dibutyltins, dioctyltins and mono substituted compounds (Sousa ACA et al., 2013). Organotin compounds are used as additives for thermal and light stabilization in PVC production to prevent its discoloration and embrittlement under the influence of light and heat, because polyvinyl chloride has the tendency to decompose upon heating (180-200ºC) or loss the HCl from the polymer with a prolonged exposure to light (Hoch, 2001). Organotin-stabilized PVC has several applications as packaging materials (e.g. food-contact plastics, packs for medical applications, packs for cosmetics, housewares and bottles); piping of potable water, wastewater and drainage water; window frames and coating materials (RPA, 2005). Another important application of organotins is their use as catalysts in the production of silicones. The most used OTs in the silicone manufacture are dibutyltins and dioctyltins. There are several products containing silicones catalysed by organotins, such as healthcare devices (e.g. breast implants, valves, tubes and bags), personal care items, textiles, construction materials, automotive and sports items (RPA, 2005; SCHER, 2006). Organotin catalysts are also used to produce a wide variety of polyurethane applications for adhesives/sealants, for the automotive industry, for the construction industry and for several appliances, binders, coatings, elastomers, footwear and furniture (RPA, 2005). According to Hoch (2001), OTs belongs to the most wide organometallic compounds group with more than 800 different organotins known and as of 2008 with an estimated annual production of about 60,000 tons (Nath, 2008).

Organotin compounds have excellent biocidal properties in particular tri-substituted organotins. In the late of 1950's, tributyltin (TBT) and triphenyltin (TPT) were used as actives

biocides in antifouling paints formulations to prevent the biofouling[2] (Sousa ACA et al., 2013). In agriculture, tri-substituted organotins were also used as pesticides, fungicides and as wood preservers (Hoch, 2001). Besides all of these applications, organotins could also be found in surface disinfectants (including the ones used in health care facilities), laundry sanitizers, rodent-repellents, scintillation detectors for ɤ- and X-rays, ballistic additives, ionophores in liquid membrane ion-selective electrodes and pharmaceuticals (e.g. anti-inflammatory and cancer treatments drugs) (see Sousa ACA et al. (2013)).

In general, mono and di-substitued organotins are used as PVC stabilizers, as catalysts in the production of polyurethane foams and in glass coating; tri-substitued organotins are used as biocides, pesticides and as intermediates in the production of other chemicals, and tetra-substitued compounds are used as intermediates in synthesis of other organic chemicals and in oil stabilizers (de Carvalho Oliveira and Santelli, 2010; RPA, 2005; Sousa et al., 2014a).

1.1.3 Regulation on organotin compounds

1.1.3.1 European legislation on organotins

A compilation on the regulations regarding organotin compounds in Europe is provided by Sousa ACA et al. (2013). The first regulation on the use of organotins was adopted as a consequence of adverse effects of tributyltin pollution. Tributyltin, and also triphenyltin, were used as active biocides in antifouling paints formulations. In 1989, the European Union (EU) introduced the Directive 89/677/EEC, banning the use of TBT and TPT on small boats (<25m). This partial ban was however not effective on reducing the levels of TBT in the environment (Sousa et al., 2013). In 2001, the International Maritime Organization (IMO) decided to ban TBT on a global scale through the International Convention on Control of Harmful Antifouling Systems on Ships (AFS Convention). This global ban entered into force in 2008. Nevertheless, in 2003, the European Union anticipated this global ban through the Directive 2002/62/EC and Regulation 782/2003 and therefore, in the EU the use of organotin compounds in antifouling paints was completely banned since 2003 (Sousa ACA et al., 2013).

Organotin compounds are also regulated in the European Union by the EC Regulation 1907/2006 on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)

[2] **Biofouling** can be defined as the settlement and growth of a variety of aquatic organisms on structures immersed in seawater, such as ships' hulls, sonar equipment, seawater cooling pipes and navigation buoys (Omae, 2003).

and their use is included in Annex XVII of the regulation that restricts the manufacture, placing on the market and use of certain dangerous substances, preparations and articles. According to the REACH directive, organotins:

1) *"Shall not be placed on the market, or used, as substances or in mixtures where the substance or mixture is acting as biocide in free association paint.*
2) *Shall not be placed on the market, or used, as substances or in mixtures where the substance or mixture acts as biocide to prevent the fouling by micro-organisms, plants or animals of:*
   a) *All craft irrespective of their length intended for the use in marine, coastal, estuarine and inland waterways and lakes;*
   b) *Cages, floats, nets and any other appliances or equipment used for fish or shellfish farming;*
   c) *Any totally or partly submerged appliance or equipment.*
3) *Shall not be placed on the market, or used, as substance or in mixture where the substance or mixture is intended for use in the treatment of industrial waters"* (cited from Annex XVII).

Due the wide commercial applications as plastic stabilizers, catalytic agents, industrial biocides, pesticides and glass coating, further restrictions on OTs usage were further adopted by the European Commission. Hence, in 2009, EC Decision 2009/425/EC restricted the use of dibutyltin, dioctyltin and tri-substituted OTs. In 2010 this decision was incorporated into Annex XVII of REACH via regulation (EU) 276/2010.

Table 2. Summary of organotin compounds requirements under European Regulations (EU) 276/2010 amending Annex XVII of REACH. The concentration in the mixture or the article, or part thereof, is greater than the equivalent of 0.1% by weight of tin. TBT: tributyltin, TPT: triphenyltin; DBT: dibutyltin, DOT: dioctyltin, PVC: polyvinyl chloride. Adapted from Sousa et al. (2013).

Substance	Products	Effective form
TBT and TPT	• Articles or part of articles	1 July 2010
	• Mixtures • Articles of parte of articles (except food contact materials)	1 January 2012

DBT	• One-component and two-component room temperature vulcanization sealants and adhesives • Paints and coatings containing DBT compounds as catalysts when applied on articles • Soft polyvinyl chloride (PVC) profiles whether by themselves or coextruded with hard PVC • Fabrics coated with PVC containing DBT compounds as stabilizers when intended for outdoor applications • Outdoor rainwater pipes, gutters and fittings, as well as covering material for roofing and facades	1 January 2015
DOT	• Textile articles intended to come into contact with the skin • Gloves • Footwear or part of footwear intended to come into contact with the skin • Wall and floor coverings • Childcare articles • Female hygiene products • Nappies • Two-component room temperature vulcanization moulding kits	1 January 2012

1.1.4 Tributyltin as a case study

1.1.4.1 TBT chemical characterization

TBT (Figure 1) is characterized by the presence of covalent bonds between a tin atom and three carbon atoms. The solubility in water is low varying according to the pH, temperature, and anions present in the water. TBT is soluble in lipids and very soluble in organic solvents (e.g. ethanol) (IPCS, 1990). The lipoaffinity of TBT is well demonstrated by its octanol-water partition constant (K_{ow}) with values between 3.49 and 5.07 depending on the TBT chemical speciation (Arnold et al., 1997).

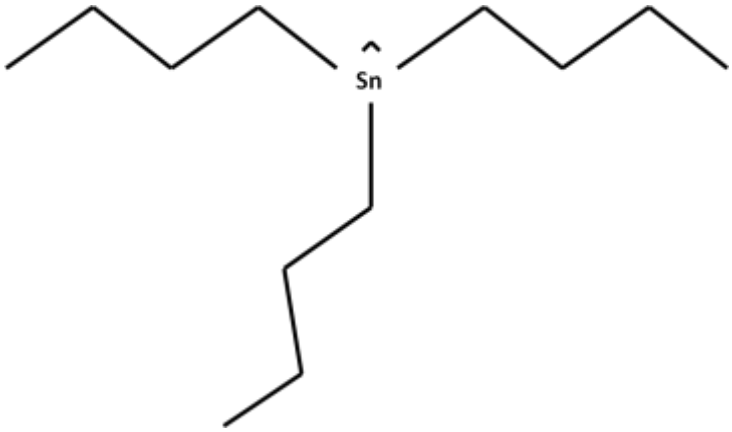

Figure 1. Chemical structure of TBT. Adapted from PubChem (www.pubchem.ncbi.nlm.nih.gov).

1.1.4.2 Bioaccumulation

Several studies have reported high concentrations of TBT in some fishes and aquatic invertebrates, such as gastropods and filter-feeding organisms (Guérin et al., 2007; Okoro et al., 2011; Santos et al., 2009). Such high concentrations were related with the massive utilization of tributyltin as biocide in antifouling paints to prevent biofouling in the hulls of ships. As a consequence of its widespread use, the inputs of TBT into the aquatic environment were huge (Sousa ACA et al., 2013). Once in the marine environment, TBT adsorves to organic matter and settles down in sediments where it can remain for decades (Fent, 2006). Several organisms at the base of the food chain have the capacity to accumulate TBT from the water and this compound is transferred to consumers and top predators, especially mammals and seabirds (Iwata et al., 1995; Takahashi et al., 1999; Tanabe et al., 1998) which lead to an increased accumulation in the food chain (Mamelona and Pelletier, 2003). In Portugal, the levels of organotins in seafood were measured during 2002 and 2003 and butyltins were detected in all the analysed groups (fish, crustaceans, bivalves and cephalopods). In bivalves, for example, TBT was detected with values up to 275 ng TBT g^{-1} wet weight (Santos et al., 2009). However, recently, the TBT levels in seafood have decreased as a consequence of the TBT global ban (Sousa ACA et al., 2013).

1.1.4.3 Absorption and distribution

The toxic effects of organotins are mediated by the tissues that serve as interface between the environment and the organism (Pagliarani et al., 2013). In vertebrates, for example, the digestive system is the primary absorption site and the liver is the main detoxification site (Pagliarani et al., 2013). In mammals, the absorption of TBT occurs in the intestinal epithelium that is the main barrier before reaching the bloodstream (Azenha et al., 2004) however the absorption through the skin in mammals also occurs (IPCS, 1990). Several studies described the absorption of TBT and other organotins *in vitro* using cell lines and *in vivo* with rats and/or mice. Evans et al. (1979) and Iwai et al. (1982) described that following TBT oral administration in rats and mice, this compound was absorbed from the duodenum, jejunum and ileum. TBT effects on the gastrointestinal tract include an increase of intestinal heme oxygenase and a reduction of intestinal P450, which suggests that TBT may affect the ability of the gastrointestinal tract to metabolize drugs or other xenobiotics (Rosenberg and Kappas, 1989).

Azenha et al. (2004) performed a series of *in vitro* studies using an intestinal cell line model to access the intestinal permeation of organotins. Their results demonstrated that butyltins including TBT have low permeability but the absorption is greatly assisted by proteins in the circulation. A study performed by Evans et al. (1979) demonstrated that when adult female mice ingests labelled ^{14}C-TBT trough drinking water, the highest quantity of the labelled compound is found in kidney followed by fat, liver, spleen, lung, brain, muscle and blood. Fifteen days after the end of the treatment, most of the tissues had lost most of the labelled TBT but the fat, lung and kidney still showed detectable levels suggestion that it is retained in this tissues/organs. In a multigenerational study with rats it was found that TBT speciation in the liver reflected the TBT degradation metabolism with [MBT] > [DBT] > [TBT], on the other hand, in the brain and fat, the quantities were reversed with [TBT] > [DBT] > [MBT] (Omura et al., 2003) which provides further evidences that the TBT detoxification occurs mainly in the liver. More recently, Cooke et al. (2008) studied the distribution and speciation of organotins in tissues of rat dams, fetuses and neonates and detected the presence of TBT in liver, brain and placenta. It was found that TBT crossed the placenta and accumulated in the fetal tissues, but the transfer of TBT to neonatal pups would be low as the levels of TBT in dams' milk were almost undetectable (Cooke et al., 2008).

Besides the *in vitro* and *in vivo* studies on absorption and distribution of organotins in rodent models, levels of these compounds were already detected in human biological tissues and

fluids. Figure 2 provides a summary of the maximum organotin levels detected in humans (for a complete list of references see: Sousa ACA et al. (2013)).

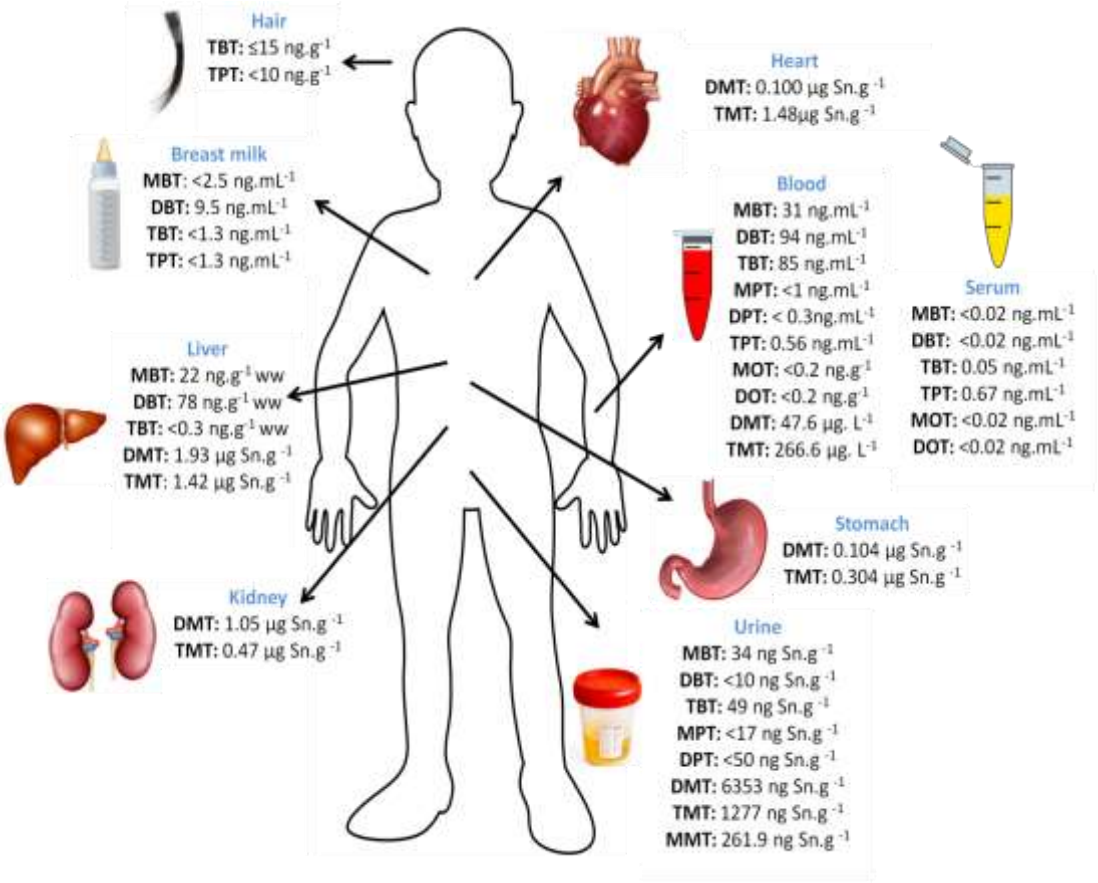

Figure 2. Summary of the highest organotin values detected in human tissues and fluids. MBT: Monobutyltin, DBT: Dibutyltin, TBT: Tributyltin, MPT: Monophenyltin, DPT: Diphenyltin, TPT: Triphenyltin, MOT: Monooctyltin, DOT: Dioctyltin, MMT: Monomethyltin, DMT: Dimethyltin, TMT: Trimethyltin. Adapted from Sousa ACA et al., (2013).

1.2 Human exposure to OTs

1.2.1 Pathways of exposure

Organotins can be found almost everywhere due its widespread use and therefore humans might be exposed to these compounds through several routes (Sousa ACA et al., 2013). Diet was traditionally considered an important source of OTs towards humans, particularly seafood rich diets where the TBT levels tend to be higher due to the global marine contamination by TBT

(Sousa ACA et al., 2013). However recent reports disclose that OTs levels in diet samples are sharply decreasing and therefore diet is no longer the major source of OTs (Sousa et al., 2014b). Several consumer products might still contain organotin compounds (see section 1.3.1.) such products include for example: pyjamas, cloths, shoes, non-allergic pillows, wallpaper, curtains, carpets and furniture (Sousa ACA et al., 2013). Beside these consumer products, silicone items may also contain OTs since they are used as catalysts during the production process. This fact is particularly important if we consider human exposure in a clinical context since healthcare devices such as breast implants and cardiac valves are made of silicone (SCHER, 2006). Drinking water transported by PVC pipes can also be considered as a way of exposure to these chemicals once organotins are used as additives for thermal and light stabilizers in PVC plastics. It was demonstrated that OTs can leach into the water flowing through PVC pipes (Adams et al., 2011; Forsyth and Jay, 1997; Sousa ACA et al., 2013). Another important source of organotins to consider is indoor dust. Organotins may leach from several appliances and consumer products with OTs in their composition and will end up in dust. As people spend most of their times in indoor environments the presence of organotins in the house dust contributes to human exposure (Fromme et al., 2005; Kannan et al., 2010; Santillo et al., 2001; Santillo et al., 2003; Sousa et al., 2014a).

1.3 Adverse OTs effects – *in vitro* and *in vivo* evidences

Organotins and TBT in particular have several adverse effects towards a wide range of organisms including bacteria and reaching mammals (see Sousa ACA et al., 2013). The first documented example of TBT deleterious effects occurred in marine organisms and it was also in marine organisms that a sexual disorder classified as imposex (superimposition of male characters into female gastropods) was described (Smith, 1971). This phenomenon soon became known as the best example of endocrine disruption in wildlife and from here the interest to study organotins toxicity, especially TBT increased. TBT is considered as a potent endocrine disruptor[3], with immunotoxic potential and recently it was also classified as an obesogenic compound. A brief overview of TBT and organotin compounds toxicity towards vertebrates using both *in vivo* and *in vitro* models is provided in the next section.

[3] According to the Endocrine Society an **endocrine disruptor** is an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action (Zoeller et al., 2012).

1.3.1 Obesogenecity

Obesogenic compounds are defined as chemicals that can have an influence on obesity development by inappropriately altering lipid homeostasis to promote adipogenesis and lipid accumulation (Grün and Blumberg, 2006, 2007, 2009). The Obesogen Hypothesis proposes that exposure to environmental chemicals during critical stages of development such as an early-life or long-term (chronic) exposures might have an influence on the promotion of metabolic diseases such as obesity and obesity-related disorders (Grün, 2014; Grün and Blumberg, 2009). Compounds with potent endocrine-disrupting properties are considered as obesogens and TBT is considered the model obesogen (Pereira-Fernandes et al., 2013). TBT is an obesogen because activates the peroxisome proliferator activated receptor gamma (PPARɣ) that is a nuclear receptor with a crucial role on the homeostatic control over energy, lipid and glucose metabolism (Grün and Blumberg, 2007). The molecular mechanism of adipocyte differentiation evolves the PPARɣ that forms heterodimers with retinoid X receptor (RXR) that regulates the expression of target genes at transcriptional level (Tontonoz and Spiegelman, 2008). Hence, PPARɣ and RXR heterodimer acts as a metabolic sensor that regulates adipocyte function, number and size (Grün and Blumberg, 2009). Various studies demonstrated that TBT induces adipogenesis *in vitro* and *in vivo* by activating PPARɣ and RXR and predisposing multipotent mesenchymal stem cells (MSCs) to differentiate into adipocytes (Grün et al., 2006; Kirchner et al., 2010). *In vitro* studies using 3T3-L1 preadipocyte cell line model, human and mouse MSCs demonstrated that TBT exposure induced their differentiation into mature adipocytes (Carfi et al., 2008; Grün and Blumberg, 2006; Kirchner et al., 2010). *In vivo* studies showed an increase of adipose mass in different vertebrate model organisms including frogs and mice (Grün et al., 2006). Moreover, the obesogenic capacities of TBT have been described in salmonids, snails and crustaceans (Janer et al., 2007; Jordão et al., 2015; Meador et al., 2011). *In utero* studies with mice further demonstrated that TBT increases adiposity of mice later in life (Grün and Blumberg, 2006). More recently, a cohort study demonstrated that placental TBT concentrations are associated with increased weight gain in human male infants at 3 months of age (Rantakokko et al., 2014).

1.3.2 Immunotoxicity

It is well known that immune system can be target of several compounds with the capacity to modify one or more immune functions resulting in adverse effects to the host's health (Corsini et al., 2013). Several studies have addressed the immunotoxic potential of organotins, and a thorough review of those studies is provided by Hendriksen et al. (2014). In this section only a brief overview of those effects is provided.

The first study that demonstrated the immunotoxic properties of organotins was conducted by Snoeij et al. (1985). In this study, rats were exposed for 2 weeks to several organotins including TBT and TPT at two different concentrations (15 and 150 ppm), and the obtained results demonstrated that both TBT and TPT reduced thymus weight in a dose-related manner while liver weights increased. These authors latter found out that red blood cells were the most resistant to OTs, and that bone marrow cells were less sensitive than thymocytes (Snoeij et al., 1986). Further evidences on the impact of OTs in thymocites continued to emerge. Raffray and Cohen (1991) for example, exposed immature rat thymocytes to TBT and they observed that low TBT concentrations (0.1 and 1 μM) induce programmed cell death (apoptosis) while higher concentrations (5 μM) leads to a cell death with necrosis characteristics.

Other *in vitro* studies using natural killer cells isolated from peripheral blood of healthy adults showed that when these cells are exposed to TBT their ability to destroy targets such as tumor cells decreases (Whalen et al., 1999). Additional studies demonstrated that TBT induces apoptosis in several target cells such as monocytes (Ghibelli et al., 1995) and lympochytes (Stridh et al., 2001; Stridh et al., 1999).

As described earlier TBT interacts with nuclear receptors PPARγ and RXR leading to adipocyte differentiation in 3T3-L1 preadipocyte cell line model, human and mouse MSCs (Grün and Blumberg, 2006; Carfi et al., 2008; Kirchner et al., 2010). Since adipocytes influence haematopoiesis (development of different blood cells) through the expression of cytokines and adhesion molecules the role of adipocyte-stimulating effect of TBT in human bone marrow cultures has also been explored. Carfi and co-workers demonstrated that TBT also induces adipocyte differentiation in human bone marrow cultures and that it induces a reduction in the number of chemokines, interleukins and growth factors which suggests that TBT induces adipocyte differentiation whilst reducing haematopoietic factors (Carfi et al., 2008). All these evidences suggests that TBT immunotoxicity may result from interference with more than one process and cell types (Carfi et al., 2010).

In humans, exposure to TBT has also been associated with induction or exacerbation of allergic diseases such as allergic airway inflammation (Kato et al., 2004).

The TBT mode of action is still under debate but the available studies suggest that calcium is involved in TBT toxicity. It has been demonstrated in several *in vitro* studies that TBT releases calcium from an intracellular source and induces calcium influx from outside the cells (See Hendriksen et al., 2014 for a complete description of those studies). This theory was further supported by *in vitro* studies using calcium chelators that decreased the toxicity of TBT. Figure 3 depicts the proposed mode of action of TBT. TBT also affect voltage-gated K^+ current in human peripherical lymphocytes (Oortgiesen et al., 1996), however as stated by Hendriksen et al. 2014, "*it remains to be assessed whether the effects of TBT on the voltage-gated K^+ current are direct effects on the channels or secondary effects, for example, caused by increased intracellular calcium levels*".

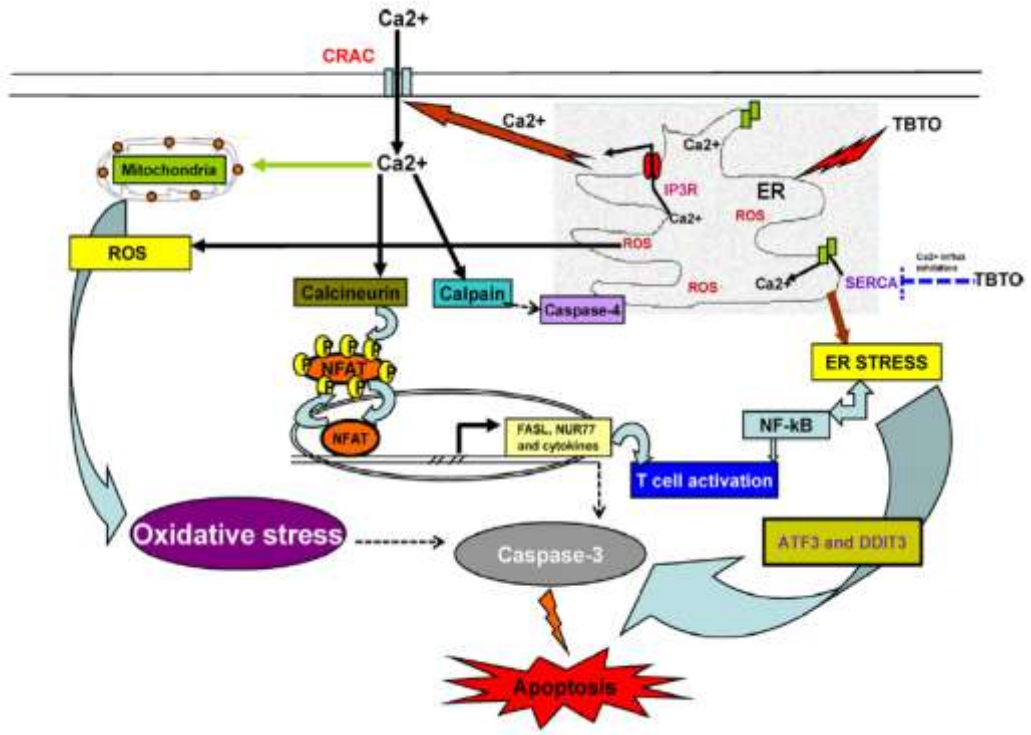

Figure 3. Illustration of the mode of action of TBTO in Jurkat T cells. TBTO initially acts on the ER and exerts ER stress inducing Ca^{2+} release from the ER lumen into the cytoplasm through the calcium channel IP3 receptor (IP3R). In addition, TBTO might inhibit SERCA and therewith blocks Ca^{2+} influx from cytoplasm to the ER which also contributes to an increased calcium level in the cytoplasm. This small cytoplasmic Ca^{2+} peak triggers a more pronounced Ca^{2+} influx into the cell through CRAC channels within the plasma membrane. The increased cytoplasmic Ca^{2+} level activates the calcium binding proteins calcineurin and M-calpain. Activated calcineurin dephosphorylates NFAT leading to its translocation to the nucleus and induction of expression of NFAT target genes (FASL, NUR77 and cytokines) resulting in T cell activation. Activation of M-calpain cleaves ER resident caspase-4. Furthermore, oxidative stress is induced by reactive

oxygen species that are produced in the ER due to ER stress and in the mitochondria due to the elevated cytoplasmic Ca^{2+} levels. Both, ER stress and increased Ca^{2+} levels activate NF-kB pathway. ER stress also induces activation of apoptosis-promoting proteins including DDIT3 and ATF3. Finally, the activated caspases induce apoptosis. Figure from Katika et al. (2012).

Overall, these changes in intracellular calcium levels may affect all cell types, since ER stress is common in all cells. However, immune cells are more sensitive to ER probably as a consequence of T-cell activation in response to Ca^{2+} released from the ER (Hendriksen et al., 2014; Kovalovsky et al., 2010). Despite these evidences supporting the Ca^{+2} dependent pathway, there are studies suggesting that the mechanism of OTs toxicity may be Ca^{+2} independent (Cima and Ballarin, 2012). Such Ca^{+2} independent mode of action include for example inhibition of oxidative phosphorylation and ATP synthase and irreversible interactions with the thiol groups of many proteins and peptides (Cima and Ballarin, 2012). Figure 4 depicts the summary of pathways involved in immunotoxic mechanisms of action of OTs (Ca^{+2} dependent and independent) according to Cima and Ballarin (2012). Divided in Ca^{2+}-dependent (red arrows) and Ca^{2+}-independent (blue arrows).

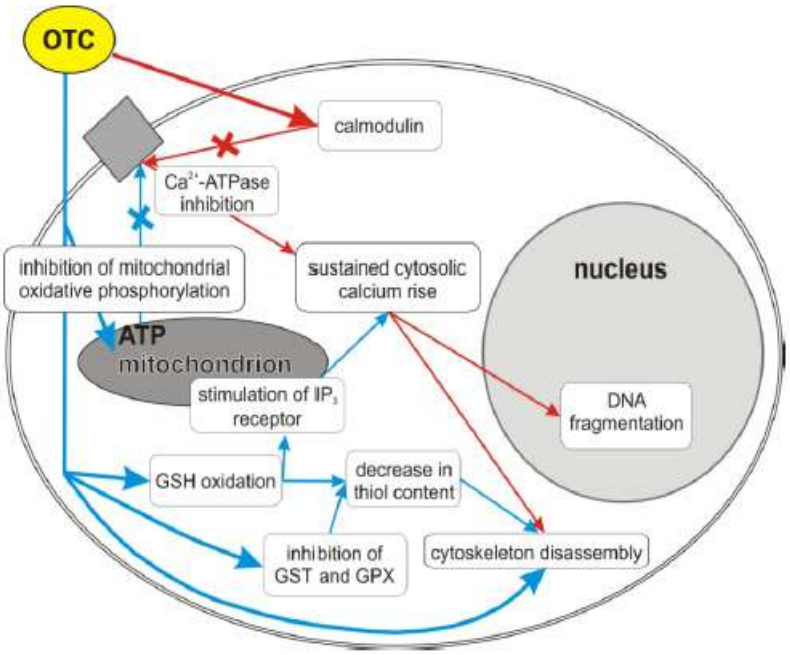

Figure 4. Summary of pathways involved in immunotoxic mechanisms of action of OTs, conventionally divided in Ca^{2+}-dependent (red arrows) and Ca^{2+}-independent (blue arrows). In Ca^{2+}-dependent mechanisms, OTs directly interact with calcium-activated calmodulin causing a complex formation preventing the regulative activity of calmodulin on the calmodulin-dependent Ca^{2+}-ATPase that leads to sustained increase of cytosolic calcium ion concentration. This takes part in a multifactorial apoptotic process, involving endonuclease activation. In Ca^{2+}-independent mechanisms, OTs impair mitochondrial oxidative phosphorylation, oxidise glutathione and inhibit several thiol-containing and GSH-dependent enzymes like glutathione S-transferase (GST) and glutathione peroxidise (GPX), making exposed organisms

more vulnerable to oxidative stress. These mechanisms are synergistic since increase in cytosolic calcium is provoked by the presence of high amount of cytosolic oxidises glutathione that causes stimulation of calcium-releasing property of IP3 receptor. Cytoskeletal protein depolymerisation is due to both cytosolic calcium increase and extensive oxidation of their thiol groups causing severe morphological change, chemotaxis failure and phagocytosis inhibition. Figure from Cima and Ballarin (2012).

1.3.3 Cardiotoxicity

Organotin compounds have been reported to interfere with the cardiovascular system in animal models (Nath, 2008). Most of the available studies investigated the effects of OTs in rats and mice, but some work dated back from 1975 also addressed the effects of OTs in rabbits, cats and dogs (see Nath, 2008 for details).

Studies on tributyltin oxide pharmacokinetics in rats, rabbits and dogs showed that TBT causes a depression of the vascular smooth muscle and a fall in the blood pressure (Truhaut et al 1976 in Nath, 2008). Rabbits when exposed to alkyltins, for example, exhibit vasodilatation (Stoner et al. 1995 in Nath, 2008). Cats when exposed to triphenyltin exhibit an increase in the blood pressure if exposed to a single dose (1mg/kg triphenyltin acetate) and arterial hypotension if the administration is repeated at 20-60 min intervals (Tauberger, 1963 in Nath, 2008).

Several *in vivo* and *in vitro* studies suggest that organic derivatives of tin, including TBT compounds, inhibit the cardiac sarcoplasmic reticulum (SR) Ca^{+2} transport and affect the Na^{+}-pump activity among other possible effects (see Nath, 2008 for a complete description of the available studies and proposed mechanisms of action).

Kodavanti et al. (1991) performed several of experiments to assess the effect of TBT, and other organotins (triethyltin and trimethyltin) on the rat cardiac SR Ca^{2+} pump as well as on SR proteins. They reported that all of the tested organotin compounds inhibited cardiac sarcoplasmic reticulum ^{45}Ca uptake and Ca^{2+}-ATPase in a concentration-dependent manner. These results suggest that these OTs may be affecting Ca^{2+} pumping mechanisms through the alteration of phosphorylation of specific proteins (such as phospholamban[4]) in rat cardiac sarcoplasmic reticulum (Kodavanti et al., 1991). These evidences are extremely important because the sarcoplasmic reticulum calcium pump together with phosphorylation of specific proteins have an important role in myocardial contraction and relaxation and if any compound alters this mechanism it obviously affects the normal functioning of the heart (Nath, 2008).

[4] **Phospholamban** in an integral sarcoplasmic reticulum protein that is expressed predominantly in cardiac muscle and in small amounts in skeletal muscle, smooth muscles and endothelial cells (Koss and Kranias, 1996; Sutliff et al., 1999).

Further *in vivo* and *in vitro* studies, reviewed by Nath (2008) also demonstrated that organotin complexes, particularly the ones derivatives of aminoacids, dipeptides, triglycine, thymidine and umbelliferrone may act as direct vasodilators on the smooth muscles on blood cells, exhibiting potent anti-hypertensive activity and therefore their use as hypertensive drugs in under appraisal (Nath, 2008).

More recently, dos Santos et al. (2012) investigated the influence of TBT (100ng/kg) on the coronary function of female rats and demonstrated that this organotin impairs the vasodilation induced by 17β-estradiol in isolated rat hearts. 17β-estradiol is a sex hormone that is involved in the protection against cardiovascular diseases by reducing the coronary artery disease via coronary vasodilation (Dubey and Jackson, 2001). Dos Santos et al., (2012) study demonstrated that the group of rats treated with TBT had decreased 17β-estradiol serum levels that were accompanied by an increase in serum progesterone levels, but maintenance in testosterone levels. The rat's heart of the TBT exposed group exhibited higher percentages of collagen in the myocardium, which according to the authors may impair cardiac function due to the presence of fibrosis. Furthermore, the TBT exposed group disclosed an arterial wall surrounded by inflammatory cells and an increase in mast cells[5] accompanied by an endothelial denudation. Based on these results the authors, concluded that TBT exposure may be a potential risk factor for cardiovascular disorders in rats (dos Santos et al., 2012). The same team continue to work on the vascular effects of TBT and studied the effect of TBT in vascular reactivity of isolated aortic rings of female rats (Rodrigues et al., 2014). Female rats were treated daily by gavage for a period of 15 days with TBT (100ng/kg). The obtained results disclosed once again a decrease in serum 17β-estradiol levels and an increase in collagen, accompanied this time with reduced α1-smooth muscle actin. Overall, this study allowed the authors to conclude that TBT decreases the vascular reactivity of female rat aorta by a mechanism that involves an imbalance in the effects of some endothelial factors as nitric oxide, K^+ channels and reactive oxygen species (ROS).

[5] **Mast cells** are involved in the first line defence against pathogens and represent important mediators in diseases. These cells are laden with granules containing a wide variety of vasoconstrictors (Bot et al., 2008).

1.4 Objectives, motivation and thesis layout

The objective of the current work is to elucidate the vascular effects of TBT trough *in vitro* experiments. This rationale is based on the fact that TBT is a widespread environmental contaminant to which humans are presumably exposed and on the fact that the prevalence of cardiovascular diseases in human population in sharply increasing. Furthermore, regulatory agencies including the Scientific Committee on Health and Environmental Risks (SCHER), recommend the study of organotin compounds toxicity towards the cardiovascular system.

The present thesis is organized in five chapters:

- **Chapter 1:** provides a brief introduction concerning organotin compounds, with special attention to tributyltin and its biological effects in mammals;
- **Chapter 2:** provides a characterization of the vascular smooth muscle tissue as well as the regulation of contraction and relaxation;
- **Chapter 3:** describes the effects of TBT in rat aorta and in the A7r5 cell line (vascular smooth muscle cell line obtained from embryonic rat aorta) in order to understand the possible impact of this compound at a vascular level;
- **Chapter 4:** provides a general discussion of the results obtained and suggests future work in order to unravel the mechanisms that regulate TBT effects at the vascular level;
- **Chapter 5:** provides the references of the thesis.

Chapter 2

Characterization of vascular smooth muscle tissue

2.1 Vascular smooth muscle tissue

The vascular smooth muscle is the muscular component of blood vessels which principal functions are the contraction and regulation of the vascular tonus, blood pressure and blood flux (Owens, 1995). The blood vessels are different due their physical dimensions, morphology, function and composition and the aspect that their all have in common is that all are lined with endothelial cells (Clark and Pyne-Geithman, 2005).

Blood vessels are classified in arteries, arterioles, capillaries and veins. The blood vessels that are exposed to greater pressures are the arteries. Arteries are responsible to carry blood from the heart and therefore the arteries are thicker than other vessels and have larger extracellular proteins such as collagen and elastin. Arteries have an important role in the function of the cardiovascular system (Clark and Pyne-Geithman, 2005).

The cells that have the responsibility to maintain the functions of smooth muscle tissue are the vascular smooth muscle cells (VSMC). The vascular smooth muscle cell is a highly specialized cell that has as primary function the contraction and relaxation of blood vessel tone-diameter, blood pressure and blood flow distribution (Owens et al., 2004). During the vascular development VSMC exhibit a high rate of synthesis of extracellular matrix components such as collagen, elastin, proteoglycans and cadherins that comprise a major portion of the blood vessel mass. At this stage of development, VSMC form abundant gap junctions with endothelial cells which is essential for vascular maturation and vessel remodeling (Hungerford and Little, 1999). In adult blood vessels VSMC proliferate at an extremely low rate and have low synthetic activities. They express contractile proteins, ion channels and signaling molecules that are necessary for the contractile function of the cells (Owens, 1995). Contrasting with the skeletal or cardiac muscle that are terminally differentiated, VSMC retain remarkable plasticity and can suffer profound and reversible phenotypic changes in response to changes in local environmental signals that normally regulate the phenotype (Owens, 1995). The plasticity of these cells can be seen during the vascular development when the VSMC plays an important role in morphogenesis of the blood vessels and have high rates of proliferation, migration and production of extracellular matrix components while acquiring contractile capabilities. In case of vascular damage these cells increase its rate of proliferation, migration and synthetic capacity playing a critical role in vascular repair (Owens et al., 2004).

VSMC have the capacity to perform different functions which is translated in a wide diversity of phenotypes, ranging from synthetic to contractile (Rensen et al., 2007). The different

functions of VSMC are notable by the diversity in morphology but also in the expression levels of marker genes, proliferative potential and migration properties. Synthetic and contractile VSMC have clearly different morphologies and this is an important parameter for the definition of the phenotype although the use of maker proteins is also used (Rensen et al., 2007). Basically, synthetic VSMC are less elongated while contractile are elongated and spindle-shaped (Figure 5) (Hao et al., 2003; Rensen et al., 2007).

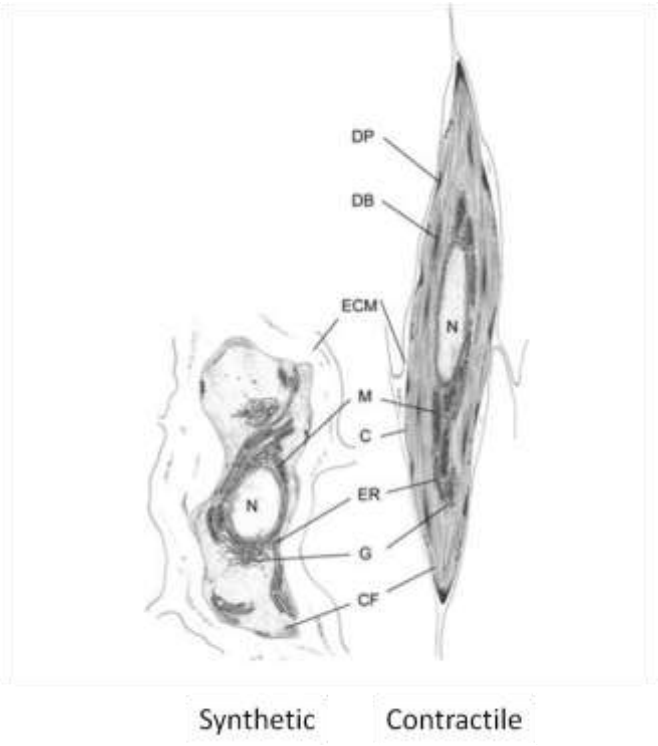

Figure 5. Structural characteristic of synthetic and contractile VSMC. DP: Dense plaque; DB: Dense body; ECM: Extracelullar matriz; M: Mitochondria; N: Nucleus; C: Caveolae; ER: Endoplasmic reticulum; G: Golgi complex; CF: Contractile filaments. Figure from Rensen et al. (2007).

VSMC are responsible for the vascular tone and their use is important to study several processes such as calcium metabolism. The entry of Ca^{2+} serves as a crucial intracellular messenger that regulates a variety of cellular processes including muscle contraction, gene expression, hormone secretion and neuronal transmission (Hofmann et al., 2014).

2.2 Contractile proteins

The contractile activity of smooth muscle is related with the contractile proteins actin and myosin (Webb, 2003). Actin is present in the thin filaments while the myosin is present in the thick filaments (Chamley-Campbell et al., 1979).

The contractile protein actin is found in all eukaryotic cells, it is a highly conserved molecule and has the capacity to spontaneously change from its monomeric form (G-actin) to a filamentous macromolecule (F-actin) (Hodgkinson, 2000). In the thin filaments of smooth muscle actin is the major component although tropomyosin and caldesmon are also present. The other proteins may be associated with the thin filaments in the cell includes filamin, vinculin, α-actinin, myosin light chain kinase and caldmodulin. Actin is responsible for the interaction with myosin to produce contraction while the other proteins modulate this interaction by imposing structural organization on the filaments. Thin filaments essentials functions include the force generation by interaction with myosin; the control of force generation by interaction with Ca^{2+} and the transmission of force to the cells to produce contraction of the muscle (Marston and Smith, 1985).

In the thick filaments the main component is myosin II, a member of the myosin superfamily of motor proteins (Krendel and Mooseker, 2005). Myosin II is a two-headed molecule consisting in two heavy chains and two pairs of light chains, the 20kDa (MLC_{20}) regulatory light chains and the 17kDa (MLC_{17}) essential light chains (Gunst, 1999). The phosphorylation/dephosphorylation of the regulatory light chains of myosin is a key event in the regulation of smooth muscle contraction and relaxation. The contraction occurs by the Ca^{2+} - and calmodulin-activated myosin light chain kinase (MLCK) and the relaxation by the myosin light chain phosphatase (MLCP) (Pfitzer, 2001).

Overall, the mechanisms that regulate the behavior of the smooth muscle are based on the physical interactions between several structural components and the cells and their integrated role in mediating the transduction of chemical energy into mechanical function (Gunst, 1999). The main process in the generation of force and shortening of muscle cells is the cyclic crossbridge interaction between the thick and thin contractile filaments which contains myosin and actin, respectively (Arner and Pfitzer, 1999). The elevation of intracellular calcium increases the Ca^{2+}-bound calmodulin which in turn activates the MLCK. For the vascular muscle contraction the MLC_{20} must be phosphorylated, predominantly at serine 19, by MLCK which results in the activation of the myosin MgATPase activity (Figure 6) (Stull et al., 1991). After phosphorylation the myosin heads (or crossbridges) interact whit actin filaments and transport them past the myosin filaments (Geeves and Holmes, 1999). The energy for contraction is originated from the hydrolysis of ATP during the process (Arner and Pfitzer, 1999). The ending of the contractile stimulation causes a decrease in intracellular calcium which in turn decreases the MLCK activity. As a result, the MLC_{20} is dephosphorylated by the action of MLCP. The reversible phosphorylation

of MLC_{20} plays an important role in the regulation of the contraction of the vascular smooth muscle (Hirano, 2007).

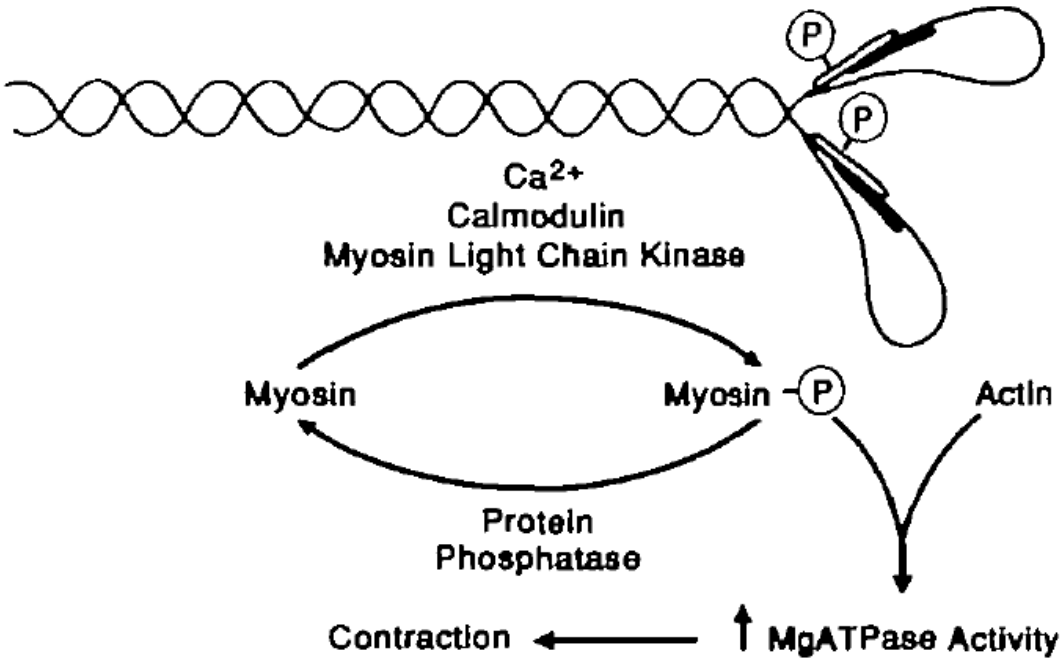

Figure 6. Illustration of myosin phosphorylation in smooth muscle. Schematic representation of the myosin molecule with two heavy chains with respective intertwined tail regions and globular head (top of the image). Associated with each head are the two types of light chains. The 20kDa (MLC_{20}) regulatory light chain is identified by "-P" and the 17kDa (MLC_{17}) essential light chain is shading. The pathway for initiating the contraction in smooth muscle is showed in the diagram. The regulatory light chain of myosin is phosphorylated (myosin-P) by Ca^{2+}/calmodulin-dependent myosin light chain kinase and dephosphorylated by a protein phosphatase. The phosphorylated myosin binds to actin, resulting in increased MgATPase activity and contraction. Figure from Stull et al. (1991).

2.3 Calcium channels

The contraction of the smooth muscle starts with an increase concentration of free calcium in the cell cytoplasm ($[Ca^{2+}]_i$), which can be originated from two different sources: the extracellular space and the intracellular stores such as the sarcoplasmic reticulum (McFadzean and Gibson, 2002). Extracellular calcium enters in the cell through the ion channels and the best characterized calcium entry pathway is the Voltage-Operated Calcium Channels (VOCC) specially the L-type Calcium Channels (LTCC) (McFadzean and Gibson, 2002). However, extracellular calcium can enter in the VSMC through other different channels in the membrane such a: Store Operated Calcium Channels (SOCC); Receptor Operated Calcium Channels (ROCC); Stretch

Activated Calcium Channels (SACC) and Nonselective Cation Channels (NSCC) (Evans and Gelband, 1999; McFadzean and Gibson, 2002; Setoguchi et al., 1997).

Calcium channels (Figure 7) are complex proteins constituted by four or five different subunits which are encoded by multiple genes (Catterall, 2000). The bigger subunit is the α_1 with 190 to 250 kDa that incorporates the conduction pore, the voltage sensor and gating apparatus and the known sites of channel regulation by second messengers, drugs and toxins. This subunit is organized in four homologous domains (I – IV) with six transmembrane segments in each (S1 – S6) (Catterall et al., 2003). Beyond this subunit there are the auxiliary subunits (β, $\alpha_2\delta$ and ϒ) that although they modulate the properties of the channels, they do not have interference in the pharmacological and electrophysiological properties since this originates mainly from the subunit α_1 (Hofmann et al., 1994).

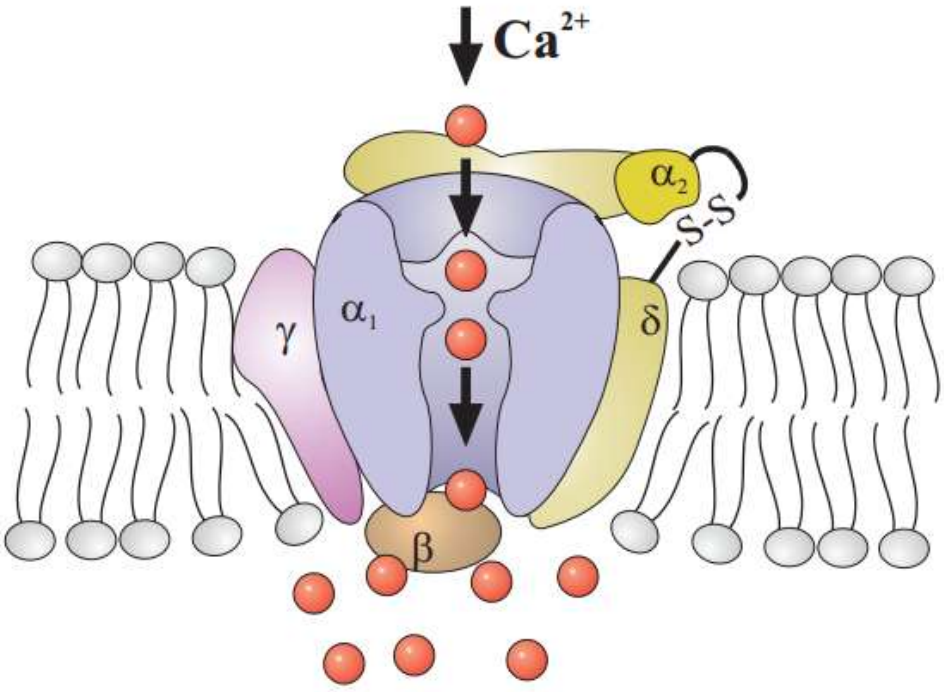

Figure 7. Schematic illustration of VOCCs with the different subunits. Figure from Lacinova (2005).

The voltage operated calcium channels (VOCCs) mediate the calcium influx in response to membrane depolarization and regulate contraction as well as secretion, neurotransmission and gene expression (Catterall et al., 2003). These calcium channels are divided into two classes: the High-Voltage Activated (HVA) and Low-Voltage Activated (LVA) (Hagiwara et al., 1975). The Ca^{2+} currents through the HVA are activated at -40 to -10 mV whilst the currents through the LVA channels are activated at lower membrane potentials (-60 to -70 mV).

The HVA Ca^{2+} channels are further divided into the L-type, N-type, P/Q-type and R-type channels whilst LVA Ca^{2+} channels only have T-type channels (Figure 8). The R-type is occasionally classified as intermediate-voltage-activated (IVA) (Yamakage and Namiki, 2002).

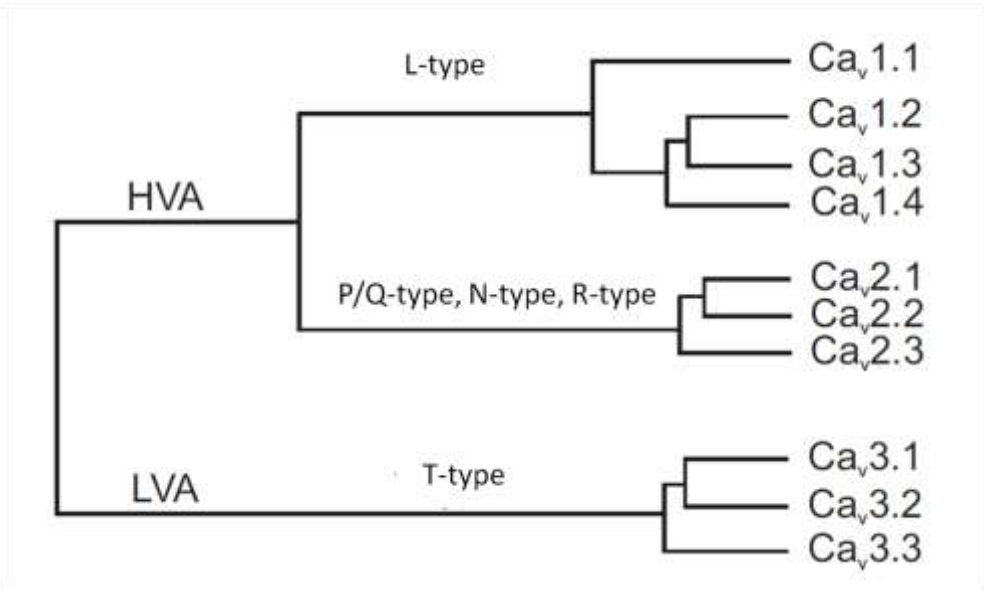

Figure 8. Dendrogram demonstrating the phylogenetic comparison of all known voltage operated calcium channels. HVA: High-Voltage Activated; LVA: Low-Voltage Activated; $Ca_v1.1$ to $Ca_v3.3$ are calcium channels subfamilies. Adapted from Lacinova (2005).

The L-type Ca^{2+} channels are considered as long-lasting and are activated at membrane potentials higher than -40mV (Evans and Gelband, 1999). They are the most important ones in both muscular and endocrine cells and can be blocked by antagonists including dihydropyridines, phenylalkylamines and benzothiazepines (Catterall et al., 2003). The N-type, P/Q-type and R-type Ca^{2+} channels are expressed in neurons where they start the neurotransmission and mediate calcium entry into cell bodies and dendrites. The T-type Ca^{2+} channels are expressed in several cells, are activated by weak depolarization and are transient (Catterall et al., 2003).

In the smooth muscle only two types of VOCCs exist: the L- and T-types (Xiong and Sperelakis, 1995) and the L-type calcium channel is considered to be the principal Ca^{2+} influx pathway (Hofmann and Klugbauer, 1996). In some VSMC the influx of calcium that occurs during the membrane depolarization is regulated by protein kinases. The phosphorylation by cAMP-dependent protein kinase (PK-A) or cGMP-dependent protein kinase (PK-G) inhibits the L-type Ca^{2+} channel activity and by this way produces vasodilatation, while the phosphorylation by protein kinase C (PK-C) stimulates the L-type Ca^{2+} channel activity and thereby produces vasoconstriction (Xiong and Sperelakis, 1995).

2.4 Regulation of vascular smooth muscle contraction

2.4.1 Ca^{2+}- dependent contraction

The activation of cell surface receptors by neurotransmitters or hormones initiates the contraction of vascular smooth muscle. This process can be trigged by the increase of calcium and is regulated mainly by the activation of the contractile proteins such as actin and myosin (Stull et al., 1991; Webb, 2003). In the resting state, the concentration of calcium is much lower inside the VSMC (i.e., 0.1µM) than in the extracellular fluid (1 – 2mM) and the activation of the contractile machinery requires that calcium concentration within cell exceeds approximately 1 µM (Akata, 2007a). The concentration of cytosolic calcium is increased by the entry of calcium from the extracellular space through the calcium channels as well as by the calcium release from intracellular storage (Webb, 2003).

The response of the cell to the binding of the substances that act as agonists (e.g. norepinephrine, angiotensin, and endothelins) is to increase phospholipase C activity via coupling to a G protein. The phospholipase C is specific for the phosphatidylinositol 4,5-bisphosphate and produces two second messengers: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to specific receptors on the sarcoplasmic reticulum leading to the release of calcium (Ca^{2+}). Diacylglicerol with Ca^{2+} activates Protein Kinase C (PKC), which in turn phosphorylates specific target proteins. Ca^{2+} binds to calmodulin causing the activation of MLCK which phosphorylates the MLC_{20}. This phosphorylated MLC_{20} in conjunction with actin leads to the crossbridge cycling, thus initiating the shortening of the smooth muscle cell (Figure 9)(Webb, 2003).

The pathways of signal transduction involving the regulation of calcium influx or release from the sarcoplasmic reticulum are implicated in the increase of cytoplasmic calcium concentrations. In the in the smooth muscle this is the principal factor for contraction (Stull et al., 1991). The activity of L-type Ca^{2+} channels is also regulated by the sarcoplasmic reticulum and there are two different mechanisms for the release calcium in the smooth muscle: Ca^{2+} -induced Ca^{2+} release (CICR) which is activated by Ca^{2+} and IP_{3-} induced Ca^{2+} release (IICR) which is activated by IP_3 (Karaki et al., 1997).

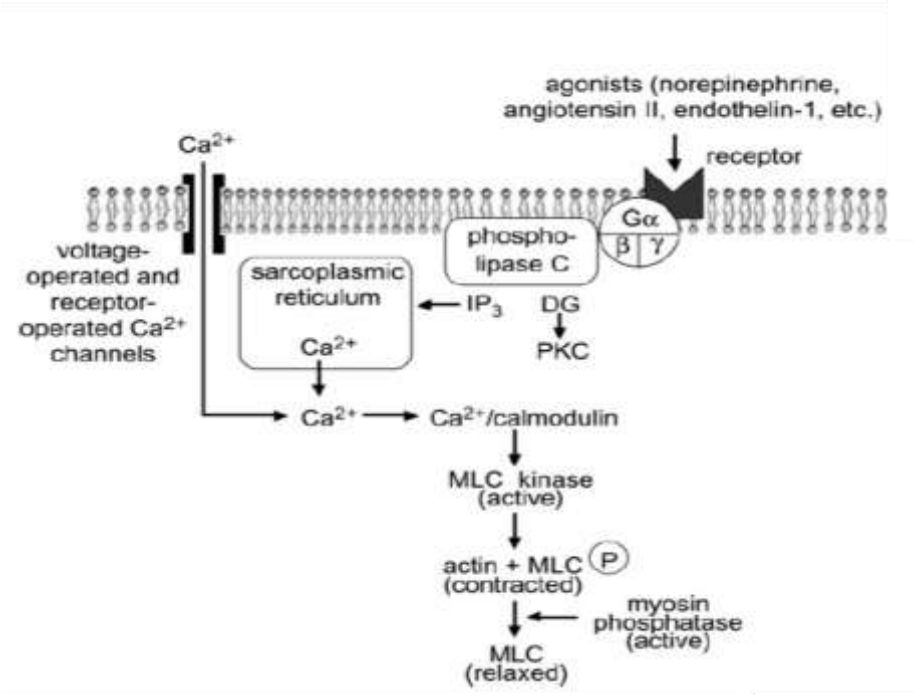

Figure 9. Illustration of the regulation of smooth muscle contraction. Adapted from Webb (2003).

2.4.2 Regulation of the membrane potential

The membrane potential is determined by the movements of ions through ion channels. The membrane potential together with the cytosolic calcium concentration regulates and modulates the influx and release of calcium through the ion channels and also the sensibility of the contractile machinery to calcium. The cells of the vascular smooth muscle express several types of channels that may be involved in the regulation of vascular tone (Jackson, 2000).

Potassium channels are the most important pathway in vascular muscle cells to conduct ions and their activity contributes to the determination and regulation of membrane potential and vascular tone (Jackson, 1998). The opening of K^+ channels results in the diffusion of this cation out of the cells and consequently in membrane hyperpolarization, on the other hand, the closure of K^+ channels has the opposite effect.

The voltage-gated Ca^{2+} channel plays an important role in the regulation of vascular tone by membrane potential. Their hyperpolarization closes the channels and leads to vasodilatation while its depolarization opens the channels and leads to vasoconstriction (Jackson, 2000). A schematic representation of the regulation of vascular tone by K^+ channels and voltage-gated Ca^{2+} channels is presented in Figure 10.

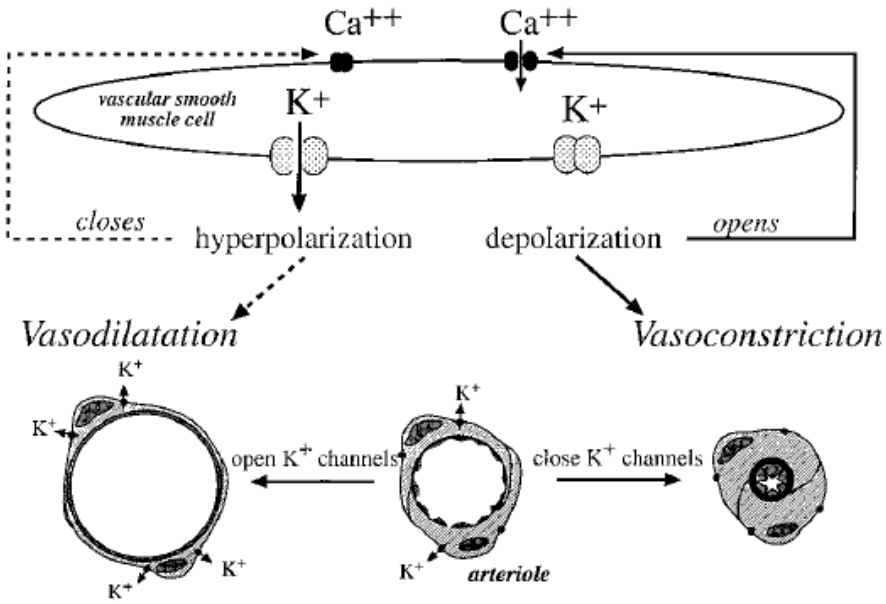

Figure 10. Schematic illustration on K^+ channels and their influence in the vascular tone. The opening of K^+ channels in the VSMC induce a diffusion of K^+ out of the cell, causing a membrane hyperpolarization and as consequence the closure of voltage-gated Ca^{2+} channels, which decreases the intracellular calcium and consequently leads to vasodilatation. The closure of K^+ channels causes membrane depolarization, which leads to the opening of the voltage-gated Ca^{2+} channels, and therefore to an increase in the intracellular calcium concentrations and consequently to vasoconstriction. Figure from Jackson (2000).

The chloride channels are a group of transmembrane proteins that allow Cl^- movement across plasma membrane and intracellular organelles (Servetnyk and Roomans, 2007). These channels have been also described as regulators of the vascular tone and their opening results in efflux of Cl^- from the vascular cells. Due to the Cl^- negative charge this efflux induces depolarization and vasoconstrion whilst the closing Cl^- channels have the opposite effect (Jackson, 2000).

2.4.3 Ca^{2+}- independent contraction

The Ca^{2+}- dependent contraction is not the only contraction mechanism in the smooth muscle, the cytoplasmic calcium concentration is further modulated by the Ca^{2+} sensitivity of the Ca^{2+} sensors (Somlyo and Somlyo, 2003). As explored above, the mechanism for contraction is through the phosphorylation/dephosphorylation of the regulatory light chain of myosin II MLC_{20}. The activation of MLCK by Ca^{2+} binding to calmodulin (CaM) leads to phosphorylation of the MLC_{20} to switch on crossbridge cycling and force contraction (Stull et al., 1991). The ratio between the

MLCK and MLCP activities determines the level of MLC_{20} phosphorylation. In situations of continuous stimulation, there is a reduction in the Ca^{2+} concentrations and in the amount of phosphorylated MLC_{20}, whilst the isometric strength increases. This suggests that non phosphorylated myosin is also involved in the maintenance of contraction. Thus, Ca^{2+}-independent modulation of the MLCK and/or MLCP activities provides additional mechanisms for the regulation of MLC_{20} phosphorylation (Somlyo and Somlyo, 2003). This mechanism generally known as calcium sensitization, is calcium independent being responsible for the alteration in the contractility even in the presence of constant concentrations of calcium.

The Ca^{2+} sensitization can be regulated either by the MLC_{20} phosphorylation-dependent or –independent mechanisms, however, the phosphorylation-dependent mechanism is considered to play the major role (Hirano, 2007).

The MLCP inhibition may occur through two different pathways: RhoA/Rho-kinases with phosphorylation of myosin phosphatase targeting subunit-1 (MYPT1) and/or PKC with PKC-potentiated phosphatase inhibitor (CPI-17) (Mizuno et al., 2008). In the first pathway Rho-kinases phosphorylates MYPT1 leading to the inhibition of MLCP activity in the smooth muscle; in the other pathway the activation of PKC phosphorylates the CPI-17 witch also potentiates the inhibition of the MLCP activity (Mizuno et al., 2008).

The modulation of Ca^{2+} sensitivity is not limited to the phosphorylation of MLC_{20} and can be also mediated by other mechanisms (Somlyo and Somlyo, 2003). The mitogen-activated protein (MAP) kinase is one of those mechanisms involved in the Ca^{2+}-independent smooth muscle activation (Dessy et al., 1998). MAP kinases are regulated by phosphorylation cascades and contains at least three protein kinases in series that culminate in the activation of a multifunctional MAP kinase (Pearson et al., 2001).

2.5 Regulation of vascular smooth muscle relaxation

The smooth muscle relaxation occurs when the contractile stimulus stops or by the direct action of a substance that stimulates the inhibition of the contractile. However, a reduction in the intracellular concentration of Ca^{2+} and a increase of MLCP activity are necessary for the smooth muscle relaxation (Webb, 2003).

The process of relaxation is the opposite of the contraction process and ends with the return to Ca^{2+} basal levels. When cytosolic calcium concentration is lower than 1µM the

dephosphorylation of MLC_{20} by MLCP occurs and consequently the inactivation of MLCK. The complex Ca-CaM-MLCK is dissociated and as a result the Ca^{2+} that was bounded to CaM is released (Arner and Pfitzer, 1999). There are, however, other ways to decrease cytosolic calcium concentrations including, for example the plasma membrane Ca^{2+}-ATPase (PMCA) pump; the SR Ca^{2+}-ATPase (SERCA) pump; the Na^{+}/Ca^{2+} exchanger (NCX) and the cytosolic Ca^{2+} -binding proteins (Akata, 2007a). PMCA and SERCA pumps are believed to play an essential role in reducing the cytosolic calcium levels during the resting condition and/or in mediating the vasodilator action of several endogenous agents. Under physiological conditions, the reduction of cytosolic calcium occurs mainly by the transference of Ca^{2+} into the extracellular space mediated by PMCA or by the uptake of Ca^{2+} into SR intracellular stores mediated by SERCA (Akata, 2007a).

The Na^{+}/Ca^{2+} exchanger (NCX), located on the plasma membrane, also plays a significant role in the regulation of $[Ca^{2+}]$ (Juhaszova et al., 1996).

Besides these mechanisms that reduces the concentrations of intracellular calcium in the VSMC, the inhibition by antagonists of the receptor-operated and voltage-operated Ca^{2+} channels located in the plasma membrane also inhibit Ca^{2+} entry in the smooth muscle cells and elicit relaxation (Webb, 2003).

The vasodilatation under physiological conditions is also regulated by the cyclic nucleotides cAMP and cGMP which are considered as key messengers in this process. The intracellular levels of these messengers are the result of the balance between the rate of their synthesis and the rate of their degradation (Morgado et al., 2012). In VSMC the elevation of both cAMP and cGMP are related with relaxation of smooth muscle tissue in response to several vasodilators (Xiong and Sperelakis, 1995).

The cyclic AMP is synthesized from intracellular ATP by adenylate cyclases which are normally activated by external stimulus (neurotransmitters, hormones or drugs) that binds to a G-protein-coupled receptor (Willoughby and Cooper, 2007).

The cyclic GMP in the VSMC may be synthesized by two guanylate cyclases that differ in their cellular localization and activation: the particulate guanylyl cyclase (pGC) that is present in the plasma membrane and that is activated by natriuretic peptides (Potter et al., 2006) and the soluble guanylyl cyclase (sGC) that is activated by nitric oxide (NO) and by NO donors (Cary et al., 2006). The intracellular levels of both these cyclic nucleotides can be degraded to their biologically inactive metabolites by phosphodiesterases (Omori and Kotera, 2007).

The actions of cAMP and cGMP are usually attributed to activation of the protein kinase A (PKA) and protein kinase G (PKG) respectively (Morgado et al., 2012). It is well established that an

increase in the cytosolic level of cAMP and cGMP in the VSMC is one of the main mechanisms for relaxation, however the mechanism is not yet fully understood and it seems to vary between different blood vessels, species and also to be dependent on the stimulus (Morgado et al., 2012). Several pathways have been proposed by different authors to explain the vasodilator effect of cyclic nucleotides (Akata, 2007b; Cogolludo et al., 2007; Karaki et al., 1997; Lincoln et al., 2001; Martel et al., 2010; Orallo, 1996; Rembold, 1992; Woodrum and Brophy, 2001). These pathways can be summarized as follows:

1) The reduction of $[Ca^{2+}]_i$ that can be achieved by the activation of Ca^{2+} uptake by the SR, increased intracellular Ca^{2+} efflux, inhibition of Ca^{2+} release from the SR and/or decreased extracellular Ca^{2+} influx;
2) The hyperpolarization of the membrane potential through activation of potassium channels, inactivation of Na^+ channels and/or inactivation of multiple channels;
3) The decrease in the sensitivity of the contractile machinery by the reduction of the $[Ca^{2+}]_i$ sensitivity of MLC_{20} phosphorylation due to a decrease in the MLCK activity and/or to an increase of the MLCP activity;
4) The decrease in the sensitivity of the contractile machinery through uncoupling from MLC_{20} phosphorylation via a thin-filament regulatory process.

Moreover, each cyclic nucleotide can also activate both the protein kinases, PKA and PKG. The PKA can be activated by cGMP but it requires a 10-fold-higher cGMP concentration than cAMP. Similarly, the PKG can be activated by cAMP although it requires a 10-fold-higher cAMP concentration than cGMP. In the smooth muscle, the levels of cytosolic cAMP are normally nearly 10-fold higher than the cGMP levels which means that elevations in the cytosolic level of cAMP could activate both PKA and PKG but cGMP only activates PKG (Rembold, 1992).

Chapter 3

Evaluation of TBT effects in the contractility of rat artery and in the L-type calcium channels

3.1 Introduction

Tributyltin (TBT) is one the most studied organotin compounds, with several described adverse effects towards a wide range of organisms, including humans. This compound was used for decades as active biocide in antifouling paints and it was responsible for a massive contamination of the aquatic environment (Sousa et al. 2014a). It acts as a potent endocrine disruptor (Sousa ACA et al., 2013), being also considered as a model obesogen due to its ability to inappropriately activate the master nuclear receptor of adipogenesis (PPARγ) and therefore to influence the development of obesity (Pereira-Fernandes et al., 2013). TBT is also considered to be immunotoxic (Hendriksen et al., 2014), neurotoxic (Mitra et al., 2013) and is also able to interfere with the cardiovascular system of mammals (Nath, 2008).

Besides being used as biocides, organotin compounds were also used as plastic stabilizers, catalytic agents for the production of polyurethane foams and silicones, pesticides and glass coatings. Due to their toxicity, the use of organotins is regulated in European Union (Decision 2009/425/EC, Regulation Nº276/2010 and Annex XVII of REACH, 2010). Accordingly, the majority of organotin compounds (TBT, TPT and DOC) had their effective ban in the 2012, whilst DBT effective ban only entered into force in the 1st January 2015 (see Sousa ACA et al., 2013 for a complete description of EC regulations on OTs). Despite the fact that organotins are already banned, the widespread contamination of the maritime environment (and consequently seafood), and their ubiquitous occurrence in consumer products grants them a constant presence in our everyday life and therefore humans may be exposed to TBT and other organotin compounds on a regular basis.

Currently, besides diet (particularly seafood from highly polluted locations), house dust is considered to be the most important pathway to human exposure to organotins, particularly TBT (Sousa et al., 2014; Kannan et al., 2010; Fromme et al., 2005). However, in specific groups of the population there is also concern of continuous exposure to OTs through medical devices (SCHER, 2006). This concern is based on the fact that organotins were employed (at least until 1st January 2015) as catalytic agents in production of silicones used in several medical devices such as breast implants, silicone valves, tubes and bags (SCHER, 2006). A couple of breast implants, for example, may contain one kg of silicone, if this silicone contains 0.1% of organotins it would represent 1 gram of OTs in the implants which may give a person of 70kg the tolerable daily intake (considering an emission factor as low as 0.000007) (SCHER, 2006).

Taking this into account, regulatory agencies including the Scientific Committee on Health and Environmental Risks (SCHER), recommend the study of organotin compounds toxicity (RPA, 2005; SCHER, 2006). Furthermore, several reports demonstrated interferences of organotins in the cardiovascular system (Cameron et al., 1991; Nath, 2008). Studies using rodent models associated TBT to cardiovascular disorders (Nath, 2008), however general consensus on the effects of TBT is far from being reached. Some studies demonstrated that TBT impairs the coronary vascular reactivity response to estradiol, produces endothelial denudation in isolated rat hearts (dos Santos et al., 2012) and has the capacity to decrease smooth muscle actin protein expression in aortic rings in rats (Rodrigues et al., 2014). It also induces endothelial dysfunction and collagen deposition (Rodrigues et al., 2014). Recently, the effect of TBT on mammalian endothelial cell integrity was studied and it was demonstrated that this compound deeply alters the endothelial profile (Botelho et al., 2015).

Based on the limited knowledge of TBT effects in the vascular system and on the recommendations of the European Commission through the Scientific Committee on Health and Environmental Risks (SCHER) to further study the impacts of organotins that may be found in heath devices, including the ones used to treat cardiovascular pathologies (e.g. cardiac valves), the present study aims to elucidate the effect of TBT in the contractibility of rat artery and its effects on the L-type calcium channels in A7r5 cells in order to understand the possible impact of TBT in vascular cells.

3.2 Methods

All the laboratory work was performed at the physiology laboratory of the Health Sciences Research Centre (CICS-UBI), University of Beira Interior.

3.2.1 Contractility Experiments

3.2.1.1 General principle of the organ bath technique

The organ bath assay (Figure 11) is a classical pharmacological tool for the evaluation of concentration-response relationships in several isolated section of contractile organs or tissues (McCallum et al., 2014). The principal characteristic of this technique is the ability to measure

concentration-dependent changes and to test the efficacy and potency of contractile agonists that can be manipulated by increasing concentrations of antagonists or inhibitors (Jespersen et al., 2015). An important characteristic of this technique is that because the tissue is alive it functions as a whole tissue maintaining physiological outcomes (e.g. contraction or relaxation) that are relevant to the body (Jespersen et al., 2015). Another important advantage is that because this technique is a synthesis of several steps, e.g. drug-receptor interactions, signal transduction, second messenger generation, change in smooth muscle excitability and change in tissue function; it allows the integrated study of all these steps contrarily to other techniques that only allow a single step characterization (Jespersen et al., 2015). Furthermore, this technique by being real time allows rapidly drawing conclusions, planning next steps, and troubleshooting during the experiment. Despite these advantages, this assay has several limitations mainly related with the protocol for sample preparation. In fact, there is the possibility that during surgical removal or during the placement of the tissue rings in the hooks, the tissues can be damaged so this is a critical step and careful handling of the tissue is required (Jespersen et al., 2015).

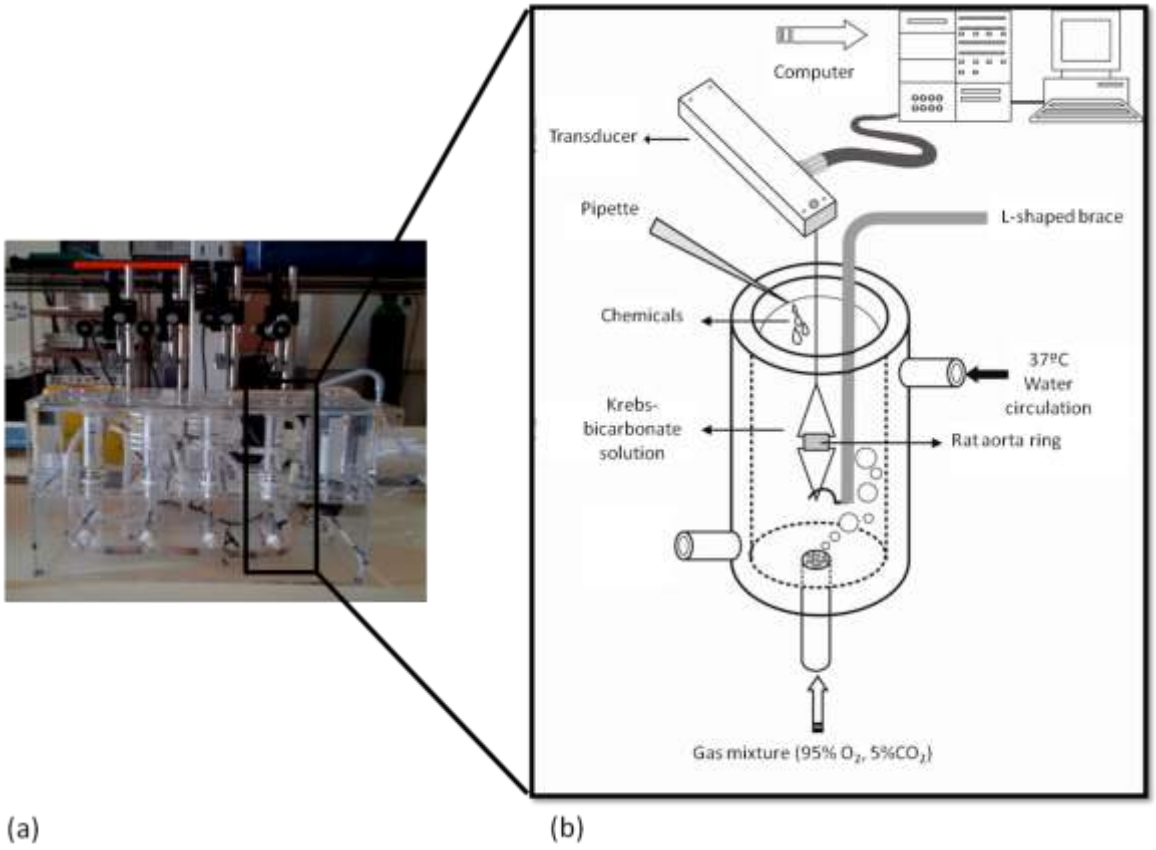

Figure 11. Organ bath system (a) Picture of the system used (b) schematic representation of the system. Adapted from Yildiz et al, 2013.

3.2.1.2 Rat Aorta Contractility Experiments

3.2.1.2.1 Experimental animals and rat thoracic aorta rings isolation

Male adult Wistar rats (Charles-River, Barcelona, Spain) weighing 400-500 g were sacrificed by decapitation and, after thoracotomy, and the thoracic aortas were removed, placed in a thermostatized (37ºC) Krebs modified solution following the standard protocol used in the physiology laboratory (Cairrão et al., 2012). The composition of Krebs modified solution was (mmol/L): NaCl 119, KCl 5, CaCl2●2H2O 2.5, MgSO4●7H2O 1.2, KH2PO4 1.2, NaHCO3 25, EDTA-Na2 0.03, L-(+)-ascorbic acid 0.6 and glucose 11 (pH 7.4). The concentration of calcium (2.5 mM) in the krebs modified solution used was based on the one previously described in similar studies (Carre et al., 2015; Rodrigues et al., 2014). The fat and connective tissue were cleaned and vascular endothelium was mechanically removed by gentle rubbing with a cotton bud introduced through the arterial lumen as fully described by Alvarez et al. (2010).

The animals used in the current experiment (n=5) were acclimatized for at least one week before the beginning of the experiments under light cycles of 12h light: 12h dark; with food and water *ad libitum*. The experiments were conducted in compliance with the protocols approved by the National Ethics Requirements for Animal Research (Decreto-Lei nº 113/2013, de 7 de Agosto) and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Union Directive number 2010/63/EU).

3.2.1.2.2 Artery tension recordings

The evaluation of the artery tension was performed following the standard protocols routinely used in the physiology lab (e.g. Cairrão et al., 2012) using in the same artery ring two different contractile agents: Noradrenaline (NA) and Potassium Chloride (KCl) (see Figure 12). These two chemicals induce contraction by different mechanisms: NA through direct binding to membrane receptors and KCl by membrane depolarization.

The rat aorta artery rings were placed in the organ bath (LE01.004, Letica) containing Krebs-bicarbonate solution at 37ºC continuously gassed with carbogen. The rings were suspended by two parallel stainless steel wires and tension measurement was performed using isometric transducers (TRI201, Panlab SA, Spain), interface PowerLab/4SP (ML750, ADInstruments), and

computerized system with Chart5 PowerLab software (ADInstruments). In the resting periods the organ bath solution was changed every 15 minutes.

Firstly, the rings were equilibrated for 60 minutes until a resting tension of 1.0 g. After this equilibrium period, the aortic rings were firstly contracted with noradrenaline (NA) at 1µM and the absence of endothelium functionality was confirmed by the lack of relaxant response to acetylcholine (Ach) at 1µM.

The arteries were then washed for 15 min three times with Krebs modified solution before the next stimuli. The rings were then contracted using noradrenaline (NA) at 1µM. When the maximum contraction was reached the effects of TBT on this contraction were analyzed. For this purpose, TBT solutions (0.1nM, 1nM, 10nM, 100nM, $1x10^3$ nM, $1x10^4$ nM and $1x10^5$ nM) were added sequentially. The artery rings and the system were then thoroughly washed with Krebs modified solution (3 times during at least 15 min each time). Afterwards, another contractile agent - potassium chloride (KCl) at 60mM - was added and the effects of TBT on this contraction were again analyzed by sequentially adding increasing concentrations of TBT. Control experiments were always performed in parallel using the same amount of ethanol used to prepare each TBT experimental solution. The concentration of EtOH was below 0.01% (v/v) in all experiments.

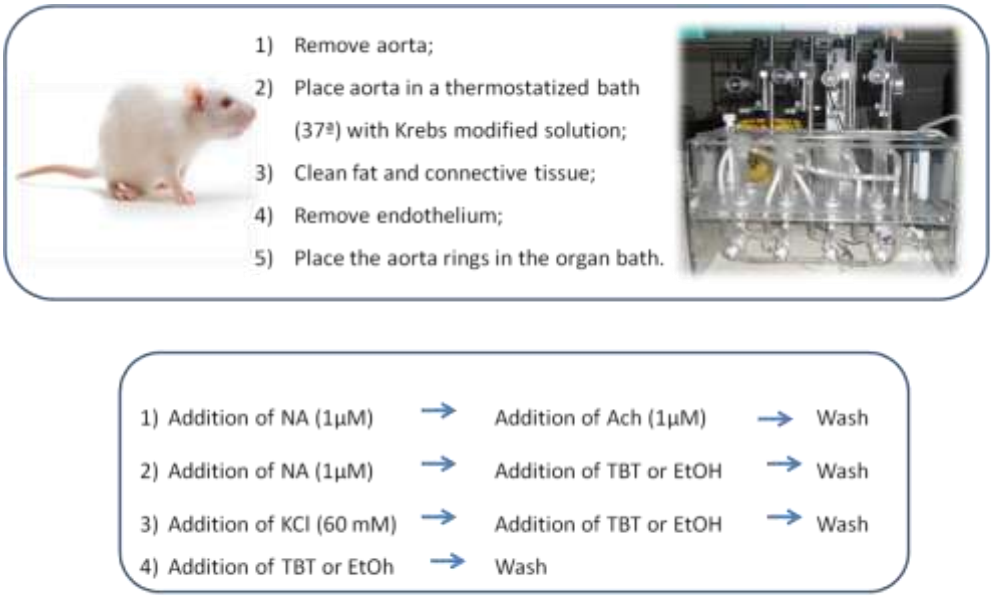

Figure 12. Schematic representation of the organ bath technique. The top panel represents the experimental preparation of the artery rings, since the removal of the aorta from the rats until the placement of the aortic rings in the organ bath. The bottom panel represents the different steps performed in order to evaluate: (1) the absence of endothelium; (2) the effects of TBT on the vasoconstriction induced

by noradrenaline (NA); (3) the effects of TBT on the vasoconstriction induced by potassium chloride (KCl); (4) the effects of TBT on the basal state. For each experiment a parallel experiment with a different artery ring was performed using ethanol, the solvent used to prepare TBT experimental solutions with the exact same conditions. All of the compounds are applied directly into the organ chamber without disturbing the tissue.

3.2.2 Electrophysiological Experiments

3.2.2.1 Cell culture

3.2.2.1.1 A7r5 cell line

The A7r5 cells are a commercial cell line derived of vascular smooth muscle obtained from embryonic rat aorta (Obejero-Paz et al., 1998b). This cell line has been extensively used as a model to study calcium homeostasis in vascular cells (Karaki et al., 1997) because A7r5 cells express the same voltage-dependent and receptor-activated calcium channels observed in freshly isolated smooth muscle cells from rat aorta (Marks et al., 1990) and the kinetics of Ca^{2+} mobilization is similar to that of differentiated smooth muscle cells (Lapidot et al., 1996). Furthermore, A7r5 cells also exhibit structural and functional properties of vascular smooth muscle (Marks et al., 1990).

In A7r5 cells the pathway of Ca^{2+} influx is voltage sensitive. This Ca^{2+} influx is a consequence of the activity of specific channels. It was demonstrated by the measurement of currents in the whole cell configuration of the patch clamp technique that these currents are due to the opening of L-type calcium channels (Marks et al., 1990; Obejero-Paz et al., 1998a). It was also demonstrated that the whole cell calcium currents recorded from these cells are often an order of magnitude greater than those recorded from freshly isolated vascular smooth muscle (Marks et al., 1990). Besides these advantages, large amounts of cells can be conveniently grown in the laboratory which enables to perform electrophysiology studies in parallel.

3.2.2.1.2 A7r5 cell culture

The A7r5 cell line used is a commercial cell line purchased from Promochem (Spain). The cells were grown in the culture medium Dulbecco Modified Eagle Medium/Nutrient Mixture F-12 Hams (DMEF-F12; Sigma-Aldrich, Portugal) supplemented with $NaHCO_3$ (1.2 µg/L), L-ascorbic acid

(20 µmg/L; Sigma-Aldrich), bovine serum albumin (0.5%; Sigma-Aldrich), heat-inactivated fetal bovine serum (FBS - 10%; Biochrom), and a mixture of penicillin (100 u/mL), streptomycin (100 g/mL) and amphotericin B (250ng/mL; Sigma-Aldrich). The cells were kept in culture at 37ºC in an atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2-3 days.

After confluence, the cells were placed in culture medium without FBS (FBS-free culture medium) for 24 hours. Trypsinization was performed and the cells were kept at 4ºC in FBS-free medium until the electrophysiological experiments (Alvarez et al., 2010).

3.2.2.2 Electrophysiological Experiments – Patch Clamp

3.2.2.2.1 Patch Clamp general principle

The patch clamp is a technique that allows the investigation of currents flowing through ion channels in several types of cells such as VSMC (Neher et al., 1978). This technique that measures ionic currents under voltage-clamp, was designed to study small patches of membrane in which near-perfect control of the transmembrane voltage can be readily achieved. Channel activity results in modifications of membrane conductance and this can be conveniently evaluated by recording membrane currents at a constant membrane voltage (Sontheimer and Ransom, 2002).

To perform patch clamp a small glass pipette is polished by heat and afterwards it is pressed against the cell membrane, forming a “seal” between the electrical surface of the pipette and the cell membrane (Figure 13). The application of a negative pressure inside the micropipette causes an increase in resistance of the “seal”, which is designated a giga-ohm seal (resistance >10GΩ). A high seal resistance is necessary because the higher the seal resistance, the more complete is the electrical isolation of the membrane patch and a high seal resistance decreases the current noise of the recording which permits a good time resolution of channel currents (Ogden and Stanfield, 1994). The cell-attached configuration allows the ionic current to flow through the open channels of the electrically isolated portion of the membrane to the micropipette. The “seal” that is formed is mechanically stable and thus it is possible to perform several settings for the study of ion channels (Neher et al., 1978).

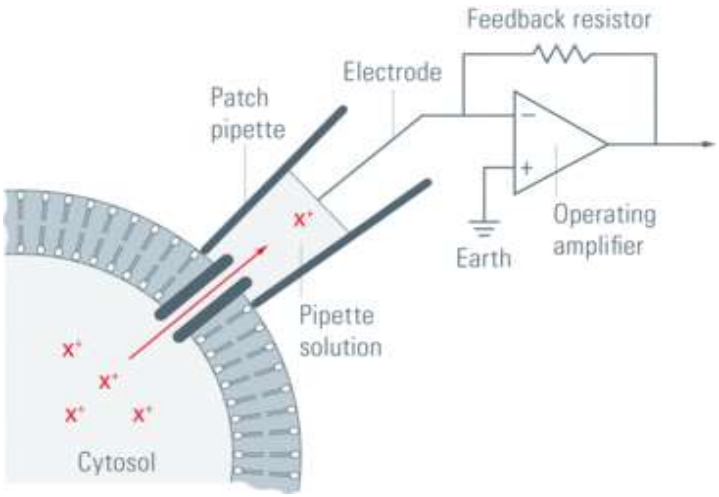

Figure 13. Illustration with the general principle of the patch clamp technique. A glass pipette that contains electrolyte solution is tightly sealed onto the cell membrane and thus isolates electrically the membrane patch. The currents fluxing through the channels in this patch flow into the pipette and can be recorded by an electrode that is connected to a highly sensitive differential amplifier. Figure from http://www.leica-microsystems.com/science-lab/the-patch-clamp-technique/.

Different configurations can be used in the patch clamp technique (Figure 14) depending on the research interest. The configuration most commonly used for cell culture and tissue preparations is the whole-cell mode (Zhao et al., 2008). With this method the patch created in the cell-attached is ruptured with a pulse of suction or voltage, thus establishing low electrical resistance and physical continuity between the cell and the pipette. The access to the cell interior allows the voltage of the whole cell to be controlled via a voltage clamp. It is possible to observe the currents from all the ion channels in the cell membrane and also to separate the individual current types by controlling the chemical composition on both sides of the cell membrane (Zhao et al., 2008).

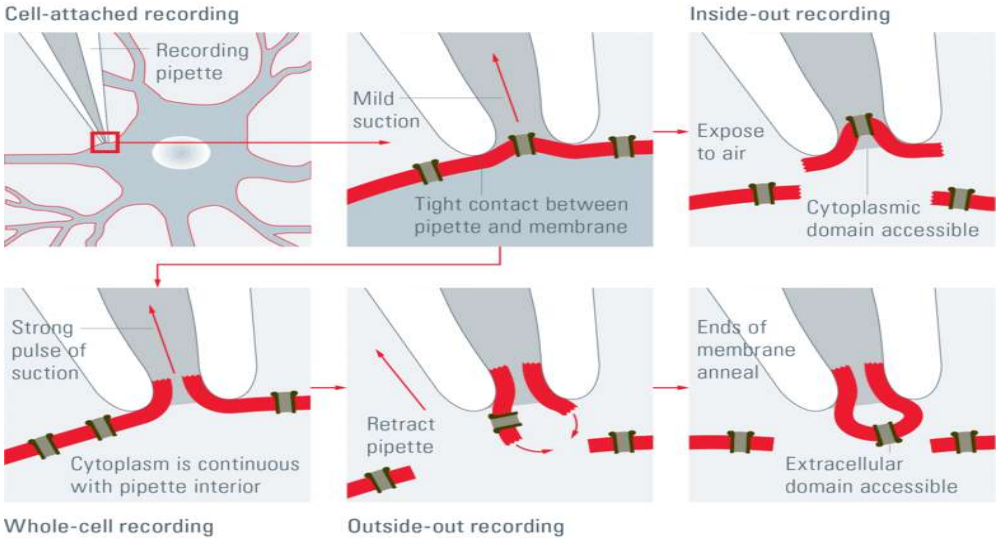

Figure 14. Illustration of the four different configurations for the patch clamp technique. **Cell-attached:** when the pipette is in closest proximity to the cell and mild suction is applied to gain a tight seal between the pipette and the membrane. **Whole-cell:** by applying a brief but strong suction the cell membrane is ruptured and the pipette gains access to the cytoplasm. **Inside-out:** when in the cell-attached mode the pipette is retracted and the patch is separated from the rest of the membrane and exposure to air. **Outside-out:** when in the whole-cell mode the pipette is retracted resulting in two small pieces of membrane that reconnect and form a small vesicular structure. Figure from http://www.leica-microsystems.com/science-lab/the-patch-clamp-technique/.

3.2.2.2.2 L-types calcium channels current measurement

The electrophysiological experiments were performed following the standard techniques routinely used in the physiology lab and fully described in Alvarez et al. (2010).

All experiments were performed at room temperature (23±2ºC). The patch clamp technique in the whole-cell configuration was used to analyze the effect of different TBT concentrations in calcium current thought the LTCC ($I_{Ca,L}$) in A7r5 cells. This study was performed over the basal calcium current without any stimulus. The basal current amplitude was measured 3 to 5 minutes after patch break to allow the equilibration between the pipette and the intracellular solutions. The composition of the control external solution in which cells were maintained during the period of the experiment and also used as control was (mM): NaCl 124.0, $CaCl_2$ 5.0, HEPES 5.0, tetraethyllammonium sodium salt (TEA) 10.0, KCl 4.7, and glucose 6.0 with a pH of 7.4 adjusted with NaOH. The solution inside the patch electrodes (2-4MΩ) - internal solution - had the following composition (mM) : CsCl 119.8, $CaCl_2$ 0.06, $MgCl_2$ 4.0, Na-ATP 3.1, Na-GTP 0.4, EGTA 5.0, HEPES 10.0, and TEA 10.0, with a pH of 7.3 adjusted with CsOH.

The cells were maintained in the petri dish with the control external solution at a holding potential of -80 mV and routinely depolarized every 8s to 0mV during 500 ms. The cells were voltage clamped with the patch electrolyte filled with internal solution using the patch-clamp amplifier Axopatch 200B (Axon instruments, USA). Currents were sampled at a frequency of 10 kHz and filtered at 0.1 kHz using the analog-digital interface Digidata 1322A (Axon Instruments, USA) connected to a compatible computer with the Pclamp8 software (Axon Instruments, USA).

For each individual cell the effects of the different concentrations of TBT (0.1nM, 1nM, 10nM, 100nM, $1x10^3$ nM, $1x10^4$ nM and $1x10^5$ nM) were tested by transferring the cell from the external solution into the inside of capillaries filled with the TBT testing solutions (Figure 15 and 16). In between each TBT concentration the cell was washed in control external solution. A similar

procedure was performed for EtOH using the same final volume of ethanol as the one used to prepare TBT experimental solutions ($7.18x10^{-9}$ to $7.18x10^{-3}$ % v/v).

The variations induced by the different TBT concentrations and their respective solvent controls in the basal $I_{ca,L}$ are expressed in percentage.

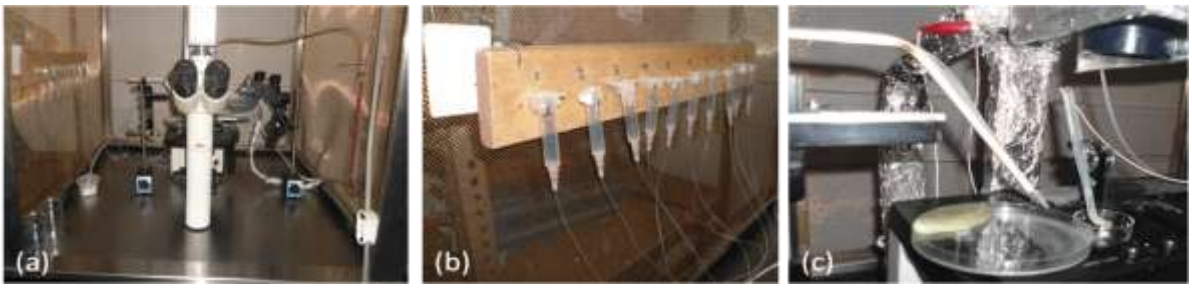

Figure 15. Patch clamp equipment (a) Picture of the equipment installed inside a Faraday cage; with the detail of the capillaries filled with the testing solutions (b) where the cells will be exposed (c).

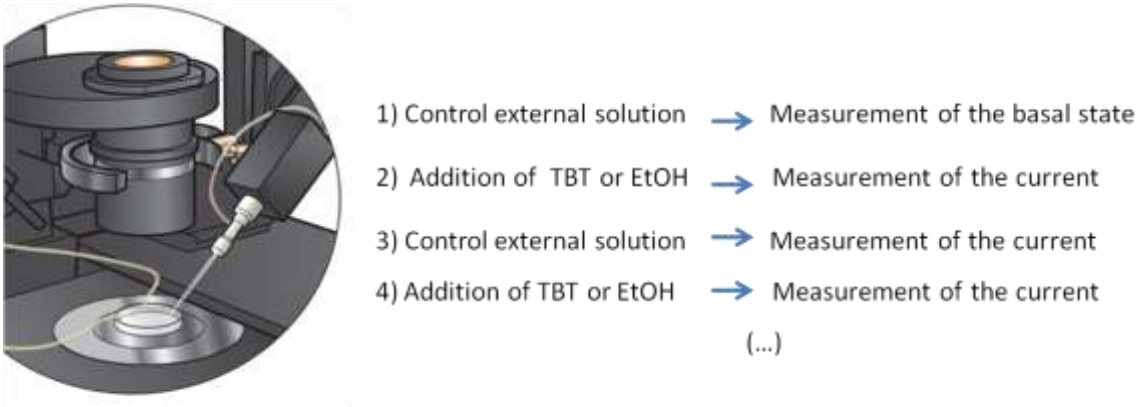

Figure 16. Schematic illustration of the steps to perform the patch clamp technique. Firstly, the basal state of the cell is measured in the control external solution. Secondly, the lowest TBT concentration (0.1nM) is tested, the effect in the calcium current is registered and after that the cell returns to the control external solution. Next, the increasing TBT concentrations are tested and between all TBT concentrations tested, the cell always returns to the control external solution. Ethanol, the solvent used to prepare the TBT experimental solutions, was tested in different cells following the exact same procedure. Figure from http://www.the-scientist.com.

3.3 Test solutions

A stock solution (1M) of tributyltin chloride (TBTCl – Aldrich, 96%) was prepared in absolute ethanol (Analytical grade, Fisher Chemicals). Experimental solutions (0.1nM, 1nM, 10nM, 100nM, $1x10^3$ nM, $1x10^4$ nM and $1x10^5$ nM) were freshly prepared every day in Krebs modified solution for

organ bath experiments or in control external solution for patch clamp experiments. Final concentrations of ethanol all experimental solutions never exceeded 0.01%.

3.4 Data Analysis

In the contractility experiments, the relaxant response induced by TBT (or ethanol) is expressed as a percentage of the maximal contraction (E_{max}= 100%) produced by the corresponding vasoconstrictor agent (NA or KCl). In the electrophysiological experiments the $I_{Ca,L}$ amplitudes were automatically calculated between the maximum current peak and the stable current plateau near the final of every 8s pulse. The variations induced by TBT are expressed as percentage. All results are expressed as mean ± standard error (SEM) of *n* experiments. In the contractility experiments *n* is the number of rings used and in the electrophysiological experiments *n* is the number of cells analyzed. Statistical analyses were performed using SigmaPlot 11.0 software. Comparison between each TBT concentration and its respective EtOH control was performed using Students t-test and Mann-Whitney Test (nonparametric test). Comparisons between the groups were performed using One Way ANOVA followed by Tukey post hoc test. Probability levels were considered significant when lower than 5% ($p<0.05$).

3.5 Results

3.5.1 Effects of TBT on rat aorta contractility

The administration of Ach (1µM) in the contracted artery rings didn't induce any relaxant response (mean tension value: -2.95±0.87 mg; n=19). This absence of relaxant response confirms the absence of endothelium in all the aortic rings used in the experiments.

The rat aortic rings without endothelium were contracted with two different contractile agents: NA (1µM) and KCl (60mmol/L). The contraction elicited by NA (1478.1 ± 141.7 mg; n=20) in rat artery rings was not significantly different (t-test, p= 0.696) from the one induced by KCl (1548.1 ± 115.5 mg; n=27).

No significant differences in the tension induced by NA (1µM) were registered in any of the different EtOH concentrations tested ($7.18x10^{-9}$ to $7.18x10^{-3}$) (one way ANOVA, p=0.574). TBT

induced relaxation (expressed as negative tensions, see Figure 17) after NA (1µM) contraction at all concentrations tested except for the lowest one (0.1nM) where a small contraction was registered (0.66%). The relaxation induced by TBT at all other concentrations was very low with a maximum relaxation of only 17.9% registered at the highest TBT concentration tested ($1x10^5$ nM) (Figure 17). This maximum effect at the highest concentration of TBT tested was also observed at its respective control (Figure 17) although to a lesser extent. In fact, the variation in tension induced by TBT was never significantly different from its respective EtOH controls (t-test, $p>0.05$). Significant differences in tension were found between the highest TBT concentration tested ($1x10^5$ nM) and the following TBT concentrations 0.1nM (Tukey test, $p=0.001$), 1nM (Tukey test, $p=0.004$), 10nM (Tukey test, $p=0.009$) and 100nM (Tukey test, $p=0.011$).

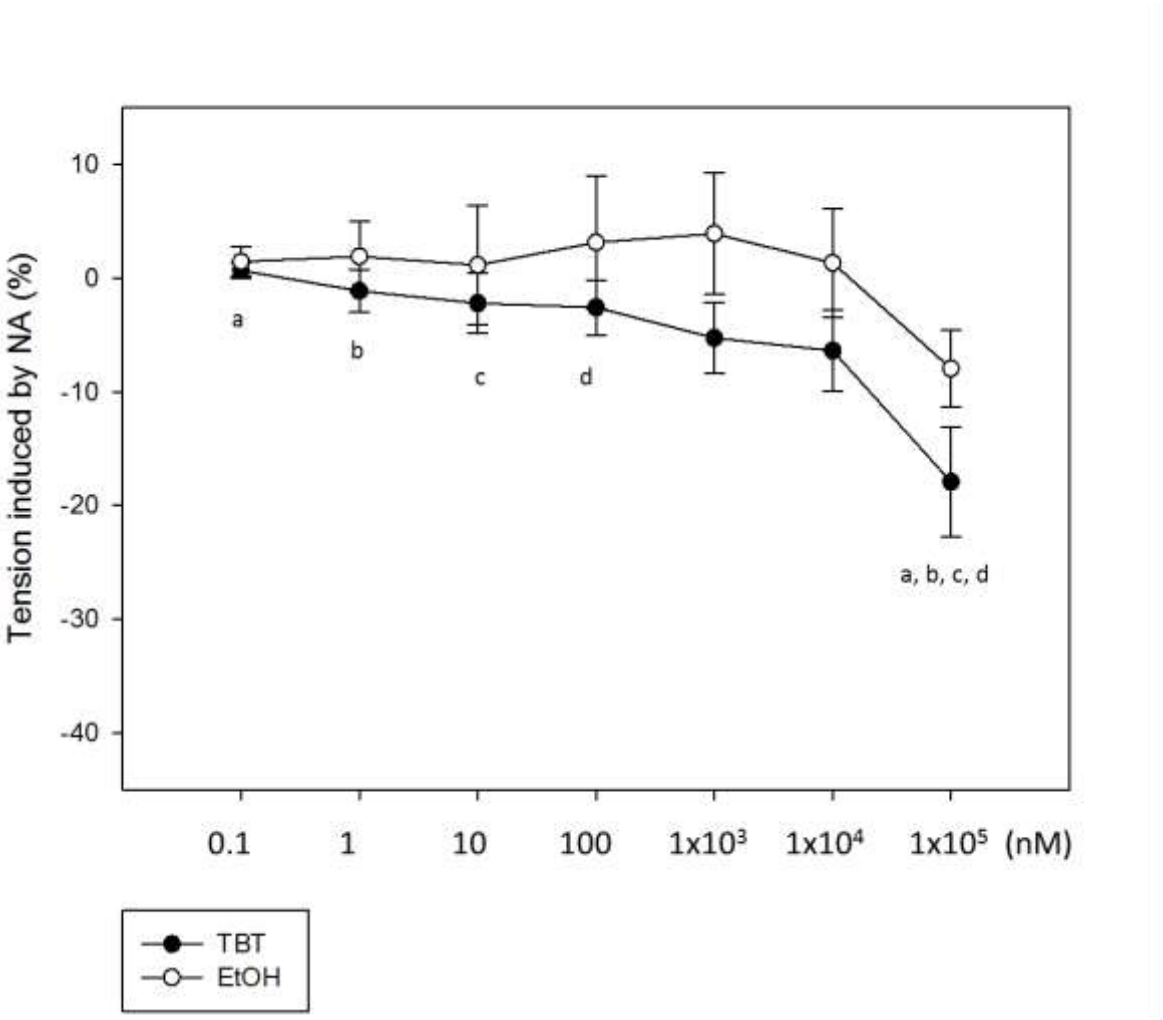

Figure 17. Effects of TBT (n=8) and respective EtOH control (n=5) on rat artery rings contracted by NA (1µM). The response induced by TBT (or ethanol) is expressed as a percentage of the maximal contraction (E_{max}= 100%) produced by NA. Negative tension values indicate relaxation whilst positive ones indicate contraction. Tension values with the same letter are significantly different. ($p<0.01$, Tukey test).

No significant differences in the tension induced by KCl (60mmol/L) were registered in any of the different EtOH concentrations tested ($7.18x10^{-9}$ to $7.18x10^{-3}$) (one way ANOVA, p=0.517). TBT induced relaxation (expressed as negative tensions, see Figure 18) after KCl (60mmol/L) at levels higher than the ones reported for NA. In fact, except for the lowest TBT concentration tested (0.1nM) the percentages of relaxation were always higher than 14%, with the highest value reaching 33%. This maximum relaxation was reached with the highest TBT concentration tested ($1x10^5$ nM). Despite this, the relaxations were still low and with no significant differences between their respective EtOH control (t-test/Mann Whitney t-test, p>0.05). No significant differences in tension could be found between the different TBT concentration tested (one way ANOVA, p=0.097).

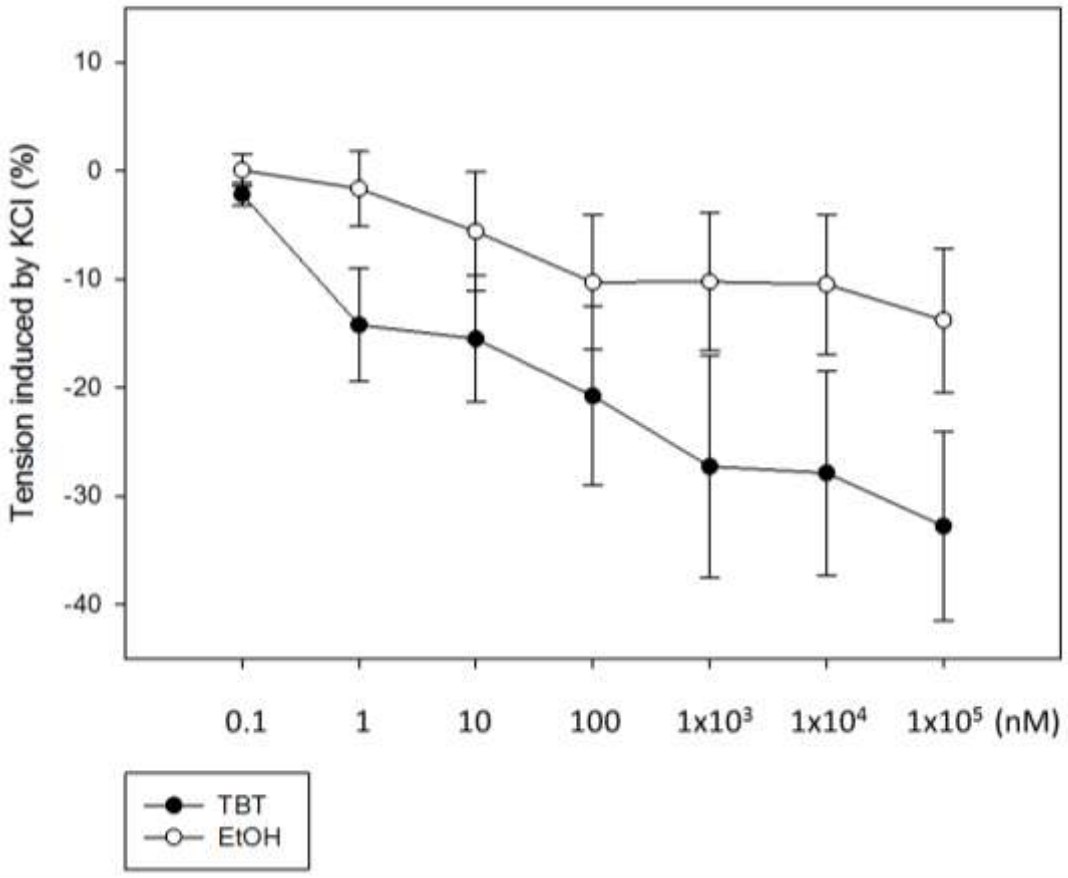

Figure 18. Effects of TBT (n=9) and respective EtOH control (n=6) on rat artery rings contracted by KCl (60 mmol/L). The response induced by TBT (or ethanol) is expressed as a percentage of the maximal contraction (E_{max}= 100%) produced by KCl). Each value represents the mean of the % of tension ± SEM from the number of experiments. No significant differences were found between the different TBT concentrations.

Figure 19 shows the comparison of TBT relaxant effects in arteries contracted with NA (1µM) and KCl (60mmol/L). Overall, TBT relaxant effects were low for both contractile agents, never reaching 50% of contraction (in fact the highest value was 33%). Significant differences between the two contractile agents were only detected between the relaxation induced by NA and KCl at TBT concentrations 1nM, $1x10^3$ nM and $1x10^4$ nM.

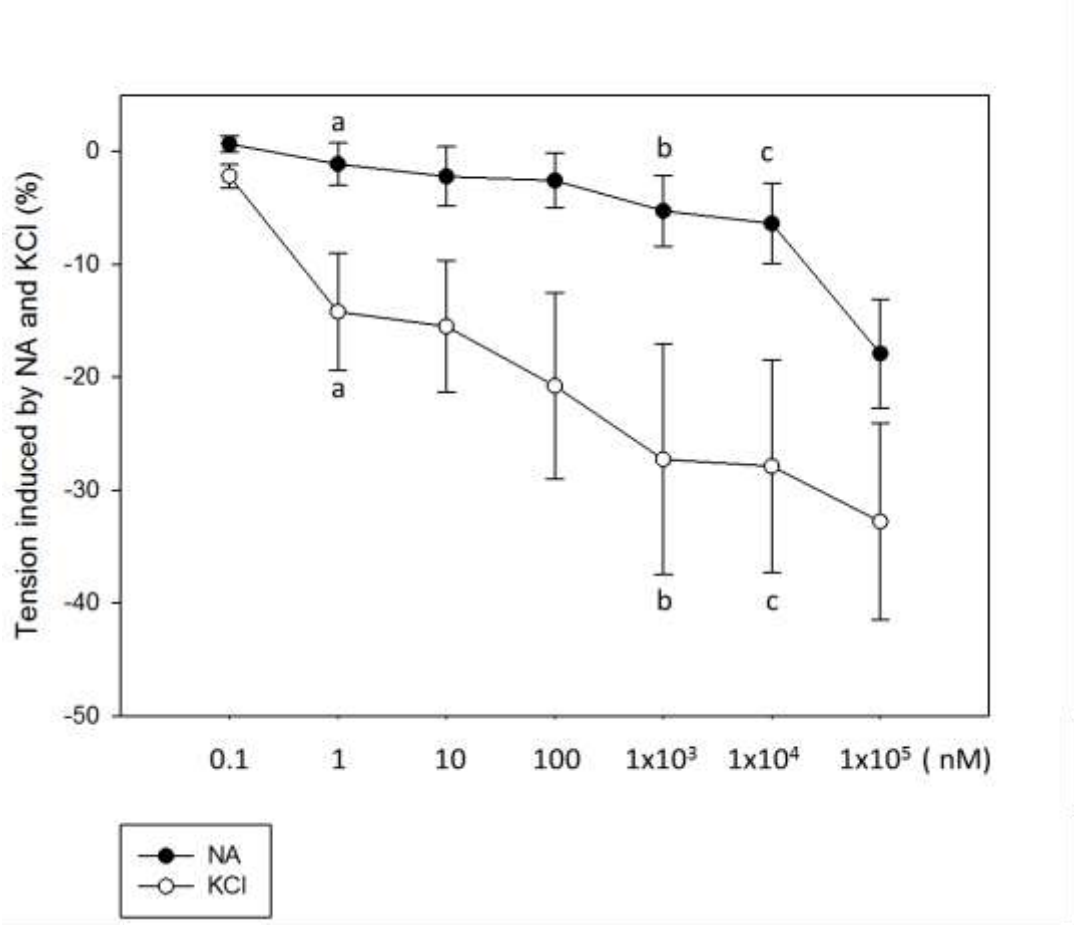

Figure 19. Comparison of the effects of TBT on the contraction induced by NA (1µM) (n=8) and KCl (60mmol/L) (n=9). The response induced by TBT is expressed as a percentage of the maximal contraction (E_{max}= 100%) produced by Na or KCl. Negative tension values indicate relaxation whilst positive ones indicate contraction. Tension values with the same letter are significantly different (t-test, $p<0.05$).

The effects of TBT and respective solvent controls on the basal tension of rat aorta without the addiction of any contractile agent, were also analyzed (Table 3). The obtained results demonstrate that TBT doesn't have a contractile effect with a maximal tension recorded of about 72 mg, far below the real tension for a contractile agent (1000 mg). Similar results were obtained for the EtOH control with a maximum tension approximately 10 folds lower that the typical tension of a contractile agent.

Table 3. Real tension (mg) of TBT (0.1 - $1x10^5$nM) and their respective solvent control. Each value represents the real tension (mg) ± SEM of the number of experiments (n). (*) The final percentages of EtOH are the same as the ones used in the preparation of the corresponding TBT experimental solution (varying from $7.18x10^{-9}$ to $7.18x10^{-3}$ and thus never exciding 0.01%).

	Real tension (mg)	
TBT Concentration	**TBT**	**EtOH(*)**
0.1 nM	-13.25 ± 37.37 (n=8)	65.43 ± 46.30 (n=7)
1 nM	29.75 ±25.99 (n=8)	128.71 ± 81.42 (n=7)
10 nM	10.75 ± 14.29 (n=8)	91.86 ±59.25 (n=7)
100 nM	31.38 ± 24.04 (n=8)	80.43 ±64.33 (n=7)
$1x10^3$ nM	22.88 ± 17.74 (n=8)	65.43± 63.56 (n=7)
$1x10^4$ nM	16.50 ± 16.04 (n=8)	86.14 ± 63.29 (n=7)
$1x10^5$ nM	71.75 ± 24.34 (n=8)	102.29 ± 70.67 (n=7)

3.5.2 Effects of TBT on L-type calcium channels of A7r5 cells

The effects of TBT and EtOH control on the inhibition of $I_{Ca,L}$ are depicted in Figure 20. Similarly to what was observed for contractility experiments with rat artery rings, no significant differences were registered in the inhibition of $I_{Ca,L}$ in A7r5 cells with any of the different EtOH concentrations tested ($7.18x10^{-9}$ to $7.18x10^{-3}$) (one way ANOVA, p=0.577).

The maximum inhibition of $I_{Ca,L}$ was observed at 1 nM of TBT with almost 15% of inhibition and the minimum was detected at $1x10^3$ nM with 1% of inhibition. The maximum inhibition was not observed for the highest TBT concentration and the same is true for the lowest inhibition.

The obtained results demonstrate that TBT inhibits the $I_{Ca,L}$ at very low levels (1% to 14%) and with no significant differences between the TBT concentrations tested and their respective controls (t-test, p>0.05) and with no significant differences within the different TBT concentrations tested (one way ANOVA, p=0.074).

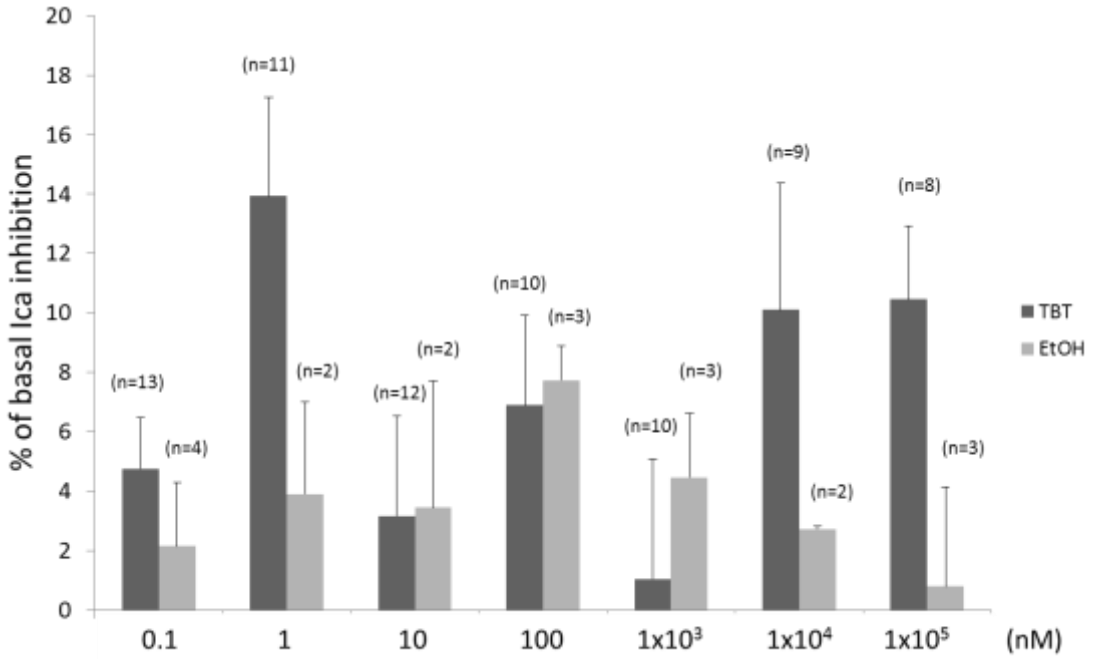

Figure 20. Effects of TBT and respective solvent control in the inhibition of $I_{Ca,L}$ in A7r5 cell line. The $I_{Ca,L}$ amplitudes were automatically calculated between the maximum current peak and the stable current plateau near the final of every 8s pulse. The variations induced by TBT or EtOH are expressed as percentage. Each column represents the mean value ± SEM from the number of experiments.

3.6 Discussion

According to the World Health Organization the cardiovascular diseases are the number one of death globally (WHO, 2015) and recent reports suggest that exposure to environmental contaminants may play a role in the etiology of cardiovascular diseases (Cosselman et al., 2015). Organotins are an important class of environmental contaminants for which limited and sometimes contradictory information on their effects into the cardiovascular system is available (e.g. Nath, 2008). Since organotins are used as catalysts in the production of silicones and medical devices that are made with silicone such as breast implants, tubes and bags, may contain organotins in their constitution, the regulatory agencies including the Scientific Committee on Health and Environmental Risks, recommend the study of organotin compounds toxicity towards humans (SCHER, 2006). In order to provide further evidences on the effects of TBT on the vascular system we investigated TBT effects on contractility of rat aorta using the organ bath technique

and on the L-type calcium channels analyzed by the whole-cell configuration of the patch clamp technique.

The results from the contractility experiments disclose that TBT had low effect of the vasoconstriction induced either by Na or KCl, the two contractile agents used in the present work. At first, the absence of endothelial cells was confirmed by the lack of relaxant response to acetylcholine in the NA contraction (about 3% of relaxation). It is well established, based on pharmacological evidences, that acetylcholine is able to induce relaxation in aortic tissues contracted by NA with intact endothelium but unable to relax these tissues without endothelium. Therefore, when endothelium is removed the relaxant response to acetylcholine is completely lost (Perusquia et al., 1996). Acetylcholine that is a neurotransmitter, when released by nerve terminals in the blood vessels wall activates the nitric oxide (NO) synthase in endothelial cells leading these cells to produce NO from arginine. The NO diffuses rapidly across membranes and in the smooth muscles cells it binds and activates guanylyl cyclase to produce cyclic GMP. The cyclic GMP triggers a response that causes the smooth muscle cells to relax (Alberts et al., 2008).

As previously mentioned, in this work two different contractile agents (NA and KCl) were used to induce contraction on the rat aorta rings without endothelium. These two agents were selected based on the fact that they illicit contraction trough different mechanisms. The calcium movements in the smooth muscle can occur by two different Ca^{2+} influx pathways: receptor-linked and voltage-dependent Ca^{2+} channels. The receptor-linked Ca^{2+} channels are opened by the release of Ca^{2+} from the sarcoplasmic reticulum induced by NA. In contrast, the voltage-dependent Ca^{2+} channels are opened by the membrane depolarization induced by high K^{+}. The opening of either these two channels leads to a continuous Ca^{2+} influx that induces contraction (Karaki et al., 1997).

The results obtained in the contractility experiments demonstrate that TBT relaxes the aorta rings, however with a very low percentage of the effect. In fact, there are no significant differences between the tested TBT concentrations and their respective solvent controls which seem to suggest that TBT does not have a significant effect on rat aorta without endothelium. Nevertheless, and despite the low levels of effects observed it was possible to conclude that when contracted with KCl the TBT effect was more pronounced (Figure 19). The fact that TBT had more relaxing effects after KCl induced contraction may suggest that TBT possible interference with the vascular tone is not mediated by receptor-linked Ca^{2+} channels as the effect after NA contraction was lower. NA is a neurotransmitter involved in the control of smooth muscle contraction by the sympathetic nervous system (Zang et al., 2006). The sympathetic nervous system is very

important for the vascular function, and the NA released from adrenergic nerves binds to α-adrenoceptors on the plasma membrane of the vascular smooth muscle cells. This initiates contraction by increasing the cytoplasmic concentration of calcium, which activates the contractile proteins (Vanhoutte and Rimele, 1982). On the other hand, high extracellular KCl concentrations induce membrane depolarization which activate voltage-dependent channels such as L-type calcium channels that increases intracellular calcium levels leading to muscle contraction (Alvarez et al., 2010). It is also well known that the opening of K^+ channels causes a membrane hyperpolarization and as a consequence closure of the voltage-gated Ca^{2+} channels, which decreases the levels of intracellular calcium and leads to the vasodilatation (relaxation) (Jackson, 2000). Nevertheless, more studies with not only Ca^{+2} but also K^+ channels are necessary.

The obtained results are somehow in accordance with the results available in the literature. In fact, published works suggest that TBT effects are mostly mediated by the endothelium (Mizuhashi et al., 2000; Rodrigues et al., 2014), and since our artery rings didn't have any endothelium this is probably the reason why in the present study we couldn't observe a higher degree of relaxation upon TBT exposure. Furthermore TBT effects are suggested to be bidirectional causing both, the contraction and relaxation of rat aortas (Mizuhashi et al. 2000).

Considering that calcium is the main responsible of the smooth muscle contraction/relaxation (Hofmann and Klugbauer, 1996) and in order to further elucidate the effects of TBT on calcium currents in the L-type calcium channels of A7r5 cells, the patch clamp technique was used. These cells were used in the contractile phenotype because is at this stage they express contractile proteins that are implicated in the regulation of the vascular contractility (Owens, 1995). It was previously demonstrated that the calcium current measured in these cells was only due the LTCC (Cairrão et al., 2012). The results obtained in the electrophysiological experiments demonstrate that TBT inhibited the basal I_{Ca} however, at a very low level and these results were not significantly different between TBT concentrations and their respective controls. Moreover, the effects obtained were not concentration-dependent with the maximal percentage of relaxant effects being observed at an intermediate TBT concentration (1nM). This can be easily understood because TBT is an endocrine disruptor and chemicals like this do not generate the standard monotonic dose-response curves. In the monotonic curves the dose and response increases together while in non-monotonic curves the higher or lowest effects can occur at the intermediate doses or the higher effect can also occur at the lowest dose (Fagin, 2012).

In conclusion, the obtained results demonstrate that TBT has no significant relaxant effect in rat aortas without endothelium contracted either with NA or by KCl, although KCl induced

higher relaxation rates. TBT seems to inhibit the L-type calcium channels although this effect was again not significant. Such results suggest that probably the mechanism by which TBT interferes with the vascular system is more complex not involving only calcium channels.

Further studies are necessary to unveil the possible mechanism of TBT in vascular cells and in the arteries. Those studies include the confirmation of the obtained results with a higher number of artery rings and cells and also the use of specific agonists and antagonists. Dihydropyridines can operate as agonistics or antagonists for the LTCC thus Bay K can be used to stimulate the calcium channel and nifedipine to block the calcium channel (Tikhonov and Zhorov, 2009). If the Bay K application stimulates significantly the calcium current above the basal level and the nifedipine significantly reduces the calcium current, this indicates that the current analyzed is in fact a LTCC current ($I_{ca,L}$). Thus, in order to completely characterize the inhibitory effects of TBT on the LTCC, the effect on the $I_{ca,L}$ stimulated by Bay K should be performed; if TBT is able to inhibit the LTCC, stimulation by Bay K will confirm it.

Chapter 4

Final discussion

4.1 Discussion and future perspectives

The main focus of the present thesis is the study of tributyltin (TBT), a chemical included in the class of organotins which are considered as one of the most important organometallic compounds produced by humans (Fent, 2006). Historically, TBT gained notoriety due its use as a biocide in antifouling paints, however it usage as biocide was globally banned in 2008. During the decades where TBT was massively introduced in the aquatic environment the scientific community was engaged in the quantification of OTs environmental levels. Afterwards most of the publications deal with the evaluation of the efficacy of the legislation on the reduction of TBT environmental levels (see e.g. review by Sousa ACA et al. (2013)). Since the description of TBT impacts in humans, particularly in the obesogenic effects of this contaminant the number of publications continue to increase (Figure 21).

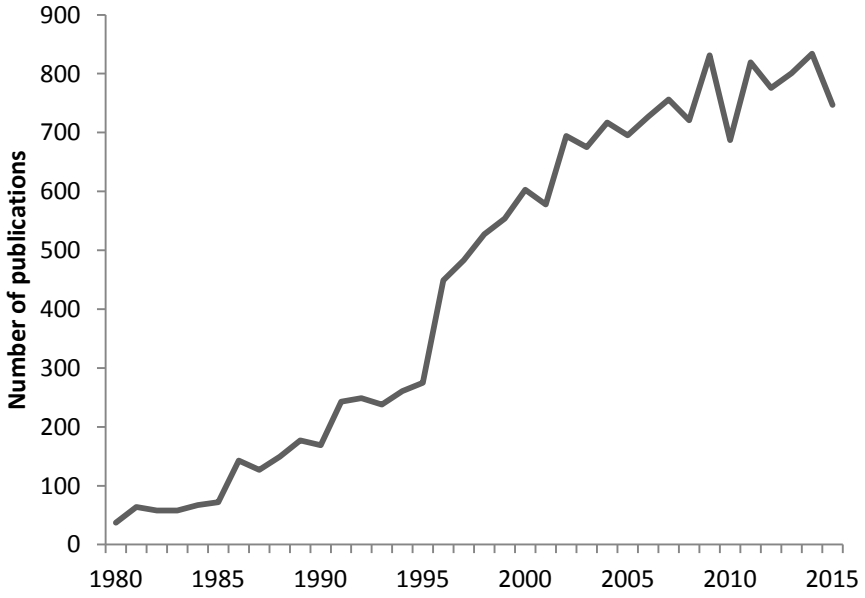

Figure 21. Number of publications on TBT per year since 1980 until 2015. The results were obtained from a search performed using in Scopus data base in December of 2015 when searching for "tributyltin" in "all fields" between 1980 and 2015. The total number of publications retrieved was 16061.

The interest in TBT has been increasing, with a minimum of 37 publications in 1980 and 2014 being one of the years where the number of publications was highest with 834 publications found in this data base.

The introduction of TBT on the marked occurred in the late of 1950's as an active ingredient in antifouling paints formulations (WHO, 1990). After several reports demonstrating the deleterious effects induced by this chemical, initiatives were taken by some countries and in 1982 France was the first country to establish restrictive initiatives. In the European Union utilization of TBT in antifouling pains was completely ban in 2003 but only in 2008 the global ban entered into force. The restrictions on TBT usage in articles or part of articles, as well as in mixtures were regulated under European Regulations and had their effective ban in 2010 and 2012, respectively (Sousa ACA et al., 2013). In the Figure 21 it is possible to observe that since the first restrictive initiative the study of TBT has gained interest for researchers and has been explored in several different areas (Figure 22). The principal area where TBT is studied is Chemistry, followed by Environmental Science; Biochemistry, Genetics and Molecular; Pharmacology, Toxicology and Pharmaceutics; and Agricultural and Biological Science.

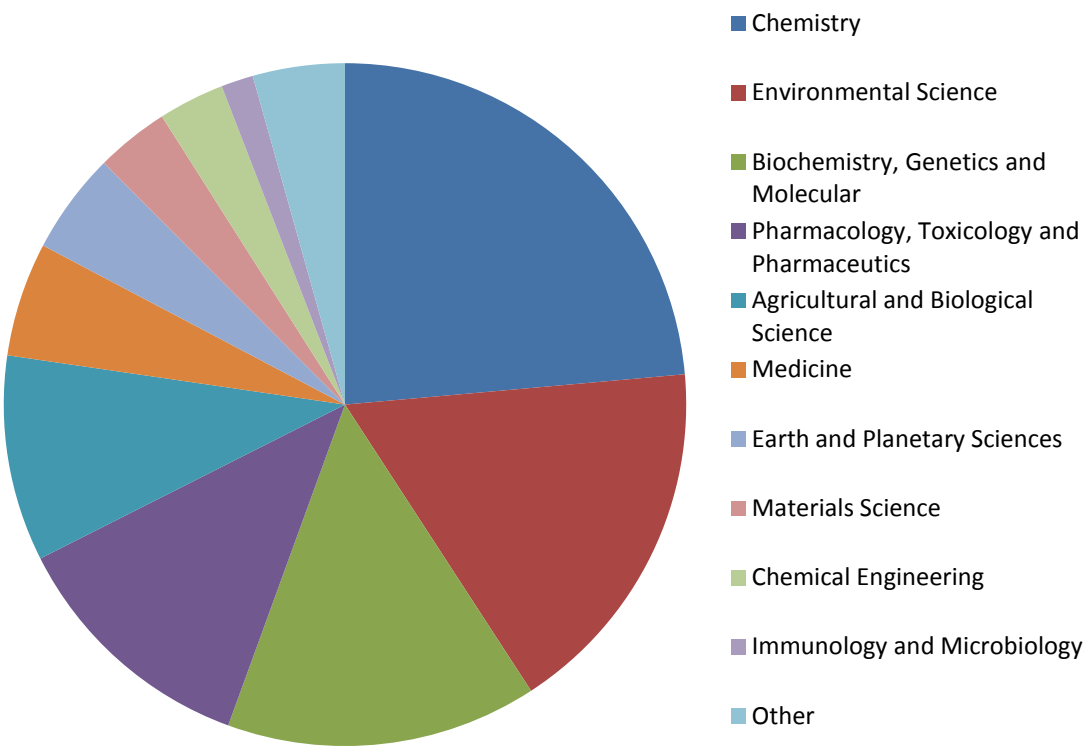

Figure 22. Percentage of documents on TBT by subject area since 1980 to 2015. Data extracted from Scopus.

Taking into account the relevance on the study of TBT, the objective of this thesis was to investigate the effect of this organotin compound in the regulation of blood flux in terms of

contractility in order to try understand the possible role of TBT at a vascular level. In this work, we analyzed the effects of TBT on rat aorta rings without endothelium by organ bath technique and on the $I_{ca,L}$ measured by whole cell voltage clamp in A7r5 cells. The obtained results seem to demonstrate that TBT induced a low percentage of relaxation on rat aortas contracted by NA and KCl, but this effect was not significantly different from the respective controls. TBT also seems to inhibit the calcium current through the L-type calcium channels but again without a significant difference from the ethanol control.

The obtained results under the framework of the present thesis are preliminary ones and in order to understand how organotins interfere at the vascular level a more complete study will be necessary. To perform a more complete study other organotins should be tested, particularly the DBT and DOT because these are the compounds mainly used in the silicone manufacture included in the healthcare devices (RPA, 2005; SCHER, 2006). In the present study arteries (aorta) from rats and the cell line also derivated from embryonic rat aortas were used. The use of tissues and cells from rats, that are considered as model and are currently used in laboratory experiments, is essential and provides a good base of results for extrapolation to humans. Nevertheless, the study of organotins in human tissues and cells should be performed in order to better elucidate their effects in human health. To conduct this experiment it will be necessary to collect human umbilical cord and to isolate human umbilical artery smooth muscle cells (HUASMC) (Cairrao et al., 2009). After this it will be possible to perform contractility experiments using a human artery and electrophysiological experiments using the HUASMC, and by this way study the organotins effects at a vascular level in human tissue and cells.

Furthermore, in the present study only rapid effects, also denominated as non genomic effects were analyzed, but for a better understanding of TBT effects it would be important to also study the genomic effects. Such study is even more important because the exposure to environmental chemicals is prolonged and the assessment of chronic effects is necessary. Another important aspect to explore the effects of this compound would be to study their effect not only in calcium channels but also in potassium channels.

The results obtained in this thesis suggest that this compound does not interfere at a vascular level at least in the parameters studied. Nevertheless, it is well known that TBT has negative effects at several levels. Our results do not allow establish any relationship with cardiovascular diseases induced by the effects of TBT but as has often been referred throughout this thesis TBT is considered as an obesogen compound with capacity to increase a number of adiposities or increase the volume of these cells and by this way contributing to obesity (Pereira-

Fernandes et al., 2013). Obesity is associated, among other factors, with hypertension, which is a prominent risk factor for the development of cardiovascular disease (Rangwala and Lazar, 2004). Hence, a possible relationship between the TBT effects and the development of cardiovascular diseases should be carefully studied considering the interplay of all these pathways.

As long as the molecular mechanism of TBT toxicity is not fully understand a greater effort must be performed in order to clarify this subject.

Chapter 5

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