Identifying new compounds for inhibition of smooth muscle contraction using organ bath: a pragmatic guide to application

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Abstract

Smooth muscle contraction is central in pathophysiology of wide-spread diseases, including increased blood pressure, lower urinary tract symptoms, and obstructive lung diseases. Accordingly, investigating mechanisms of contraction, and identification of compounds for inhibition of contraction are of high interest. The key technique for these purposes are measurements of smooth contraction by organ bath studies, which allow to induce and quantify smooth muscle contractions of intact tissues in vitro. In fact, organ bath studies were an essential preclinical step in development of medications for smooth muscle-based diseases (e. g., antihypertensive drugs, asthma sprays, and drugs for prostate- and bladder-related symptoms), but are poorly known among academic scientists. Apart from the high scientific relevance, organ bath is an attractive approach for young researchers, as it provides quick and functional results, is easy to learn, and associated with low costs. Here, we describe our procedures and protocols for isometric tension measurements aiming to examine effects of drug interventions on smooth muscle contractions of lower urinary tract tissues and blood vessels.

1. Introduction

Smooth muscle contraction plays a major role in pathophysiology and treatment of wide-spread diseases, including hypertension and cardiovascular disease, obstructive lung diseases, and lower urinary tract symptoms (table 1). In addition to increased blood pressure, locally increased vascular smooth muscle tone accounts for life-threating, but common complications in several disorders, including impaired kidney function in diabetes, portal hypertension in liver cirrhosis, or pulmonary hypertension. Other conditions are less harmful, but account for large decreases in quality of life, including lower urinary tract symptoms in mild to moderate stages or erectile dysfunction, and have high social and economic impact.

organ	function	disease	drugs
Blood vessel (peripheral) [1]	Blood pressure, blood flow (systemic, regional)	Arterial hypertension [1]	AT_1R antagonists (sartans), ACE inhibitor, Ca^{2+} antagonist
Airway	Respiration	COPD, asthma	β-agonist
Prostate	Reproduction	Voiding symptoms, BPH	α_1-blockers, PDE5 inhibitor
Bladder	Micturition	Storage symptoms, incontinence	Anticholinergics, β_3-agonist
Kidney [1]	Renal function	Diabetes	
Corpus cavernosum [1]	Reproduction	Erectile dysfunction	PDE5 inhibitors

Table 1: Smooth muscle, function and diseases. [1]vascular smooth muscle, [2]plus pulmonary hypertension, portal hypertension (liver cirrhosis)

More specifically, *vascular smooth muscle* in blood vessels and of pericytes is induced by neurotransmitters, humoral and paracrine factors, including noradrenaline (inducing contractions via α_1-adrenoceptors), endothelins, angiotensin-II (inducing contractions via angiotensin-II type 1 receptors, AT_1R), thromboxane A_2, serotonin, and several others and in addition to mechanical stimuli. Increased smooth muscle tone in walls of resistance vessels contributes to increased blood pressure and increased systemic vascular resistance in arterial hypertension. Medications are applied to reduce vascular smooth muscle tone by preventing angiotensin-II-induced contractions (sartans, ACE inhibitors, calcium antagonists) or by decreasing blood flow by reducing cardiac output (β-blockers). In addition to arterial hypertension, further conditions related to abnormal increases of vascular smooth muscle tone are of high clinical relevance as well. Thus, impaired kidney function represents a common, and severe complication of diabetes, and is caused by increased pressure of the intrarenal vasculature. In liver cirrhosis, increased intrahepatic vascular resistance causes portal hypertension, together with prehepatic, splanchnic vasodilation, finally resulting in formation and life-threating bleeding of esophageal varices. Pulmonary hypertension is a life-threating condition. Phosphodiesterase-5 (PDE5) inhibitors were recently approved for medical treatment of pulmonary hypertension, and may act by vasorelaxtion of pulmonary arteries. Previously, PDE5 inhibitors were available for treatment of erectile dysfunction, where they improve erections by inducing vasorelaxation in the corpus cavernosum. *Airway smooth muscle* contraction is induced by cholinergic (muscarinic) receptors, while activation of β-adrenoceptors induce relaxation. Accordingly, β-adrenoceptor agonists represent the first line treatment of obstructive lung diseases, including asthma and chronic obstructive pulmonary disease (COPD). In the lower urinary tract, cholinergic (muscarinic) smooth muscle contractions in the *bladder wall* cause bladder emptying during micturition. Abnormal, unvoluntary contractions cause urgency, or incontinence in advanced stages. Consequently, anticholinergics and β_3-agonists are applied for medical treatment, which may both inhibit bladder smooth muscle contractions. In the *prostate*, increased smooth muscle

tone may compress the urethra in benign prostatic hyperplasia (BPH), what impairs bladder emptying and voiding. At least partially, prostate smooth muscle contraction is induced by α_1-adrenoceptors, so that α_1-blockers emerged to the first line option of medical treatment of prostate-related voiding symptoms. Smooth muscle contractions in the *gastrointestinal tract* occur cholinergic.

Individual, clinical, and economic consequences of these diseases are enormous (table 2). Worldwide, cardiovascular disease, obstructive lung diseases, and lower urinary tract symptoms affect billions of patients. Annual expenses for drug treatment amount to billions USD worldwide, while follow-up costs (including care, absence at work, etc.) range to trillions, and cardiovascular diseases account for millions of deaths each year.

Organ system	disease	Number of patients	costs
Cardiovascular system	• Arterial hypertension • „cardiovasuclar disease"	• 3.5 Mrd (ww) >110-115 mmHg • 874 Mio (ww) >140 mmHg • 9.4 Mio death/a	1 Trillion USD ww/a
	Liver cirrhosis: portal hypertension	100 Mio ww	
Lower urinary tract	LUTS: • prostate (BPH) • bladder (OAB)	1 Mrd (ww, 2018) (=OAB+BPH)	• BPH, medications: 4.8 Mrd $, ww/2009 • OAB, only USA: 65.9 Mrd $ (medical + non-medical)
Airways	Asthma, COPD	358 Mio asthma 174 Mio COPD (ww)	19.3 Mrd €/a (only asthma in Europe)

Table 2: Smooth muscle-based diseases: Prevalence, clinical & socioeconomical relevance, costs (Mio, 10^6; Mrd, 10^9, ww, wordwide).

According to the role of smooth muscle contractions for these diseases, inhibition of smooth muscle contraction is a central strategy of medical treatment. Development of most, if not all of these drugs involved organ bath studies as a critical, inevitable step in preclinical stages. In these studies, contractions of designated smooth muscle tissues (arterial rings, airway tissues, or tissue strips from the prostate or urinary bladder) are induced in vitro (by application of endogenous, contractile agonists, by neurogenic stimulation, or mechanical stimuli), so that the impact of drugs on contraction can be easily and quickly assessed (fig. 1). Apart from identification and pharmacological characterization of candidate compunds, organ bath studies allow target validation (using tissues from knockout animals), research about pathophysiology (using tissues from animal models with experimentally-induced diseases, or from patients), and finally basic research addressing general mechanisms of smooth muscle contraction and relaxation in physiology and pharmacology. Consequently, organ bath studies are performed mostly at institutions for pharmacology, physiology, internal medicine and urology, and in the pharmaceutic industry. Notably, it is rather poorly known among academic scientists, although the advantages of the organ bath technique in academic context are easily obvious. Thus, it provides rapid and functional results, is easy to learn (within two weeks), running costs and required space are quite low, and sources of tissues which can be used are manifold. Although the translational value is limited for most compounds, results can be discussed and published in any context of pathophysiology, pharmacology.

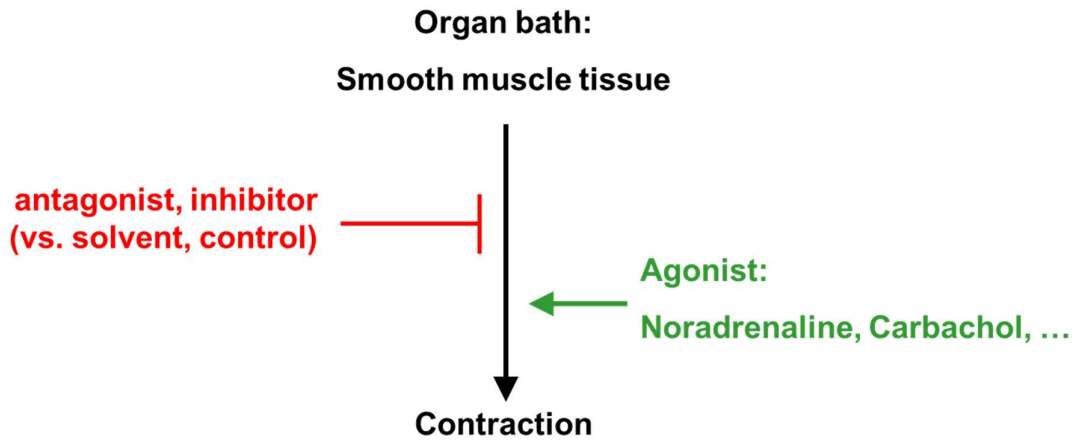

Figure 1: Principle strategy to assess inhibitory effects of candidate compounds on contraction of smooth muscle tissues in organ bath experiments, as applied in our lab.

2. Research interests and models in our lab

In our lab, located at the Department of Urology of the University Hospital Munich-Großhadern (Ludwig-Maximilians University Munich, Germany), we perform extensive organ bath studies using **human tissues from the prostate and the urinary bladder**. These tissues are obtained from radical prostatectomy and radical cystectomy, performed for prostate and bladder cancer at our department. Following surgery, sampling from organs is performed by pathologists, and tissues are examined in the organ bath at the same day. This research is performed in the context of **lower urinary tract symptoms (LUTS),** which represent the most important clinical condition in non-oncologic urology. LUTS occur in two forms, including voiding symptoms caused by the prostate, and storage symptoms caused by the urinary bladder.

Voiding symptoms (alternatively termed „obstructive symptoms“) are caused by **benign prostatic hyperplasia (BPH)**, where increased prostate smooth muscle tone and enlargement of the prostate may both contibute to compression of the urethra, resulting in impaired bladder emptying and voiding (fig. 2). Accordingly, symptoms („LUTS suggestive of BPH“) are characterized by weak or interrupted urinary flow during micturition, incomplete bladder emptying or the feeling of incomplete emptying, and delayed onset of voiding. First-line options for medical treatment include α_1-adrenoceptor antagonists (α_1-blockers) and the phosphodiesterase-5 inhibitor tadalafil, which inhibit prostate smooth muscle contraction and are believed to improve symptoms by smooth muscle relaxation in the prostate. However, their efficacy is limited to improvements around 50 %. Consequences are discontinuation of medication due to disappointing efficacy, disease progression, complications, and finally hospitalization and surgery. In our organ bath experiments using human prostate tissues, we aim 1) to uncover reasons for the limited efficacy of available medications, and 2) to identify new compounds showing inhibition of prostate smooth muscle contraction.

Storage symptoms (alternatively termed „irritative symptoms“) are caused by an **overactive bladder (OAB)**, where involuntary, exaggerated contractions of smooth muscle in the urinary blader wall (detrusor) cause urgency, and incontinence in advanced stages (fig. 2). Under normal conditions, bladder emptying during micturition is caused by cholinergic detrusor contractions (fig. 2). Consequently, but although detrusor overactivity in OAB is presumably caused by non-cholinergic contrations, medical treatment is based on muscarinic receptor antagonists (antimuscarinics). As an alternative, β_3-adrenoceptor agonists are available and act by inhibition of detrusor contractions as well. However, both options show insufficient improvements, which is paralleled by unbalanced side effects, resulting in discontinuation rats as high as 90 % one year after first prescription. In our organ bath experiments using human bladder tissues, we aim to identify new compounds showing inhibition of detrusor smooth muscle.

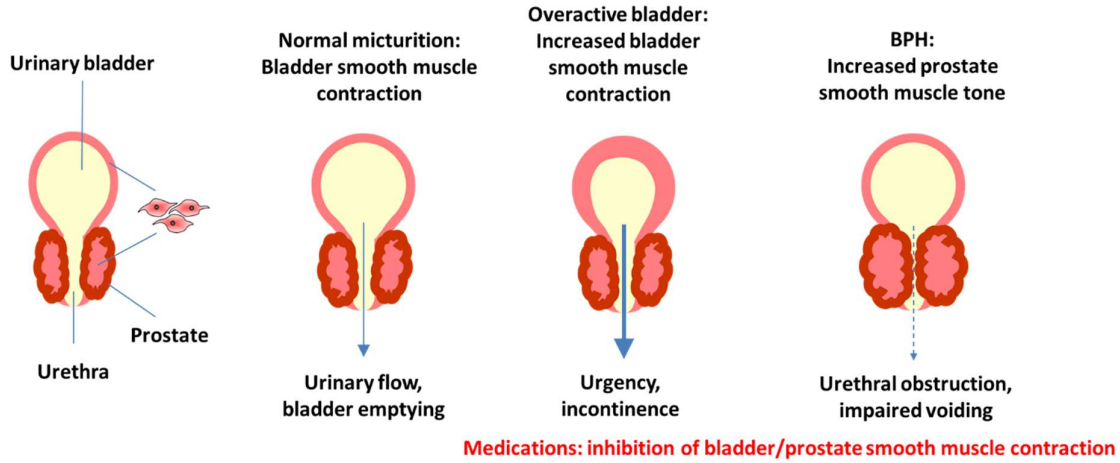

Figure 2: Micturition and lower urinary tract symptoms (LUTS). LUTS include prostate-related voiding symptoms, and bladder-related, storage symptoms. In humans, the prostate surrounds the urethra, and normal micturition is caused by smooth muscle contractions in the bladder wall (detrusor). Consequently, urethral compression due to increased prostate smooth muscle tone and enlargement in BPH impairs voiding, while unvoluntary detrusor contractions in OAB cause weak-bladder symptoms. According to the role of smooth muscle contractions for both kinds of LUTS, medical treatment aims to inhibit contractions in the prostate and bladder. However, available options are characterized by insufficient efficacy. In organ bath studies with human prostate and bladder tissues, we aim to identify novel compounds for inhibition of smooth muscle contractions, and to understand the reasons for the currently still limited efficacy.

During the covid pandemic, the availability of human lower tract tissues became limited for us, due to security reasons related to access to operation room, and due to reduced numbers of cancer surgeries. In order to continue with organ bath-based research, we changed to investigation of **vascular smooth muscle contraction** in 2020. We use **renal, interlobar and coronary arteries obtained from pig kidneys and hearts**, which were easily available from a local butcher. In these studies, we examined the effects of compounds, from which we expected an inhibition of vascular smooth muscle contraction and which showed inhibition of contractions in our previous studies using lower urinary tract tissues. Investigation of their effects in vascular smooth muscle appeared interesting to us for several reasons. First, some of the compounds showing activity in the lower urinary tract emerged to attractive candidates

being tested in clinical trials addressing lower urinary tract symptoms, where cardiovascular side effects are important and may limit drug treatment. Secondly, some of the drugs showing activity in lower urinary tract tissues in our previous studies point to an involvement of targets, which have never been described before in the context of smooth muscle contraction of any organ, so that it is meaningful and interesting to test these compounds in other smooth muscle types, in addition to the lower urinary tract. Third, using pig arteries from kidneys and hearts in organ bath experiments was an attractive approach to continue with pharmacological research in this situation.

3. Protocols and procedures

3.1 Principle strategy

Most of our organ bath experiments are performed to examine the effects of novel compounds (e. g. kinase inhibitors or inhibitors for monomeric GTPases) on smooth muscle contractions and follow a standardized protocoll, which is designed to examine the possible inhibition of contraction by these compounds. The workflow and essential steps of experiments aiming to assess inhibitor effects on agonist-induced smooth muscle contractions are shown in fig. 3. These steps include 1) mounting of tissues, 2) equilibration period to obtain a stable and defined pretension, 3) assessment of highmolar potassium chloride- (KCl-)induced contraction, 4) washout of KCl, 5) application of inhibitors and solvent (for controls without inhibitors) and incubation, and 6) construction of concentration response curves for contractile agonists or of frequency response curves by electric field stimulation (EFS).

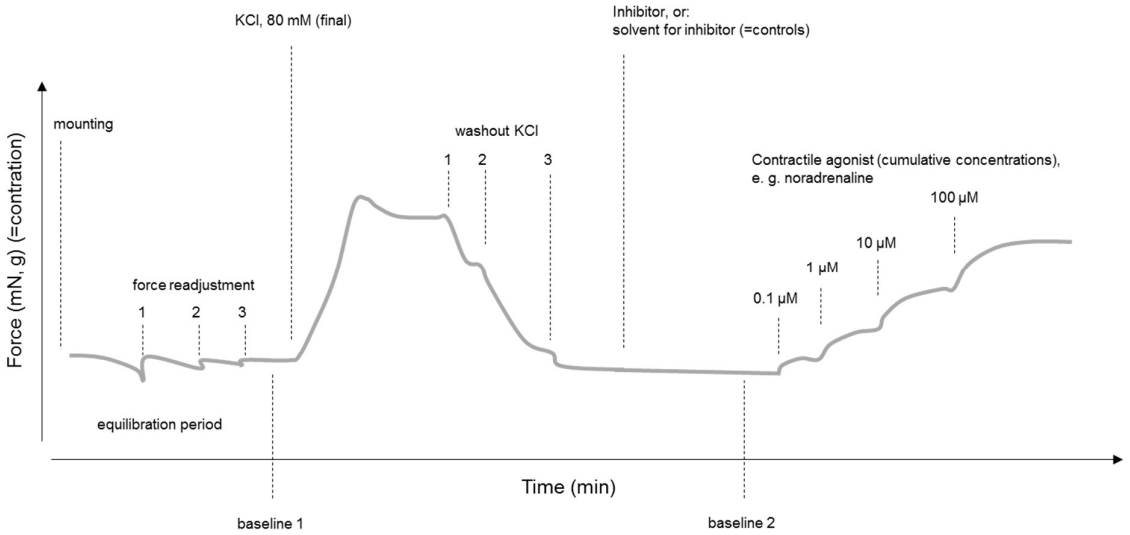

Figure 3: Schematized organ bath experiment, designed to explore drug effects on agonist-induced contractions. One of such experiments takes approximately 90-120 min. Alternatively to agonist-induced contractions, EFS-induced contractions can be examined at the end.

3.2 Normalization to highmolar KCl

Normalization of agonist- and EFS-induced to KCl is required in our experiments, where tissues showing high individual heterogeneity are used. In other experiments, e. g. if tissues from normal rats, without experimentally-induced diseases or long-term drug treatment are used, normalization may not be necessarily required, and contractions may be directly expressed in force (or mass) units (i. e., mN or gram), as long as the tissues in different channels have the same (or similar) sizes. However, human tissues are characterized by high heterogeneity. Apart from any natural individual variation, souces of tissue heterogeneity include the degree of disease, inflammation, and tissue composition, by different cell types and non-cellular components, such as binding tissue or collagens.

Exemplarily, heterogeneities of human prostate tissues used in our studies can be easily demonstrated by Western blot analyses. Fig. 4 shows, how the content of different markers varies between prostate tissues from different patients. The varying content of prostate-specific angiten (PSA) reflects the highly divergent degree of BPH. The varying content of calponin illustrates strong variations in smooth muscle content, and the cytokeratin variations point to a varying content of glandular compartments between the tissues. Clearly, varying degree of smooth muscle content, but also varying degree of inflammation due to BPH will directly affect contractility. Thus, if a comparison of agonist-induced contractions between tissues treated with inhibitor or solvent is aimed, in order to assess the putative inhibition of contraction by the drug, knowing the overall contractility of the tissue sample is important, what can be obtained by assessment of KCl-induced contractions. High concentrations of potassium induce receptor-independent contractions, so that KCl-induced contraction of a tissue sample depends on size, smooth muscle content, and condition (smooth muscle phenotype, disease, traumatization during preceding surgery or sampling, etc.).

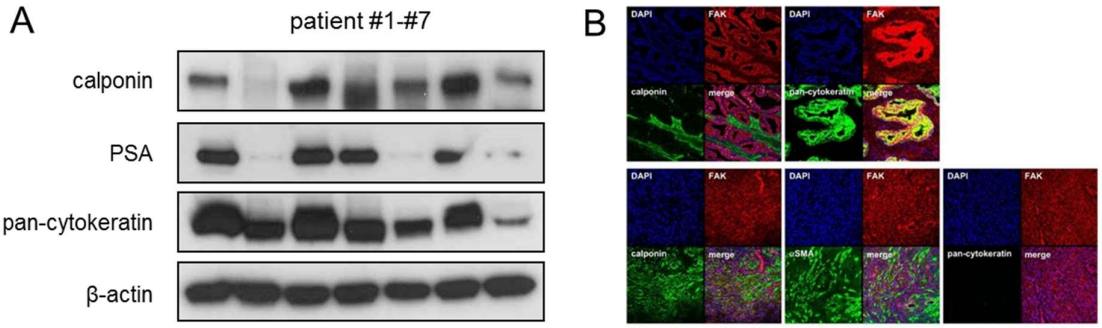

Figure 4: Heterogeneity of human prostate tissues. Varying smooth muscle content is reflected by different content of calponin, shown by Western blot analyses in A. Different smooth muscle content may impart different overall contractilities of tissue samples, so that normalization to this content is required to compare agonist-induced contractions between different samples (e.g., if comparisons between drug-treated and control groups are aimed). Prostate tissues with typical architecture include glands (with pan-cytokeratin-positive epithelia), which are embedded in the stroma, where smooth muscle cells are the predominant cell type (upper panels in B). However, tissue composition varies, even within the same organ, so that areas containing only stroma and smooth muscle are common (lower panels in B).

The need of normalization becomes again obvious by realization of the following scenarios. Thus, if two samples from the same organ have the same smooth muscle content in the same condition, the agonist-induced contractions may be similar, and inhibition of these contractions by drugs will result in lower forces and easy to see (fig. 5). However, in human (and non-human) tissues, the smooth muscle content may even differ between different samples from the same human organ, in particular in the prostate, which is composed by smooth muscle and glands. Consequently, it is clear, that contractions may be lower in a drug group, if the smooth muscle content is low, or if the smooth muscle is defective. This may pretend an inhibition, even if there is no inhibition (fig. 5). Vice verca, an inhibition by a drug can be covered, if the smooth muscle content in the drug-sample exceeds the content in the control sample. All these problems can be overcome by normalization of agonist-induced and neurogenic contractions to potassium-induced contractions.

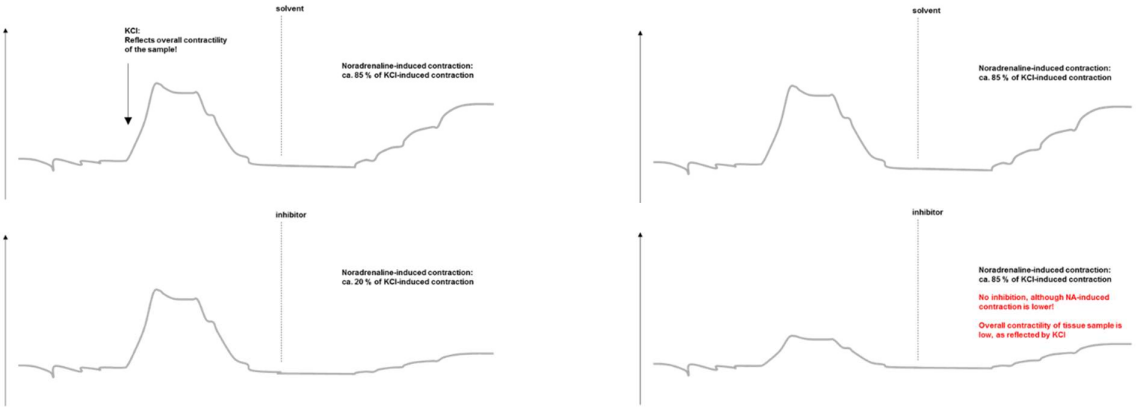

Figure 5: Realization of the need for normalization, if examination of drug effects on agonist-induced contractions are aimed. Left: if the overall contractilities are similar in samples of the control and drug group, an inhibition by the drug becomes easily obvious. Right: if the overall contractility of the sample in the drug group is lower than in the control group, this may pretend an inhibition of agonist-induced contraction even if there is no such inhibition.

Alternatively to these procedures, variations can be eliminated if two curves are recorded with each samples, i. e. by two cycles of concentration response curves with each tissue. First, agonist- or EFS-induced contractions are induced without drug and solvent in all samples,. Subsequently and after washout of agonists, solvent (controls) or drug are added, and a second concentration response curve is recorded. Finally, second curves are compared with first in both groups. Although this is fact possible and we published such results, we prefer the other option described above, as the alternative is prone to artefacts and adverse reactions (e. g., if first and second curves differ even in the control groups, what needs to be explained).

3.3 Protocols and procedures

Specifically, isometric tension measurements in our **experiments addressing drug effects on agonist- and EFS-induced smooth muscle contractions follow the protocolls** below, using the myograph model 720M (DMT, Denmark):

- Switching on the device, including heating (37 °C), washing chambers with Krebs-Henseleit solution (KH) (for preparation of KH, see below), filling with KH (10 ml/chamber, according to the chamber volume of and heating to 37 °C.
- Mounting of tissues: solid tissues (prostate, bladder) are spiked on the pins/needles, while tissues with ring structure from tube-like organs (arterial rings from renal interlobar or coronary arteries, or rings from ureter) are placed so, that they surround both pins. In our experiments, all four chambers of one device are filled with samples from the same tissue.
- Adjustment of first pretension, by increasing the distance between mounting pins, until the desired pretension is attained (4.9 mN for human prostate, human bladder wall, human trigone, 9.8 mN for pig interlobar arteries, 19.6 mN for pig coronary arteries).
- Within the next 45 min: readjustment of desired pretension, three times, as the initial pretension will spontaneously decline. Readjustment results in a mostly stable pretension after this so-called equilibration period.
- Start recording of tension, using LabChart, by recording of a baseline (ca. 5-10 min, =the tension before adding KCl).
- Add 400 µl of 2 M KCl solution (resulting in a final potassium concentration of approximately/little bit more than 80 mM).
- Wait, until a stable plateau contraction has been reached; i.e. tension will rise, reach a peak, and subsequently either remain ±stable or again decline, but then reach a ±stable tension.
- Wash, three times with fresh KH, until a stable resting tension has been obtained again, ranging ±at the first baseline tension.
- Add drug to two of the four chambers, and equivalent amounts of solvent (as controls without drugs) to the other two chambers.
- Waiting 30 min („incubation with drug/solvent“).
- Construction of concentration response curves for contractile agonists, or of frequency response curves by EFS. For concentration response curves, add agonist dilutions in cumulative amounts, according the pipetting plans below. If now reactions occur within approx. 5 min (e. g., following administration of the lowest concentrations), continue with the next dilution/concentration. Obtaining full contraction levels may take aournd 5 min using α_1-adrenergic agonists, or longer (10-20 min) for other agonists (U4669, endothelins).

Our **concentration response curves** are constructed according to following plans (referring to a volume of 10 ml in organ bath chambers) (grey: these high concentrations are only applied, if we assume that a competitive antagonism may occur):

α_1-adrenergic agonists (noradrenaline, phenylephrine, methoxamine), muscarinic agonists (carbachol, methacholine), serotonin		
Concentration of added dilution	Added volume	Final concentration in organ bath chamber
0.1 mM	10 µl	0.1 µM
	20 µl	0.3 µM
1 mM	7 µl	1 µM
	20 µl	3 µM
10 mM	7 µl	10 µM
	20 µl	30 µM
	70 µl	100 µM
	200 µl	300 µM
	700 µl	1000 µM

U46619		
Concentration of added dilution	Added volume	Final concentration in organ bath chamber
0.01 mM	10 µl	0.01 µM
	20 µl	0.03 µM
0.1 mM	7 µl	0.1 µM
	20 µl	0.3 µM
1 mM	7 µl	1 µM
	20 µl	3 µM
10 mM	7 µl	10 µM
	20 µl	30 µM

endothelin-1, -3		
Concentration of added dilution	Added volume	Final concentration in organ bath chamber
0.4 mM	2.5 µl	0.1 µM
	5 µl	0.3 µM
	17.5 µl	1 µM
	50 µl	3 µM

Construction of frequency response curves by EFS is performed using a generator (CS4, DMT, Denmark) and electrodes from the same manufacturer, and using the software MyoPULSE for setting the generators parameters. Electrodes are placed to chambers, so that mounted tissue strips are then placed between the electrodes. Following connection to the generator and choosing the „continuing pulse“ mode and monopolar pulse shape, five trains

of pulses are applied, using different frequencies (2, 4, 8, 16 and 32 Hz), a constant pulse voltage of 20 V and a pulse width of 1 msec. The duration of applied pulse trains depends on the tissue response. Thus, pulse trains are applied, until the tension starts to decrease again (i. e., following the initial EFS-induced descrease), what takes 10-20 sec in most cases. Subsequently, and following an interval of 30 sec, the next train with higher frequency is applied, until the highest frequency has been applied.

Assessing drug effects on KCl-induced contractions is sometimes asked during peer review, and performed according to the following protocoll:

- Induction of KCl-indued construction („1st KCl"), as described above and following adjustment of pretension.
- Washout (see above).
- Administration of solvent or drug.
- Again induction of KCl-induced contraction („2nd KCl"), 30 min following administration of drug or solvent.

Finally, the 2nd KCl (=tension after KCl minus baseline) is expressed as percentage of the 1st KCl (=tension after KCl minus baseline). For presentation and discussion of results, 2nd KCl may be compared with the first KCl, or the 2nd KCl-induced contractions can be compared with each other. We prefer the second method, as the 1st KCl is automatically always 100% (with an SD =0), prepresenting a kind of normalization, which is not in line with requirements for all pharmacological journaly. Thus, the second method will result in data such as „2nd KCl = 123% after solent and 134 % after drug" (with SDs).

3.4 Stock solutions, dilutions

Stock solutions and dilutions of α_1-adrenergic agonists, muscarinic agonists, serotonin, and endothelins are prepared with water. Stock solutions of U49919 are prepared with ethanol. Stock solutions of drugs are prepared according to information in data sheets provided by manufacturers (mostly DMSO). Stock solutions and dilutions of α_1-adrenergic and muscarinic agonists are freshly prepared before experiments (and can be used for one day, if tubes are stored on ice during). Stock solutions of other agonists and of drugs are frozen as aliquots, with volumes of aliquots corresponding to the amounts required for one experiment (including four channels), and are thawed before the experiment.

10 mM stock solutions of cheap agonists, which are acquired in large amounts (>100 mg) (i. e., α_1-adrenergic and muscarinic agonists), are prepared as follows:

- Weigh in any random amount, ranging around 1-3 mg (if the compound has an Molecular weight (MW) of something like 200-300 g/mol).
- Multiply the weighed-in mass (measured in mg) by one-hundredth of the MW of the compound.
- Result =volume in ml, which you have to add, to obtain a solution of 10 mM.
- Example: your weighed in 1.78 mg phenylephrine. Phenylephrine has a molecular mass of 211 g/mol. Thus, to obtain a stock solution of 10 mM phenylephrine with your random amount, you have to add 1.78/2.11 = 0.844 ml = 844 µl of water.
- Prepare dilutions (1:10), as required and according to the plans above.

10 mM stock solutions of drugs, which are supplied in small amounts (e. g., 10 mg) should be prepared by the same way. Alternatively, and to simplify the procdure, the content of flasks containing 10 mg (at least according to the manufacturers labeling) may be dissolved at once. In doing so, the required amount of solvent can be calculated by same formula as described above, assuming the manufacturer's information is right (i. e.: content = 10 mg). While dissolving the whole amount at once without own weighing my result in some (but sometimes acceptable) inaccuracies, this is the only possible option for U4669, which is not solid, but a viscid to pasty compund. According to the molecular weight of 350,49 g/mol for U46619, 2.853 ml of ethanol (≥99%) need to be added to flasks containing 10 mg U4669, resulting in a stock solution of approximately 10 mM.

Endothelins are expensive, supplied in small amounts, and have very high molecular weights (because they are peptides). To a vial containing 1 mg (according to the manufacturer's information/labeling) endothelin-1 or -3, we add 1 ml of water, resulting in a stock solution of 0.4 mM. In addition to the plan described above, one dilution (to 0.04 mM) is required, if lower concentrations are aimed to be included in concentration response curves. Endothelin-2 has a lower solubility, so that we dissolve 1 mg in 3 ml of water, and the plan has to be adapted accordingly (here not described again in detail).

Other agonists and drugs, not mentioned here, are prepared by the same approaches and applied by equivalent plans.

Krebs Henseleit solution used in our lab is prepared by ourselves, as follows.

- Mix 40 ml stock solution „KH1" with 920 ml water.
- Bubble for 20 min with carbogen (95% O_2, 5% CO_2), while the flask is placed in a water bath (37°C).
- Add 40 ml stock solution „KH2", and 1.5 g glucose.
- Continue carbogen bubbling and storing at 37°C.
- If the solution becomes hazy (slightly milky, whitish, due to fall out of calcium carbonate), discard and start new. Mostly, this results from insufficient carbogen supply.
- This solution may used for one day. Prepare fresh each day.
- Preparation of KH1: 172.5 g NaCl, 8.75 g KCl, 9.36 g $CaCl_2x2H_2O$, 4.05 g KH_2PO_4, 7.34 g $MgSO_4X7H_2O$, aqua dest. ad 1000 ml.
- Preparation of KH2: 52.5 g $NaHCO_3$, aqua dest. ad 1000 ml.
- KH1 and KH2 can be stored for weeks, but at 4°C.

3.5 Handling of tissues

Custodiol® is an organ-protective solution, designed for transport and storage of organs in the context of organ transplantation. We use custodiol for transport of prostates and bladders from the surgery room to the pathology department (where sampling is performed), for any subsequent transport and storage of tissues, and preparation/cutting of samples directly before the organ bath experiment. Any temporary storage of tissues between sampling and

experiment in custodiol is performed at 4°C. For experiments with pig arteries, kidneys and hearts are transported from the butcher to the lab, where arteries are prepared as soon as possible in custodiol solution. Subsequently, arterial tissues are temporarily stored at 4°C in custodiol solution, until used in experiments.

3.6 Calculation of results

Together, the calculation of results includes three steps (fig. 6):

1. calculation of agonist- or EFS-induced tensions in each channel, for each agonist concentration or each frequency;
2. mean values of channels in each experimiment, resulting in double determinations for both groups (control and drug) in each independent, single experiment;
3. means of all independent experiments performed for one series, resulting in concentration response curves, or frequency response curves.

Optional, EC_{50} and E_{max} values can be calculated, by curve fitting of all single experiments.

For reasons explained above, all agonist- and EFS-induced contractions in our studies are expressed percentage of KCl-induced. Consequently and more specifically, results in step 1) are calculated as follows:

- Determine tension after stable, KCl-induced contraction (see above) in each chanel (units may be anything, e. g. mN or gram), and substract the baseline tension before adding KCl. Resulting value is the KCl-induced contraction.
- Determine tensions after each agonist concentration or after each frequency during EFS, and substract the baseline tension before starting the concentration response curves or frequency response curves.
- Calculate the tensions at each agonist concentration or frequency (tension minus baseline) as percentage of KCl-induced contraction (tension at KCl minus baseline), for each channel: (agonist tension – baseline 2) / (KCl tension – baseline 1) x 100.

Then, perform steps 2) and 3) as described above. Diagrams with concentration response curves or frequency response curves (including standard deviations) can be created using Excel or GraphPrism. In concentration response curves, logarithmic scaling at the x-axis is required for agonist concentrations. Typically, diagrams in our study contain two curves, including the control curve without drug and the curve recorded in the presence of drug, which were both obtained using tissues from the same organs (paired samples). Using GraphPrism, EC_{50} values and E_{max} values for agonist-induced contractions can be calculated for each independent experiment. Mean values and standard deviations, and mean differences with 95% conficence intervals can be calculated and may presented as scatter plots, together with concentration response curves.

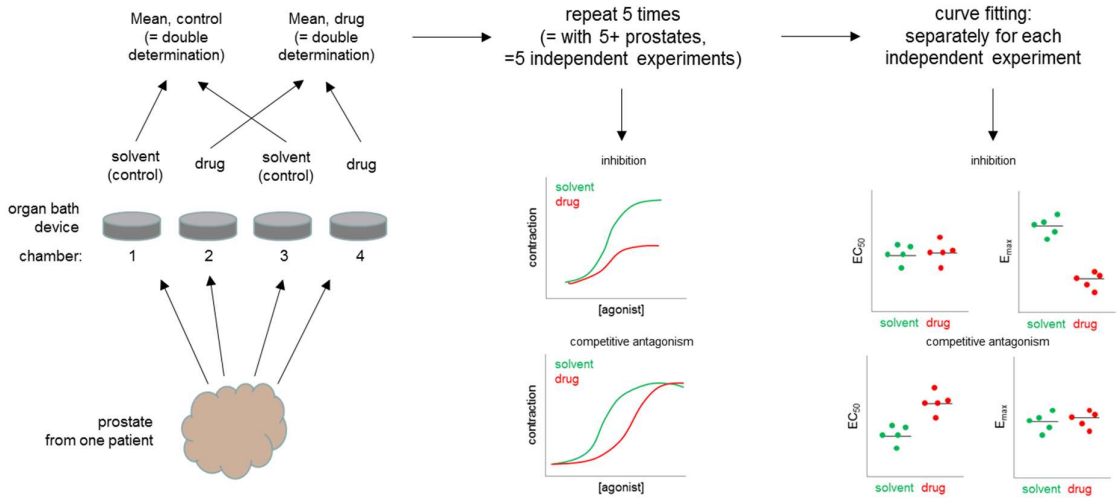

Figure 6: Schematized allocation of tissues, group formation, and steps for presentation of data in our studies. In each single (independent) experiments, effects of drugs and solvent are assessed in double determination. This is repeated at lest five times, resulting in group sizes of n=5 and paired values. Agonist-induced contractions are shown as concentration response curves, EFS-induced contractions as frequency response curves (not shown here), and EC_{50} and E_{max} values (calculated separately for each single experiment by curve fitting) as scatter plots. Examples show competitive antagonism (including rightshifts of curves, increased EC_{50} values and unchanged E_{max}), and inhibition without features of competitive antagonism („inhibition“).

3.7 Remarks to data presentation, statistical analyses, and reporting of procedures

Statistical analyses, correct presentation of data, and appropriate descriptions of data analyses, group formations, study design and so on are emerging topic, currently undergoing seminal changes, in order to improve the reproducibility of preclinical findings by novel guidelines and publication ethics. In fact, irreproducibility of scientific results is an increasing concern, as it impairs drug development and causes enormous financial damage, ranging to billions of USD per year alone in the USA. Consequently, a novel programm aiming to improve the reproducibility of preclinical research has been raised by the NIH, which is increasingly implemented by scientific journals. Thus, new guidelines for reporting of data, data analysis, statistics and study design are currently promoted by a NIH committee, and are now embedded as requirements for publication and in author instructions by an increasing number of journals. These guidelines follow two overall strategies to improve reproducibility in preclinical research: First, they aim to increase the transparency of data and procedures. Secondly, authors are encouraged to describe in detail their procedures regarding study design, group formation, data analyses. Accordingly, large attention should be given on effect sizes, and the focus should be moved away from p values. Thus, reporting and presentation of data is now even possible without p values (see examples in references). Specifically, we strongly recommend to declare studies as „explorative“ instead of hypothesis-testing, as explained in available guidelines. The advantage is, that any p value is then only descriptive (or may be even omitted), and that flexible group sizes are possible (provided, these procedures are adequately explained in the methods section). Although it is commonly assumed, that science is always

„hypothesis-testing“, most preclinical studies are not hypothesis-testing at all, but are highly explorative.

These new guidelines are published in particular by pharmacological journals (see references), which implement these guidelines by including these terms in their instructions for authors for manuscript preparation and are consequently required for publication in these journals. We have highly positive experiences with these guidelines. Examples, how these can be easily and pragmatically implemented, including appropriate descriptions of all procedures in the „Data Analysis“ sections and information, which tests can be used for which data sets, can be found in our recent publications.

4. Recommended References

Seminal review articles, describing **fundamental mechanisms of smooth muscle contraction**:

- Somlyo AP, Somlyo AV. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev 2003;83(4):1325-58.
- Somlyo AP, Somlyo AV. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J Physiol 2000;522 Pt 2(Pt 2):177-85.

Own review articles describing **mechanisms of smooth muscle contraction in disease-specific, or other specific context**:

- Hennenberg M, Trebicka J, Sauerbruch T, Heller J. Mechanisms of extrahepatic vasodilation in portal hypertension. Gut 2008;57(9):1300-14.
- Hennenberg M, Stief CG, Gratzke C. Prostatic α1-adrenoceptors: new concepts of function, regulation, and intracellular signaling. Neurourol Urodyn 2014;33(7):1074-85.
- Li B, Wang R, Wang Y, Stief CG, Hennenberg M. Regulation of smooth muscle contraction by monomeric non-RhoA GTPases. Br J Pharmacol 2020;177(17):3865-3877.

Articles containing **novel guidelines for reporting of data and data analyses**, and the need of **normalization of contractions**:

- Michel MC, Murphy TJ, Motulsky HJ. New Author Guidelines for Displaying Data and Reporting Data Analysis and Statistical Methods in Experimental Biology. Mol Pharmacol 2020;97(1):49-60.
- Erdogan BR, Karaomerlioglu I, Yesilyurt ZE, Ozturk N, Muderrisoglu AE, Michel MC, Arioglu-Inan E. Normalization of organ bath contraction data for tissue specimen size: does one approach fit all? Naunyn Schmiedebergs Arch Pharmacol 2020;393(2):243-251.
- Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA, Gilchrist A, Hoyer D, Insel PA, Izzo AA, Lawrence AJ, MacEwan DJ, Moon LD, Wonnacott S, Weston AH, McGrath JC. Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 2015;172(14):3461-71.
- Motulsky HJ, Michel MC. Commentary on the BJP's new statistical reporting guidelines. Br J Pharmacol 2018;175(18):3636-3637.

Own articles, giving **examples for implemenation of latest guidelines for reporting of data and data analysis**, mostly addressing drug effects on smooth muscle contractions:

- Spek A, Li B, Rutz B, Ciotkowska A, Huang R, Liu Y, Wang R, Strittmatter F, Waidelich R, Stief CG, Hennenberg M. Purinergic smooth muscle contractions in the human prostate: estimation of relevance and characterization of different agonists. Naunyn Schmiedebergs Arch Pharmacol 2021, in press.
- Wang R, Yu Q, Wang X, Li B, Ciotkowska A, Rutz B, Wang Y, Stief CG, Hennenberg M. Rac1 silencing, NSC23766 and EHT1864 reduce growth and actin organization of bladder smooth muscle cells. Life Sci 2020;261:118468.
- Li B, Wang X, Wang R, Rutz B, Ciotkowska A, Gratzke C, Herlemann A, Spek A, Tamalunas A, Waidelich R, Stief CG, Hennenberg M. Inhibition of neurogenic and thromboxane A 2 -induced human prostate smooth muscle contraction by the integrin α2β1 inhibitor BTT-3033 and the integrin-linked kinase inhibitor Cpd22. Prostate 2020;80(11):831-849.
- Li B, Yu Q, Wang R, Gratzke C, Wang X, Spek A, Herlemann A, Tamalunas A, Strittmatter F, Waidelich R, Stief CG, Hennenberg M. Inhibition of female and male Human detrusor smooth muscle contraction by the Rac Inhibitors EHT1864 and NSC23766. Front Pharmacol 2020;11:409.
- Wang X, Wang Y, Gratzke C, Sterr C, Yu Q, Li B, Strittmatter F, Herlemann A, Tamalunas A, Rutz B, Ciotkowska A, Waidelich R, Liu C, Stief CG, Hennenberg M. Ghrelin aggravates prostate enlargement in rats with testosterone-Induced benign prostatic hyperplasia, stromal cell proliferation, and smooth muscle contraction in human prostate tissues. Oxid Med Cell Longev 2019;2019:4748312.
- Li B, Wang X, Rutz B, Wang R, Tamalunas A, Strittmatter F, Waidelich R, Stief CG, Hennenberg M. The STK16 inhibitor STK16-IN-1 inhibits non-adrenergic and non-neurogenic smooth muscle contractions in the human prostate and the human male detrusor. Naunyn Schmiedebergs Arch Pharmacol 2020;393(5):829-842.