Human Detrusor Smooth Muscle Metabolism and the Pathophysiology of Detrusor Instability in Women

JOANNE SUSAN HOCKEY

A Thesis presented to the University of London

in fulfilment of the regulations for the M.D. degree.

The Department of Gynaecology

The Elizabeth Garrett Anderson Hospital and

The Institute of Urology and Nephrology, U.C.L. Hospitals.

1999

ABSTRACT

The pathophysiology of abnormal detrusor function and the development of unstable detrusor contractions is a significant clinical problem, particularly in women. The treatment available is often ineffective and is associated with many uncomfortable side effects. Often the unstable contractions occur together with damaged bladder neck support following pelvic floor damage sustained in childbirth. In women these unstable contractions are not usually associated with bladder outlet obstruction such as occurs in men with prostatic hypertrophy. Most of the human and animal research has concentrated on abnormal bladder function as a consequence of bladder outlet obstruction and this study sought to investigate possible mechanisms of the development of abnormal bladder function in the absence of such obstruction.

Abnormal detrusor smooth muscle metabolism as a result of bladder ischaemia has been proposed as a pathophysiological process contributing to the development of abnormal bladder function and unstable bladder contractions. In this work the role of cellular hypoxia, specifically, in this pathophysiological process was studied in vitro using biopsies from human stable and unstable bladders, either as isolated multicellular preparations or isolated single myocytes.

Cellular hypoxia was found to reduce the force of a carbachol induced contraction in isolated detrusor muscle strips, the biopsies from control bladders being more affected than those from unstable bladders. Isolated single cell experiments demonstrated that cellular hypoxia attenuated the intracellular Ca^{2+}-transient released from the sarcoplasmic reticulum in response to muscarinic stimulation, however cellular hypoxia had no effect on the surface membrane electrophysiological properties.

ACKNOWLEDGEMENTS

Contents

Discussion 150

Index of Figures

11

Index of Tables

List of Abbreviations

The following convention applies to all ionic species

Ca	Total calcium. This term is also used to describe the concentration of calcium salts e.g. $CaCl_2$.
[Ca]	Total calcium content.
Ca^{2+}	Free ionised calcium.
$[Ca^{2+}]$	The concentration of free ionised calcium.

ATP	Adenosine triphosphate.
BCECF	2',7'-bis(carboxyethyl)5-(6)-carboxyfluorescein.
$[Ca^{2+}]_i$	Intracellular calcium ion concentration.
$[Ca^{2+}]_o$	Extracellular calcium ion concentration.
CO_2	Carbon dioxide.
Cyclic AMP	Cyclic adenosine monophosphate
EC_{50}	Half maximal concentration.
E-C coupling	Excitation-contraction coupling.
EGTA	Ethylene glycol bisaminoethylether tetracetic acid.
Fura-2 AM	Fura-2 acetoxymethyl ester.
H^+	Hydrogen ion.
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.
I.C.S.	International Continence Society.
IP_3	Inositol triphosphate.
K^+	Potassium ion.
K_d	Dissociation constant.
ms	Milliseconds.
n	Number of observations.

Na^{2+}	Sodium ion.
NaCN	Sodium cyanide.
pEC_{50}	-log EC_{50}.
PO_2	Partial pressure of oxygen.
pH_i	Intracellular pH.
PMT	Photomultiplier tube.
r	Correlation coefficient.
R	Ratio of emitted wavelengths.
rps	Revolutions per second.
sd	Standard deviation.
SR	Sarcoplasmic reticulum
TTX	Tetrodotoxin

INTRODUCTION

The urinary bladder has to equate the continuous production of urine produced by the kidney with the need to expel urine from the body at an appropriate time in a suitable place. This dual role requires the bladder to be both highly compliant to enable it to store urine without a high rise of intravesical pressure and also to be highly contractile, capable of generating sufficient pressure to empty completely. The lower urinary tract also needs an efficient sphincteric mechanism, which in women is dependent on healthy surrounding anatomical structures such as the pelvic floor musculature in order to function successfully. Abnormalities of the lower urinary tract, the surrounding structures or its controlling mechanism may lead to distressing symptoms which can dramatically reduce that individual's quality of life.

Normal lower urinary tract function can be divided into the passive filling and active voiding phases. In the passive phase urine fills the bladder and is stored without an appreciable rise in pressure, against a closed urethral sphincter; during the active phase the detrusor muscle contracts, causing an increased intravesical pressure exceeding the pressure in the relaxed urethral sphincter, and rapidly expels urine. The relationship between the volume of urine in the bladder and the intravesical pressure in the storage phase, together with the relationship of detrusor voiding pressure and urinary flow through the urethra can be studied by urodynamic investigation.

Urodynamic investigation provides valuable information about the functioning of the lower urinary tract. In the 1960's Hodgkinson noticed abnormalities of detrusor contraction in the urodynamic studies of a small group of patients who had failed to benefit from incontinence surgery and termed this detrusor dyssynergia (Hodgkinson 1963). The pivotal work was performed later by Bates (1971) who described a retrospective study of

1,400 patients examined by videourodynamics and proposed that the "unstable bladder" was a possible reason for some operative failures of incontinence surgery (Bates 1971).

In the early 1970's with the formation of what would become The International Continence Society attempts were made to standardise the terminology of urodynamic methods and the interpretation of study findings (Bates *et al* 1981). The standardisation document was published in 1988 (Abrams *et al* 1988) and has enabled comparisons to be drawn between different clinical units in the investigation, definition and treatment success of specific lower urinary tract problems.

Conventional urodynamics, involves the simultaneous measurement of vesical and abdominal pressure (and by subtraction, the detrusor muscle pressure), in relation to both bladder filling and voiding. Bladder filling may be physiological or by the introduction of fluid (usually sterile water or normal saline) through a urethral catheter. Clinical problems may be categorised in terms of the underlying urodynamic abnormality and may be subdivided into those occurring during filling and those during voiding.

Urodynamic filling or storage phase abnormalities are said to be most common in women, voiding dysfunction being more usual in men (Abrams 1997). These storage problems include, stress incontinence, sensory urgency, detrusor instability and detrusor hyper-reflexia. Voiding disturbances may be as a result of hypotonic contractions of the detrusor or urethral obstruction which may lead to incomplete emptying of the bladder which may then result in storage phase abnormalities. These storage and voiding abnormalities can be described using the I.C.S. standardised urodynamic descriptions (Abrams *et al* 1988).

Storage Disorders

a) Stress Incontinence

The term stress incontinence describes both a lower urinary tract symptom and a clinical sign. The patient will suffer from leakage of urine during episodes of increased abdominal

pressure e.g. during coughing, sneezing, lifting and during sexual intercourse. Weakness of the urethral sphincter with ineffective transmission of intra-abdominal pressure to the urethra resulting in urinary leakage without concomitant detrusor pressure rise is termed genuine stress-incontinence, the term "genuine" being applied if there are no other abnormalities during the urodynamic study.

b) Sensory Urgency

Sensory urgency is a symptom complex and describes a syndrome, of as yet unknown aetiology, characterised by the sensation of pain during bladder filling leading to frequency of micturition as a result of reduced functional bladder capacity. There are by definition, no phasic detrusor contractions during the filling phase of urodynamics.

c)Urgency and urge incontinence

Urgency and urge incontinence are distressing symptoms, incontinence being demonstrable as a clinical sign. The underlying urodynamic abnormality is an involuntary rise in intravesical pressure due either to an alteration in the pressure-volume relationship (compliance) of the bladder or an uninhibited phasic rise in pressure. Such phasic increases were termed detrusor instability (Bates 1971) in the absence of neurological disease. If neurological disease is present it is termed detrusor hyper-reflexia (Bates *et al* 1981).

Voiding disturbances

a) Hypotonic detrusor activity

This term describes the failure of the detrusor muscle to generate sufficient intravesical pressure to overcome urethral resistance to initiate, sustain and complete bladder emptying. Its clinical significance is dependent on the activity of the urethral sphincter ranging from poor flow and mild incomplete emptying in the incompetent sphincter to urinary retention if present in association with urinary sphincter dyssynergia.

b) Increased outflow resistance

Voiding disturbances may arise from an increase in the bladder outflow resistance. This resistance may be anatomical or physiological. An anatomical example is prostatic hypertrophy in the male, increased outflow resistance occurs less commonly in the female as the urethra is shorter (Masters & Ramsden 1997). A rare cause of urethral sphincter hypertrophy in some women with polycystic ovarian syndrome has also been described (Fowler *et al* 1988). This syndrome is characterised by the presence of continuous repetitive discharges on E.M.G. testing. Physiological obstruction may occur as a result of repeated conscious attempts to inhibit leakage by prolonged contractions of the pelvic floor.

c) Uncoordinated voiding

An aberration of the neuronal control of voiding may result in failure of the urethral shincter to relax when detrusor pressure is consciously elevated to allow the emptying of the bladder. This deviation is a feature of multiple sclerosis and spinal cord injury and can lead to bladder overdistension.

In clinical practice lower urinary tract symptoms are often multiple and a single diagnosis may be difficult. It has been argued that the term detrusor instability merely describes a urodynamic observation and may not imply underlying pathology (Griffiths 1998). The standardisation of lower urinary tract function produced by the International Continence Society defines the "unstable detrusor muscle as one that is shown objectively to contract, spontaneously or on provocation, during the filling phase while the patient is attempting to inhibit micturition", (Abrams *et al* 1988). Such contractions may be associated with a feeling of urgency and cause incontinence or they may be asymptomatic and of no clinical significance. Ambulatory urodynamics has demonstrated asymptomatic detrusor contractions during physiological bladder filling in 69% of clinically normal women (Van Waalwicjk-van Doorn *et al* 1991) and 72% of men with benign prostatic hypertrophy

(Robertson *et al* 1996). The diagnosis of detrusor instability should therefore be used with reference to the clinical significance of the patients symptoms and signs and should not rest with the demonstration of phasic detrusor contractions on urodynamic studies alone (Zinner 1998).

It has been stated that "the subject of detrusor instability is arguably the single most notable development of the urodynamic era" (Mundy 1988) however the interest in this condition has not yet produced effective medical management based at the prevention of the pathophysiological processes that underlie its development. The medical management of disorders of micturition is still far from satisfactory (Harriss 1995), this may be due to the difficulties in accurately categorising the different types of detrusor instability as the clinical condition itself is heterogeneous.

1.The Clinical Problem

Urinary incontinence is defined as involuntary loss of urine which is objectively demonstrable and a social and hygienic problem (Abrams *et al* 1988). The prevalence of urinary incontinence in the community is reported to be 8.5% in women and 1.6% in men aged 15-64, years rising to 11.6% in women and 6.9% in men aged 65 and over (Thomas *et al* 1980). Fewer than a third of the people admitting to incontinence were undergoing investigation or treatment at the time of that questionnaire. In the hospital outpatient population, 70% of women referred to the Bristol Urology unit in 1995 were suffering from incontinence (Abrams 1997). 85-90% of urinary incontinence in women is due to genuine stress incontinence or detrusor instability and as these are both common problems they often coexist (Cardozo 1991). Up to 10% of the female population may have detrusor instability, some with multiple symptoms of urgency, urge incontinence, enuresis, frequency and nocturia. Patients with frequency and urgency, but without leakage of urine, were bothered as much by their symptoms as were patients with daily incontinence (Norton 1990), so detrusor instability in varying degrees of severity could be implicated in producing a reduction in the quality of life for those sufferers. Those women who have

22

both detrusor instability and poor sphincter function, for example from damage to the pelvic floor following childbirth, are less able to manage their symptoms of urgency and frequency as their capacity to "hold on" by utilising the pelvic floor for continence is reduced. Their problems are accentuated by being unsuitable for bladder neck surgery as this may reduce voiding efficiency and increase outflow, thus worsening their instability. These women need to adjust psychologically to their condition and these abstract feelings can be measured by "Quality of Life" questionnaires. This is a useful measure of disease severity and success of treatment as urinary incontinence has little or no impact on mortality. These questionnaires are able to take into account the patient's perception of the inconvenience of her condition, are a useful adjunct to objective measurements and have shown that bladder overactivity has a profound impact on the quality of life of those affected (Jackson 1997).

Outflow obstruction has only been found to be an aetiological factor in the development of detrusor instability in men with prostatic hypertrophy (Mundy 1988). Most of this instability is cured by the removal of the obstruction (Abrams *et al* 1979). Genuine outflow obstruction in women is relatively rare (Masters & Ramsden 1997) and there are few published series (Farrer *et al* 1976, Farrer & Turner-Warwick 1979, Massey & Abrams 1988). More commonly iatrogenic urethral obstruction occurs with bladder neck surgery for genuine stress incontinence and is associated with a rate of 18% of patients developing detrusor instability (Cardozo *et al* 1979). However the incidence of detrusor instability in both sexes is similar so as many of the cases of detrusor instability in women are non-obstructive they are termed idiopathic. Treatment is not straightforward in this group, the treatments available can be conservative or surgical.

The conservative treatments include habit retraining in the form of bladder drill, biofeedback, hypnotherapy or acupuncture. The aim of such approaches is to gradually increase the length of time between voids. These treatments can improve symptoms in 80%

(Cardozo 1991), but they are time-consuming and require patients to be highly motivated. Drug treatments are aimed at inhibiting bladder contractions, their efficacy can be measured by improvements in voiding frequency, episodes of incontinence and voiding volumes. The main drug treatments are concerned with antimuscarinic action and include oxybutynin hydrochloride and tricyclic antidepressants. The anticholinergic effects of these drugs are however non-specific and associated with poor patient compliance due to unwanted side-effects. A newer more bladder-specific antimuscarinic agent, tolterodine, has undergone extensive trials and is as effective as oxybutynin in relieving symptoms and reducing the number of incontinent episodes but is much better tolerated and therefore more likely to improve patient compliance (Appell 1997). The elderly population has been shown to demonstrate a greater relief of symptoms when prescribed tolterodine than the younger groups with respect to the reduction in the number of incontinent episodes and increases in voided volumes (Malone-Lee 1997).

The symptoms of enuresis and nocturia can be improved by the use of the synthetic vasopressin analogue desmopressin, this treatment being usually reserved for occasional use to prevent potential kidney damage or fluid overload.

Patients with chronic problems of urge incontinence, urgency/frequency and voiding dysfunction refractory to medical treatment may benefit from electrical neuromodulation of the sacral nerve. This can either be temporary (used to test effectiveness) with a wire placed near the sacral nerve roots and connected to an external stimulator or if successful, can be changed to a permanent implantable system. Improved urodynamic parameters and relief of symptoms occur in up to 80% of subjects (Van Kerrebroeck 1998). Sacral nerve root stimulation has also been reported to be more successful in the treatment of symptoms in patients with idiopathic detrusor instability compared to patients with multiple sclerosis (Bristow *et al* 1988).

For those women with severe instability which is not amenable to conservative treatment, surgery may be required. Conventional bladder neck surgery, e.g. the Burch colposuspension, if stress incontinence is also present leads to a high rate of operative

failure, 74% of patients diagnosed as having unstable bladders preoperatively continued to suffer from leakage compared to 25% of those in whom no unstable bladder contractions were noted preoperatively (Jorgensen *et al* 1988) . Bladder nerve transection procedures were described by Ingelman-Sundberg in 1959 and modified in the 1970's for the treatment of detrusor instability in women (Ingelman-Sundberg 1975;1978). Although initially helpful they were associated with the development of vesico-vaginal fistula and so were discontinued. Transvesical phenol injections of the pelvic plexuses (Blackford *et al* 1984) has also been described. This procedure made the bladder acontractile by destroying nerve endings and was associated with a high complication rate and so is not now used in clinical practice .

The augmentation cystoplasty is a more invasive procedure and involves the incorporation of a segment of bowel into the bladder, usually ileum or occasionally the sigmoid colon (Mundy *et al* 1985). This procedure has been assessed over the last 15 years and long term results indicate a 78% continence rate in patients with neuropathic disease and a 93% continence rate in patients with detrusor instability (Venn and Mundy 1998). 63% of the patients were between 10 and 40 years of age and were selected for their suitability for major surgery and were all highly motivated. 6% of patients with detrusor instability and 60% of patients with neuropathic bladders required clean intermittent self-catheterisation postoperatively. The other complications of this procedure include bacteriuria, absorption disorders, stones and the potential risk of malignancy from the proposed carcinogenic properties of urinary tract nitrosamines, produced by the bacteria that colonise the cystoplasty.

At present, detrusor instability may be considered to be potentially curable, if severe enough to warrant major surgery, or suitable for invasive neuromodulation and if one prefers the problems associated with the treatment to those of the disease. However none of the described treatments is aimed at reversing or preventing the processes that underlie the development of the disease itself. It is therefore important to continue to try to discover

possible factors in the development of the disease in order to be able to devise more effective treatments for the different clinical presentations of the condition.

Before discussing the pathophysiology of detrusor instability it is necessary to understand the normal physiology of micturition.

2. Normal Bladder Physiology

Continence is partly automatic and partly under conscious regulation. It is automatically regulated until the desire to void is felt. From then on the reflex micturition process is consciously inhibited. When this is no longer desirable or possible the sequence of urethral relaxation and detrusor contraction automatically takes place. Continence thereafter takes place by conscious squeezing with striated muscles and then by external mechanical compression of the urethra (Klevmark & Kulseng-Hanssen 1991). In an uninhibited system, in simple terms, normal bladder function is organised on the basis of a reflex arc, i.e. during filling the bladder remains quiescent until a critical bladder volume or intravesical pressure is reached, reflex stimulation then occurs resulting in detrusor contraction to increase luminal pressure, the bladder neck widens and the urethral resistance is lowered and voiding proceeds. Efficient voiding demands the integrated activity of these components. Uncoordinated activity between these components will cause incomplete voiding so that it is convenient to consider the whole bladder/urethral sphincter system as a combined voiding unit. Efferent fibres from both the autonomic and somatic nervous systems play a role in this process.

It is useful to discuss the efferent and afferent arms of the neuronal control, the voiding reflexes and their control in the context of both voiding and filling of the bladder and in the mechanisms of continence.

2.1. Neurophysiological pathways regulating micturition

Voiding is co-ordinated in the normal subject by the stimulation of afferent nerves prompted by a rise in detrusor pressure. This initiates a series of reflexes resulting in the relaxation of urethral sphincter muscle, a significant rise in detrusor pressure and the opening of the bladder neck. As soon as the bladder has only a negligible residual volume of urine, voiding is complete and continence is restored by a gradual increase in urethral resistance from the external sphincter to the more proximal parts of the urethra.

In studying the neurophysiology of the micturition process it is necessary to look at the efferent and afferent neuronal arms. If the peripheral efferent nerves which are responsible for the motor aspect of voiding are discussed first then their modulation by the sensory, afferent nerves and central nervous system may be considered later.

2.1.(i)The efferent arm

The motor neurones supplying the detrusor in the human are almost all parasympathetic.(De Groat 1997) The final common autonomic efferent pathways to the bladder and urethral sphincter unit are in the pelvic plexus (inferior hypogastric plexus) which lies either side of the rectum and genitourinary tract. The anteriomedial relation of this plexus in the female is the upper vagina, at which point it lies in the base of the broad ligament. Within the plexus synaptic contacts are made or fibres run to more distant connections in the bladder wall. A network of nerves derived from this plexus is formed around the bladder extending to its lateral surfaces. Ganglia are present both on the bladder surface and also within the bladder wall. It is not thought to be possible to transect the nerves distal to these terminal ganglia to cause denervation in a manner similar to that described in the formation of the non-obstructed pig model of detrusor instability, (Sethia

27

et al 1990). Branches of the vesical plexus penetrate the muscle coat and the smaller branches pursue a tortuous course in the bladder muscle so that they are not damaged by the stretching of the bladder during filling.

Pre-ganglionic fibres originate from the spinal cord at the level of S2-S4. There is little sympathetic noradrenergic stimulation in the bladder; those sympathetic nerves that do exist are to be found mainly around the bladder base and urethra, originate in the intermediolateral column of the spinal cord from T10-L2 and travel mainly in the hypogastric nerve. Sympathetic fibres probably also inhibit the post-ganglionic parasympathetic fibres during the filling phase of micturition (De Groat 1997). Sympathetic fibres are not believed to play a role in regulating detrusor contractility and any which do innervate the dome of the bladder will influence the local vasculature. These efferent connections are illustrated in figure I1.
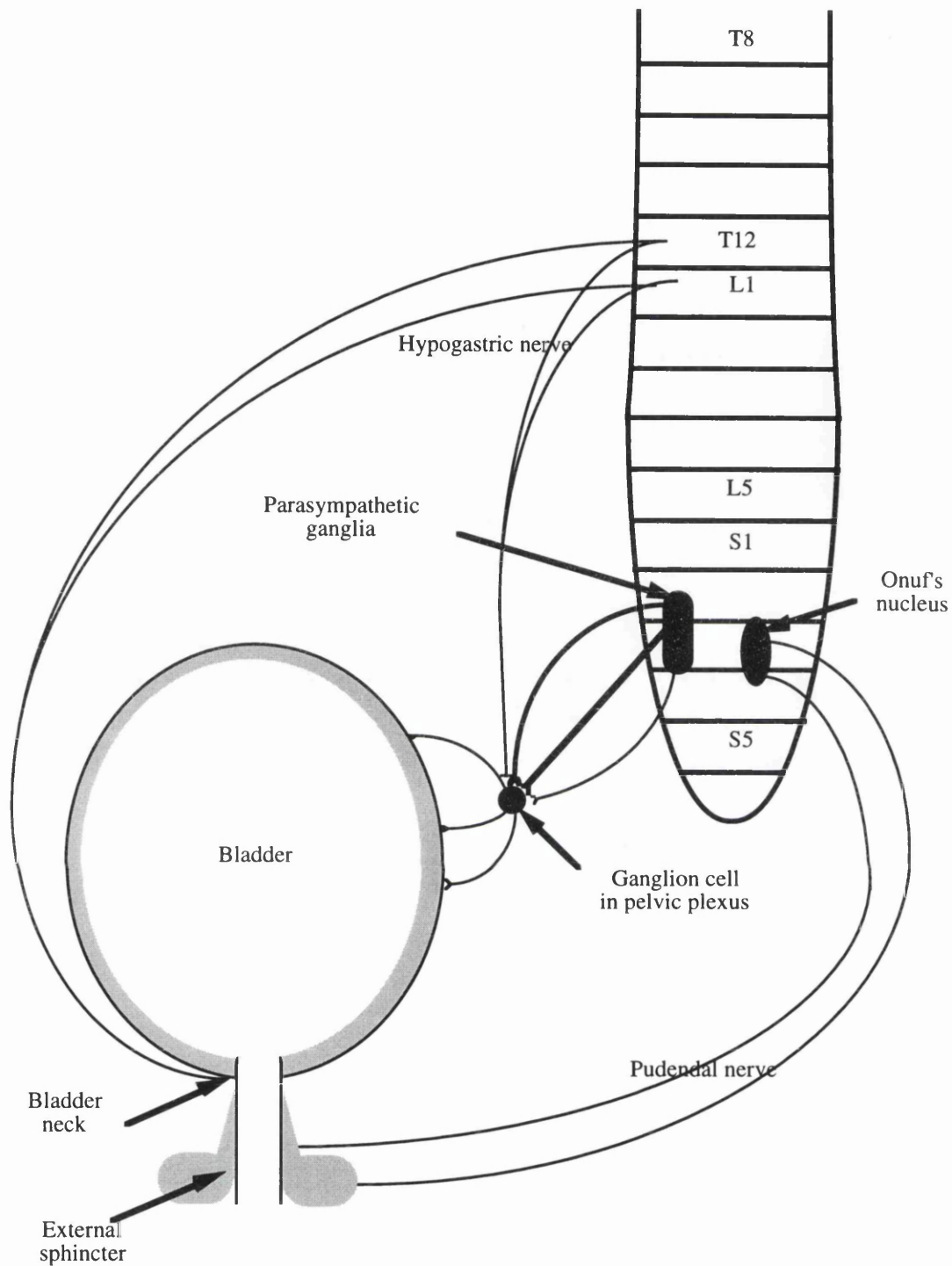
Figure I.1.

A diagram of the efferent connections to the lower urinary tract.

In addition to the autonomic fibres which innervate the lower urinary tract somatic nerves originate at the S2-S4 level and travel in the pudendal nerve to the striated muscle in the external urethral sphincter. These alpha motorneurones originate in a region of the sacral

spinal cord termed Onuf's nucleus, which also supplies motor fibres to the pelvic floor and levator ani muscles including the anal sphincter.

The excitatory neurotransmitters involved in this efferent pathway are discussed in section 5 pages 45-49.

2.1.(ii). The afferent arm

Afferent nerve endings situated in the bladder wall are mainly bare ended fibres running beneath the urothelium or between muscle bundles and respond to increases in wall tension rather than bladder volume. These mechanosensitive afferents travel in parasympathetic nerves to the sacral dorsal root nerves and convey sensations of bladder fullness and pain which lead to the perceptions of desire and urgency.

Sensory impulses reach the spinal cord and higher centres mainly via the pelvic nerves to sacral segments S2-S4. Some sensation also relays via the hypogastric nerves, running with sympathetic fibres T10-L2 spinal segments (see section 2.1.(iv).). Those few afferents that cross to T10 are active only during marked distension of the bladder. These afferents allow patients who have total transection of the spinal cord below the level of T10 to have the sensation of being catheterised and overdistended whilst having no sensation of voiding. Once in the spinal cord bladder afferents cross over to the opposite side and ascend in the spinothalamic tracts.

The sacral afferents are either myelinated A-δ fibres or unmyelinated C-fibres. The A-δ fibres have a threshold of excitation when bladder pressures reach about 10-15 cm H_2O. Some of these c fibres do not appear to fire at the normal range of stimuli and are called "silent afferents" (De Groat 1997) . These are thought to be important in pathological bladder conditions such as sensory urgency and may respond to stimuli such as inflammation. Partial or complete degeneration of some bladder afferents, particularly those unmyelinated fibres can be achieved using Capsaicin (an extract of chilli peppers) intravesically (De Groat 1997), this has shown to be effective in cases of hyper-reflexia by increasing functional bladder volumes and decreasing urinary frequency although is of little

30

or no benefit in cases of sensory urgency where treatment is not well tolerated (Fowler 1994).

2.1.(iii). Higher connections and control of voiding

Voiding is initiated once the bladder afferents are sufficiently excited to exceed the stimulation threshold at the level of the ganglia so that action potentials are transmitted to the spinal cord. Voiding is not simply a spinal reflex and needs to be controlled by higher centres. Some reflex voiding does develop in patients following spinal transection but this involves plastic changes to the spinal connexions.

The micturition reflex is co-ordinated in the pontine micturition centre (Kuru 1965). Efferent impulses from this centre run in the reticulospinal tracts and stimulate parasympathetic and somatic motor neurons situated in the lateral cell column and in the anterior horn at level S2-S4. These connections are illustrated in figure I2.
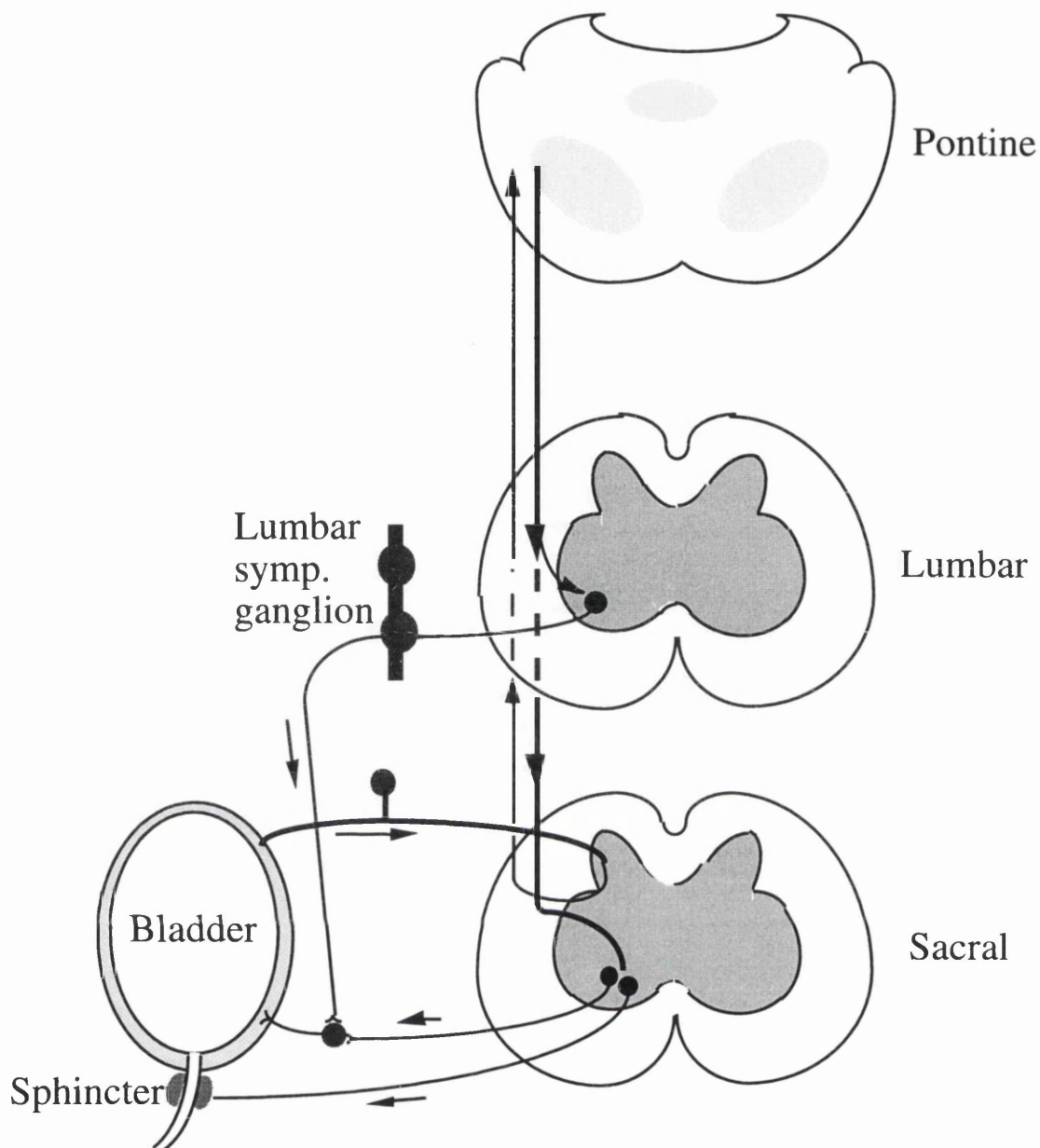
Figure I 2

The nervous reflexes associated with co-ordinated voiding.

Voiding i.e. the simple mechanism of co-ordinating a decrease in urethral pressure with an increase in detrusor pressure requires an intact spinal cord and connections to the pontine area of the brainstem. The most important co-ordination centre for bladder and urethral function lies in the reticular formation of the pons, the nucleus coerulus. This area

represents the final common pathway for normal bladder filling and voiding affecting both efferent and afferent activities. Lesions below this area can lead to uncoordinated voiding, e.g. detrusor dyssynergia when the detrusor will contract against a closed urethral sphincter. Lesions above this area will lead to involuntary co-ordinated voiding.

In order for co-ordinated voluntary voiding there needs to be an intact axis above the level of the pons. Humans and some animals require conscious control to determine the convenience of micturition at a given time and the ability to postpone voiding if necessary. The pontine micturition centre is controlled by both stimulatory and inhibitory cerebral areas. It is difficult to accurately map the areas of the cerebral cortex involved in regulation of the pons as much of the work has been done using animals or by studying gross lesions or surgical injury in humans.

Voluntary control of voiding seems to require connections between the paracentral lobule and the brainstem as well as between the medial surface of the frontal lobe and the septal and preoptic areas of the hypothalamus. Loss of these connections removes the inhibitory control of these cortical centres on bladder reflexes, interferes with the processing of sensory information from the bladder and removes inhibitory control of the hypothalamic area where voiding behaviour is organised. Such lesions reduce awareness either of urgency prior to a void or sensation of voiding.

Motorneurones influencing the urethral sphincter can also be modulated by descending extrapyramidal pathways in the same way as motorneurones to the lower limbs. This can result in an inability of the urethral sphincter to relax and is seen in multisystem atrophy.

2.1.(iv). Filling and storage of urine

During natural filling in humans at an average rate which is 1ml/hr/kg, there is no appreciable bladder pressure rise in the healthy bladder (Klevmark & Kulseng-Hanssen

1991). During early bladder filling the collapsed, folded bladder wall is unfolded thereafter further filling results in distension of the bladder with stretching of the bladder wall. The rise in pressure, for a given volume filled is determined by the compliance of the bladder wall i.e. the ratio of the rise of intravesical volume per unit increase of bladder pressure. During normal volume filling of the normal bladder the increase in detrusor pressure is less than 15cm of water, the high degree of compliance is due to stretching of elastic elements, and stress-relaxation of the viscoelastic elements. In normal bladders with high compliance the passive stretching during filling causes the muscle bundles in the bladder wall to undergo reorganisation and the muscle cells can become elongated up to four times their minimum length (Uvelius 1980). Abnormally low compliance or fast, non-physiological filling results in greater increases of intravesical pressure (Finkbeiner & O'Donnell 1990, German et al 1994).

In addition to the visco-elastic properties of the bladder wall itself there are a number of neurophysiological mechanisms which suppress the increase in bladder wall tension and hence the micturition reflex . These mechanisms suppress postganglionic efferent nerves until a threshold of excitation is reached.

Inhibitory neurones are situated in the intermediolateral column of the spinal cord and these inhibit parasympathetic activity repeatedly during filling. In addition to this there is further suppression of parasympathetic efferents by visceral afferents from pelvic organs and somatic afferents from the perineum. The ganglia also act as filters by blocking low levels of preganglionic activity again until a certain threshold at normal functional capacity is reached (de Groat 1968). In addition the hypogastric nerve is also involved by the modulation of efferent activity via a negative feedback process (section 2.1.(ii) page 30). Afferent stimulation from the bladder and urethra does not cause efferent activity in postganglionic fibres before a critical level of stimulation occurs (Zinner et al 1980). This feedback loop can itself be modulated by descending fibres from the nucleus coeruleus, so that during filling the loop is active but during voiding the loop is suppressed

The bladder neck and proximal urethra are also closed by increased sympathetic stimulation to these regions. The mechanism of continence in the female is largely dependent on these neuromodulations but they are not wholly responsible for continence particularly in the parous woman.

2.2. Mechanism of continence in the female lower urinary tract

Continence in women is a balance between three variables, (i) urethral pressure, (ii) detrusor pressure and (iii) abdominal pressure in conjunction with bladder neck position.

2.2.(i) Urethral pressure

This is produced by the urethral sphincter mechanism including the urethral wall. The sphincter is a complex group of co-ordinated muscles comprising smooth and striated fibres, under autonomic and somatic control as described above. The smooth muscle of the urethra is continuous with the smooth muscle of the trigone and detrusor but is histologically and functionally different from them. It consists of an inner longitudinal layer and an outer circular layer. The muscle is thought to be under parasympathetic cholinergic control and its fibre orientation and innervation suggests its main function is to shorten and widen during micturition (Gosling *et al* 1981)

At the level of the mid-urethra it is surrounded by a sleeve of circular striated muscle, the rhabdosphincter or external urethral sphincter which is thicker anteriorly and separated from the periurethral muscle by connective tissue. The muscle cells are slow twitch types and are capable of maintaining tone for long periods of time.

The extrinsic striated periurethral muscle is slightly more distal and is synonymous with the pubococcygeus part of the levator ani, which lies lateral to the urethra. It is at its widest at the junction of the middle and lower thirds of the urethra. It consists of large diameter fibres of slow and fast twitch types: the latter are believed to contribute an additional occlusive force on stress and exertion. It is innervated by motor fibres from the pudendal nerve and these fibres also provide a guarding reflex, that is, an increased afferent activity

in the bladder results in an increase of efferent pudendal and inferior hypogastric nerve activity to the striated musculature of the urethra and so increasing urethral resistance. The cell bodies of these neurones are situated in Onuf's nucleus which also receives sensory bladder afferents and descending tracts which can inhibit the guarding reflex and reduce outflow resistance.

Enhancing the occlusive forces of the muscle elements of the sphincter is connective tissue which allows apposition of the mucosa; this connective tissue contains collagen, elastin and a rich plexus of blood vessels and lies within the submucosa. The latter are believed to contribute at least a third to the urethral resistance (Raz *et al* 1972). The mucosa contributes to continence by the nature of its highly folded epithelium which is oestrogen dominated and deteriorates with age. Urethral pressure measurements also show that the transmission of intrabdominal pressure to the upper half of the urethra and bladder neck help to maintain a positive pressure gradient (Enhorning 1961). The amount by which the urethral pressure exceeds the detrusor pressure when multiplied by 100 is known as the pressure transmission ratio (PTR) and this should always exceed 100% in a continent woman, except during voiding.

Decreases in the urethral pressure occur in the following situations:

a) associated with a rise in detrusor pressure during normal voiding or with detrusor instability when the urethral pressure will fall.

b) urethral instability. At rest, the urethral pressure has been shown to fall significantly in patients with urethral sphincter incompetence.

c) sustained response to stress. Following several acute rises in abdominal pressure, the urethral pressure may show a 50% reduction over a variable period of time.

d) small variations in pressure due to vascular pulsation have been detected. (Enhorning 1961).

2.2.(ii) Detrusor pressure

This is normally less than urethral pressure at rest and whilst the bladder is filling. Detrusor pressure may increase in detrusor instability, due to low bladder compliance, in outflow obstruction with overdistension retention of urine following anaesthesia or in hyper-reflexia. In the presence of a normally functioning pelvic floor musculature an increase in detrusor pressure may be perceived as urgency and the individual does not always suffer incontinence.

2.2.(iii). Abdominal pressure and bladder neck position.

Enhorning (1961) has shown that normally a rise in abdominal pressure is transmitted equally to the bladder and proximal urethra, producing the positive closure mechanism in the urethra. Excessive rises in abdominal pressure may temporarily lead to the detrusor pressure exceeding the urethral pressure especially where there is imperfect transmission of this rise to the proximal urethra as well. This occurs when the bladder neck descends on straining.

To function effectively as a sphincter mechanism the sphincter itself needs to be correctly positioned in relation to the main body of the bladder and the symphysis pubis so that sudden abdominal pressure on a closed sphincter augments urethral pressure. The structures which maintain the bladder neck in an elevated position include the posterior pubourethral ligaments, the pubocervical fascia and pubococcygeus fibres of the levator ani muscle.

The posterior pubourethral ligaments are made of dense connective tissue which arise primarily from the vagina and periurethral tissues, to attach laterally to the pelvic wall. The pubocervical ligaments however are delicate, comprised of smooth muscle and probably assist in bladder neck opening rather than providing support for it (DeLancey 1989).

The pubocervical fascia maintains support for the bladder neck and also helps in the closure mechanism. A precervical arc consisting of collagen, elastin and smooth muscle fibres, runs transversely across and above the bladder neck and is attached to it in the middle: at either end the arc is attached to a fibrous band- the arcus tendinus fasciae pelvis.

When the pelvic floor contracts, the anterior fibres of levator ani lift the vaginal wall anteriorly, compressing the proximal urethra and bladder neck against the precervical arc (DeLancey 1988).

If the pelvic floor becomes damaged and is unable to lift the anterior vaginal wall adequately the bladder neck will lie below the area of maximum pressure and the result will be pressure transmission to stored urine above the bladder neck and leakage of that store.

During normal micturition following suitable efferent activity in a co-ordinated way a detrusor contraction occurs leading to a rise of intravesical pressure and a decrease in intraurethral pressure as a consequence of relaxation of urethral and periurethral striated muscle; in the female the bladder neck exhibits mobility and forms a funnel shape as a consequence of detrusor contraction assisting voiding (Mostwin1991, DeLancey 1990). Stimulation of the motor nerve supply to the detrusor results in neurotransmitter release, excitation and then contraction.

With the exception of the bladder neck and the trigone, the musculature in all regions of the bladder possesses similar histological and histochemical characteristics (Gosling 1979). The detrusor muscle consists of numerous interlacing bundles which connect with each other in an intricate fashion, an arrangement resulting in the formation of a complex meshwork of smooth muscle. This arrangement is ideally suited to cause reduction in all dimensions of the bladder on contraction, thereby enabling the bladder to completely empty its contents.

In order to stimulate the detrusor to contract and initiate voiding the parasympathetic nerves supplying the bladder release neurotransmitter at the neuromuscular junction. To understand possible pathophysiological processes it is important to have a knowledge of current opinions concerning detrusor smooth muscle physiology and in particular its contractile mechanism and how this is coupled to excitation.

3. Tension generation

Tension in bladder smooth muscle, in common with other smooth muscles, is generated by activation of the cellular contractile machinery. Shortening of the muscle cell occurs by sliding of thick and thin filaments in the cell past each other. The filaments are arranged in parallel in the cell, and sliding is achieved by the formation of crossbridges between the filaments. Thick filaments are composed of myosin molecules, each of which have double projecting heads (the crossbridge) which can attach to sites on the thin actin filaments. The myosin heads have enzymatic activity (ATPase) which is triggered by interaction with the actin molecules, and is able to break down ATP to liberate energy which is used to power a change in the crossbridge conformation leading to pulling of the two filaments past each other (Brading 1987).

Actin filaments in smooth muscle insert either onto the plasma membranes through "dense bands" seen at intervals along the membranes, or onto "dense bodies" in the sarcoplasm. The thick filaments lie in the sarcoplasm in parallel with the length of the cell. The thick filaments allow the thin filaments to slide their whole length allowing the myocyte to produce active force over a great range of lengths (Craig & Megerman 1977).

An increase of the $[Ca^{2+}]$ from about $0.1\mu M$ to $1\mu M$ in the sarcoplasm is responsible for the activation of the contractile proteins to form crossbridges and hence generate tension by the phosphorylation of these proteins. The myosin filament is activated by Ca^{2+} in smooth muscle, whereas in striated muscle the actin filament is Ca^{2+} sensitive. The Ca^{2+} sensitivity of contractile proteins is later discussed in detail.

The crossbridges are only active when two small protein components (light chains) on the myosin heads are phosphorylated. The detailed mechanism is summarised in figure I3. Phosphorylation occurs as a result of sarcoplasmic Ca^{2+} binding to the soluble specific Ca^{2+}-binding protein calmodulin in the sarcoplasm, a cascade is initiated resulting in the phosphorylation of a light chain of the contractile protein myosin.

39

$$Ca^{2+} + calmodulin$$

$$\downarrow$$

$$Ca^{2+} - calmodulin\ complex$$

$$\downarrow$$

Inactive
myosin light
chain kinase $\longrightarrow$ Active
myosin light
chain kinase

$$\downarrow$$

Myosin light
chain   ATP   ADP   Phosphorylated
myosin light chain

$$\downarrow$$

Acto-myosin
interaction
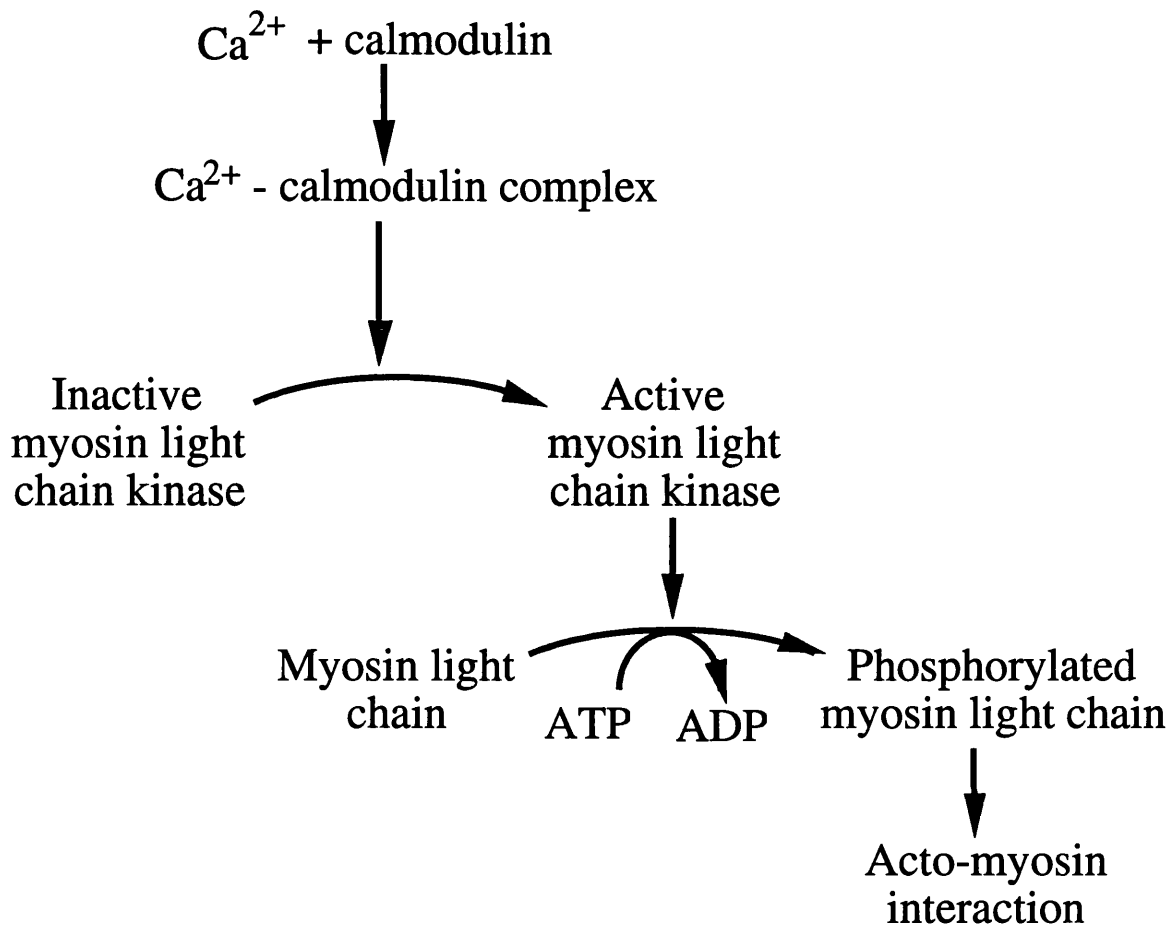
Figure I3 The Ca^{2+}-calmodulin cascade. Sarcoplasmic Ca^{2+} combines with a soluble protein calmodulin which initiates a chain of reactions leading to interaction between actin and myosin producing initiation of contraction resultant in the generation of muscle force.

In smooth muscle the activation of the formation of myosin crossbridges occurs when the Ca^{2+} -calmodulin complex causes the phosphorylation of the myosin heads by activating myosin light chain kinase. The number of crossbridges that are phosphorylated at any one time depends on the relative activities of the enzyme, myosin light chain kinase and the inhibitory enzyme myosin light chain phosphatase which causes dephosphorylation of the

myosin light chain heads. The phosphatase is independent of the Ca^{2+} concentration in the sarcoplasm.

One additional form of regulation that operates in smooth muscle during prolonged periods of tension (and thus may not be appropriate in the consideration of detrusor smooth muscle) is the conversion of the crossbridges into a "latch" state in which crossbridge turnover and ATP utilisation is considerably reduced without significant loss of force (Askoy *et al* 1982). This may be an energy conserving mechanism in smooth muscles such as blood vessels. Another mechanism involves an inhibitory protein, caldesmon, which may act in a similar way in some respects to troponin in skeletal muscle (Marston & Smith 1985). It is associated with the thin filaments, and prevents the interaction of actin and myosin molecules except when it is bound to a Ca^{2+} -calmodulin complex, which causes it to release its inhibition.

More recently, work has been published investigating the sensitivity of the contractile proteins in detrusor smooth muscle (Wu *et al* 1995). The Ca^{2+} dependency of contractile protein activation is in principle a means whereby the force of contraction could be modulated either by experimental, therapeutic or pathological means. For example, a reduction in the contractile protein Ca^{2+} sensitivity would mean that for a given increase of the sarcoplasmic $[Ca^{2+}]$ the contractile proteins would be activated less completely and less tension would be generated. Wu et al (1995) used guinea-pig detrusor smooth muscle strips permeabilised to small molecules by alpha-toxin from Staphylcoccus aureus to investigate the effect of changing the $[Ca^{2+}]$ in the vicinity of the contractile apparatus at different pH values. They showed that the contractile proteins are sensitive to concentration changes of Ca^{2+} in the range of $0.1\mu M$ to about $1\mu M$ and this would be adequate to activate the smooth muscle machinery. The sensitivity of the contractile proteins was demonstrated to be decreased at raised pH, which in intact detrusor muscle causes a negative inotropic effect. A decrease in contractile protein sensitivity has also been demonstrated by the stimulation of beta-adrenergic receptors in guinea-pig teania coli which demonstrated a fall in tension without a decrease in intracellular $[Ca^{2+}]$.

In summary, the contractile machinery is activated by an increase in intracellular $[Ca^{2+}]$ in detrusor smooth muscle in common with other smooth muscle types. In normal conditions a change in the intracellular $[Ca^{2+}]$ from $0.1\mu M$ to $1\mu M$ is adequate to activate the contractile proteins in detrusor smooth muscle.

Therefore, in order to elicit a contraction, the detrusor smooth muscle cell must initiate a transient rise of $[Ca^{2+}]$ from submicromolar levels to micromolar concentrations, i.e. from its resting Ca^{2+} level to its active level. This is achieved by various methods, namely the excitation of the detrusor cell and by coupling this excitation with a series of intracellular events in order to maximise intracellular $[Ca^{2+}]$ to the level required for contraction. Before studying the mechanisms of producing the Ca^{2+} transient rise it is necessary to explain Ca^{2+} homeostasis in the resting smooth muscle cell.

4. Intracellular Ca^{2+} in relaxed smooth muscle

To maintain a relaxed state in smooth muscle the intracellular free $[Ca^{2+}]$ has to be kept at submicromolar levels. This is achieved by the extrusion of Ca^{2+} across the cell membrane, binding of Ca^{2+} to intracellular sites and uptake into intracellular organelles. Ca^{2+} extrusion out of the cell occurs against both potential and concentration gradients, and requires energy. There are at least two known mechanisms, an ATP-dependent Ca^{2+} pump and a Na^+- Ca^{2+} exchange mechanism. The latter exchange uses the potential energy of the inwardly directed Na^+ gradient to power extrusion against its electrical and chemical gradients; however the importance and even the existence of the latter in detrusor smooth muscle is disputed (Brading 1987).

The total intracellular Ca^{2+} in smooth muscle has been estimated from studies in guinea-pig taenia coli (Brading & Widdicombe 1977) and is probably of the order 0.5 millimol/kg wet tissue weight, but most of this Ca^{2+} is either bound to cellular proteins or membranes, or

sequestrated inside organelles. The sarcoplasmic reticulum of smooth muscle consists of vesicles often found in asssociation with the caveoli at the surface of the cell, and also deeper in the cytoplasm (Daniel 1985). It has been shown that Ca^{2+} can be pumped into these vesicles by an ATP-dependent Ca^{2+} pump that differs somewhat in its properties from that found in the plasma membrane. The sarcoplasmic reticulum stores of Ca^{2+} have been of increasing interest to investigators as it is now thought that the Ca^{2+} stored here is responsible for much of the generation of the transient rise in intracellular Ca^{2+} required for activation of the contractile machinery of smooth muscle.

4.1. Intracellular Ca^{2+} stores

The evidence for intracellular Ca^{2+} stores has been gathered for several smooth muscle types. In guinea-pig portal vein smooth muscle the contractions generated in the absence of Ca^{2+} in the superfusate have been investigated (Bond & van Wart 1984). The ability of the smooth muscles to contract repeatedly and maximally in Ca^{2+} free solutions demonstrates that the intracellular recycling of Ca^{2+} released from an intracellular store occurs. The demonstration that in vascular smooth muscle such stores may be responsible for smooth muscle contractions in the absence of extracellular Ca^{2+} resulted in a series of experiments to identify if the same process was apparent in detrusor smooth muscle (Mostwin 1985).

Mostwin (1985) used guinea-pig taenia coli and bladder strips and investigated the hypothesis that these types of smooth muscle would show similarities to vascular smooth muscle. It had been shown in earlier work by others that depolarised vascular smooth muscle could further contract in response to stimulation by agonists suggesting that there might also be voltage-independent mechanisms for activating contraction. The bladder and taenia-coli strips were exposed to carbachol in a solution either containing calcium or one which was Ca^{2+} free. Tension recording showed that in the Ca^{2+} free solution tension generated by the muscle did not decline immediately but responded to repeated stimulation with agonist gradually diminishing over a period of 30 minutes. The force generated by stimulation of the strips by a depolarising solution (40mM KCl) diminished in the calcium free solution much more rapidly when compared to the decline of the carbachol stimulation

43

in the same circumstances. It was concluded that the response of the bladder to depolarisation depended primarily on the extracellular calcium, the response to carbachol appeared to involve the release of stored intracellular calcium. Thus bladder, like other smooth muscles, appears to contain agonist releasable intracellular calcium stores.

There must be several characteristics of an intracellular Ca^{2+} store to render it capable of being an integral part of the formation of the transient rise in free sarcoplasmic $[Ca^{2+}]$ responsible for activation of the contractile machinery. These include a mechanism to release Ca^{2+} following appropriate stimulation (i.e. receptor mediated release), a process of re-uptake following release and repletion of stores during relaxation.

The intracellular stores release Ca^{2+} after stimulation with caffeine (Ganitkevich & Isenberg 1992, Himpens & Somlyo 1988) and this may be measured using intracellular fluorescent Ca^{2+} indicators e.g. Indo-1 and Fura-2. The transient rise in Ca^{2+} occurred very quickly after stimulation (1s) and following caffeine washout fell rapidly to subresting levels i.e. an undershoot occurred (Ganitkevich & Isenberg 1992). Subsequent caffeine applications produced attenuated transient peaks suggesting the caffeine sensitive Ca^{2+} store had lost a part of its releasable Ca^{2+}. Depolarisation restored the height of the transient suggesting Ca^{2+} influx through L-type Ca^{2+} channels was important to replenish the stores once depleted. The accumulation of Ca^{2+} by the stores was prevented by ryanodine, an alkaloid which causes slow release of Ca^{2+} in the sarcoplasmic reticulum, and the caffeine evoked intracellular Ca^{2+} transient was absent in the presence of ryanodine (Gantikevich & Isenberg 1992).

Studies on neonatal rabbits, in comparison with mature animals demonstrates a developmental aspect to the intracellular Ca^{2+} stores. Intracellular binding and secondary Ca^{2+} release are low in neonatal bladder smooth muscle and increase with maturation, the neonatal bladder is more dependent on the influx of extracellular Ca^{2+} for smooth muscle contraction than the more mature rabbits (Zderic et al 1993,1994).

Initiation of contraction involves a number of steps which culminate in the release of Ca^{2+} from intracellular stores. With detrusor smooth muscle the primary regulated step is via autonomic motor nerves, with postganglionic fibres embedded in the muscle mass. Excitation of the motor nerve releases a neurotransmitter which will subsequently activate intracellular Ca^{2+} release.

Intracellular Ca^{2+} stores are linked to these membrane events by coupling mechanisms. In order to understand the pathophysiology of unstable detrusor contractions it is necessary to discuss the normal process of excitation and its coupling to contraction.

5. Excitation

Excitation of the detrusor cell occurs as a result of agonist binding to membrane bound receptors. This could lead to i) opening of Ca^{2+} channels and an influx of Ca^{2+} into the cell or ii) the activation of a second messenger system leading to release of intracellular Ca^{2+}, or a combination of the two.

Investigation of the nervous excitation of the urinary bladder has generally focused on the study of isolated smooth muscle strips in organ baths, where the extracellular environment can be controlled. Contractions can be elicited by tetanic pulse trains and if each pulse train is short (<0.1ms) the motor nerves, rather than the muscle cells themselves are excited. This selective nervous excitation can be achieved because the membrane time constant of the nerve is much less than that of the smooth muscle cell, so that short stimulating pulses will depolarise the nerve membrane more rapidly than the detrusor cell membrane. Excitation of the motor nerve releases a transmitter which will subsequently activate the detrusor smooth muscle cell.

Much of the work in this area has been performed on small mammals and certain differences are noted when the results are compared to work completed using human biopsies. A discussion of the results from both sources of tissue is included.

5.1. Cholinergic Transmission

The principal neurotransmitter from the autonomic motor nerves supplying the bladder in mammals is acetylcholine. This neurotransmitter together with its group of analogues was shown to be the most effective in producing tension in isolated human detrusor smooth muscle in a comparison with other non-specific pharmacological agents, (Todd & Mack 1969). Acetylcholine binds to muscarinic receptors of which the M_3 subtype plays a key role in activating detrusor contraction.

Muscarinic receptors at the neuromuscular junction have been extensively studied and have been categorised into M_1, M_2, M_3 and M_4 subtypes based on their affinity for cholinergic ligands. Muscarinic receptors occur widely throughout the body and thus are responsible for many of the side effects of current anti-cholinergic therapy used for the treatment of unstable detrusor contractions. The desire to alter detrusor contractility has led to a search for muscarinic agonists that are relatively selective for the bladder. Radioligand binding studies confirm that the bladder body and base contain muscarinic receptors (Nilvebrant *et al* 1985, Levin *et al* 1988).

Cholinergic transmission is mediated by M_1 and M_2 receptors within the central nervous system whereas the human bladder has predominately M_2 and M_3 receptors, of which the M_3 type is functionally the more important. The binding properties of muscarinic receptors in the bladder are altered as a result of smooth muscle hypertrophy or neurological injury (Lepor 1989). Levin has demonstrated a deterioration in the binding properties of muscarinic receptors a few hours after bladder neck destruction (Levin *et al* 1988).

Evidence for the importance of a muscarinic mediation has been gathered from the effect of atropine on human detrusor smooth muscle. Atropine, an anti-muscarinic agent, inhibits contraction in electrically stimulated isolated detrusor smooth muscle. Kinder (1985) reported the effect of atropine on isometric tension produced in response to electrical field stimulation. This tension was reduced by 94.1% using the neurotoxin tetrodotoxin,

suggesting the contractions were nerve stimulated. After the separate application of atropine these contractions were also reduced, by 92.7%, indicating that nerve mediated stimulus in human detrusor is cholinergic.

In other studies atropine resistance has been investigated. Residual contractions when human detrusor was field stimulated in the presence of atropine have been demonstrated (Sjogren et al 1982). These experiments were performed using diseased (unstable and obstructed) bladder biopsies. Nergårdh & Kinn (1983) however found that when comparing atropine sensitivity in normal and hypertrophied bladder biopsies there was equal sensitivity, particularly when stimulated at low frequencies.

Atropine resistance has been noted in detrusor muscle from small animals and has fuelled speculation that alternative neurotransmitters function via the detrusor in abnormal bladders.

In animal studies of detrusor smooth muscle, atropine sensitivity has been shown to vary between species. The rabbit and pig demonstrated greater atropine sensitivity, an 80% reduction of the magnitude of the electrically stimulated contraction in control conditions, than the guinea-pig detrusor preparation which was subject to a 70% reduction in force in the presence of atropine (Brading 1987) . The magnitude of atropine resistant responses is frequency dependent. Other authors report reduced atropine sensitivity in detrusor from other small animals e.g. the rat (Hammerstrom & Sjostrand, 1984; Carpenter 1977)

Detrusor smooth muscle biopsies taken from normally functioning and diseased human bladders have shown no atropine insensitivity (Kinder & Mundy, 1987; Palfrey et al, 1984; Chen et al ,1994; Speakman et al ,1985 and Sibley 1984). However atropine insensitivity has been reported by some authors in biopsies from diseased detrusor, e.g. interstitial cystitis (Ruggieri et al ,1990; Palea et al, 1993), obstructed detrusor instability (Nerdgårgh & Kinn 1983; Sjogren et al ,1982) and detrusor hyper-reflexia (Saito et al 1993). There has been no demonstration of atropine insensitivity in idiopathic detrusor instability (Kinder & Mundy 1987).

The controversy regarding atropine insensitivity in human pathological detrusor smooth muscle has fuelled the search for non-cholinergic transmitters in the bladder.

47

5.2. Non- cholinergic nerve transmission in the bladder

There has been much speculation regarding non-cholinergic bladder stimulation. The secondary neurotransmitters fall into two types, excitatory and inhibitory transmitters. Adrenergic transmission, although not considered to be a "secondary" agent will be considered with other inhibitory modulators.

5.2.i) Excitatory neurotransmitters

Possible non-cholinergic excitatory neurotransmitters include a number of small proteins including substance P, neurokinin A and arginine-neurokinin which have all been investigated in relation to the stimulation of human detrusor muscle (Maggi *et al* ,1988). Neurokinin A was found to be the most potent. Substance P has also been investigated in the context of infravesical obstruction (Andersson *et al* ,1988) but there was found to be no difference in effect of this agent on normal or hypertrophied bladders and thus its role in disease was unproved.

ATP is the most likely of these secondary transmitters and purinergic transmission was initially suggested by Burnstock (Burnstock *et al* ,1972) when he tested the effects of several purine nucleotides on bladders of rats and guinea-pigs. It was shown that most of these substances caused contraction of detrusor smooth muscle, ATP was the most potent and at the correct dose its application could match the electrically stimulated atropine-resistant contractions. The addition of quinidine blocked the ATP evoked contraction and the electrically stimulated atropine resistant contraction, although the response to acetylcholine in the absence of atropine persisted. Later work from this group (Burnstock 1981) using the guinea-pig bladder showed that ATP was released when the bladder was stimulated, and that the release was blocked by TTX and was also Ca^{2+} dependent. The effects of various analogues suggested that the receptors involved were of the subclass P_2, in particular the P_{2x} subtype. Purinergic transmission was demonstrated in the human urinary bladder in isolated strips in 1990 (Burnstock 1990). A radioligand binding assay

specific for purinergic receptors in the bladder has been described (Ruggieri *et al* 1990) and used to identify such receptors in the human bladder. Identification of purinergic transmission in isolated single cells was performed in guinea-pig single cells (Schneider *et al* 1991) and its association with Ca^{2+} influx observed. ATP gated channels were thought to be responsible for this observation, and earlier ATP was described as being a fast excitatory transmitter in guinea-pig detrusor (Inoue & Brading 1990). Human isolated detrusor cells were demonstrated to respond to ATP application (Wu *et al* 1995) and such application produced a transient rise in intracellular Ca^{2+}.

5.2. ii) Inhibitory neurotransmission

Adrenergic stimulation with beta-adrenergic agonists causes relaxation of the urinary bladder (Morita *et al* 1993), this relaxation seems to be as a result of the stimulation of beta 2 receptors on excitatory ganglia and is shown in both rabbit and man. An interaction between cholinergic and adrenergic stimulation mediated by inhibitory muscarinic receptors on adrenergic nerve terminals has also been mooted (Mattiason *et al* 1987) although the physiological significance has not been established. The interaction of the sympathetic nervous system inhibiting the parasympathetic nervous systems has been discussed earlier (see section 2.1. (ii) p 30).

Another neurotransmitter that seems to cause relaxation of the detrusor muscle is vasoactive intestinal polypeptide. The results of studies are varied. V.I.P was shown to relax electrically stimulated contractions in isolated muscle preparations in rabbit, pig and also in human detrusor biopsies (Klarskov *et al* 1984; Klarskov 1987a,b,) but in another study to relax contractions in small mammals only, with no effect on human detrusor (Sjogren *et al* 1984). In human samples from diseased and normal bladders V.I.P. relaxed spontaneous contractions only, with no effect on electrically stimulated contractions (Kinder & Mundy 1985). The role of V.I.P. in diseased bladders has also been investigated more specifically but there was found to be no correlation between the relaxation effect of V.I.P. in normal rats to those with infravesical obstruction (Andersson *et al* 1988). Another inhibitory neurotransmitter Gamma-amino butyric acid, GABA has

been shown to exert a relaxant effect on electrically stimulated isolated preparations from human detrusor (Chen *et al* 1994) and is thought to act via a $GABA_B$ receptor on the post ganglionic nerves. The importance of this observation has yet to be established.

6. The link between membrane events and intracellular Ca^{2+} release.

Application of acetylcholine, or its analogue carbachol, to isolated detrusor muscle elicits a contraction and a transient rise of the intracellular $[Ca^{2+}]$. The half-maximal concentration of carbachol is between 1-10μM for these phenomena (Speakman *et al* 1987) and the intracellular Ca^{2+} "transient" exhibits an undershoot suggesting that the intracellular $[Ca^{2+}]$ falls below the resting value following excitation. This picture is similar to an undershoot following transients evoked by caffeine and can be explained by uptake of sarcoplasmic Ca^{2+} into intracellular stores, the sarcoplasmic reticulum. It is generally accepted that both the contraction and the rise of intracellular Ca^{2+} upon application of cholinergic agonists can be blocked by atropine suggesting mediation via a muscarinic receptor. The link between surface receptor activation and the release of intracellular Ca^{2+} could be mediated either by the production of second messengers, which diffuse to the intracellular Ca^{2+} stores, and/or depolarisation of the cell membrane to activate inward ionic currents, carried at least in part by Ca^{2+}. Several functional muscarinic subtypes (M_1-M_4) have been identified (section 6.1. p51) with the M_1 and M_3 subtypes coupled to hydrolysis of membrane phosphoinositides and the M_2 and M_4 subtypes involved in the inhibition of adenylate cyclase activity (Yang 1991).

The membrane events are linked to intracellular events by coupling systems. These can be pharmacological or electrophysiological. Pharmacological coupling includes the activation of second messenger systems and electrophysiological systems include depolarisation and opening of voltage regulated Ca^{2+} channels in the membrane. These processes may be

linked or able to influence each other and this is discussed in consideration of electromechanical coupling.

6.1. Intracellular messengers

Stimulation of M_3 muscarinic receptors in the urinary bladder drive the stimulation of phosholipase C. Phospholipase C catalyses the hydrolysis of membrane bound phosphoinositides with subsequent formation of inositolphosphates and 1,2,-diacylglycerol (DG). Inositol 1,4,5,-trisphosphate (IP_3) is released into the cytoplasm and this second messenger is thought to be responsible for the mobilisation of Ca^{2+} stores located in the sarcoplasmic reticulum (Andersson *et al* 1991).

Inositol 1,4,5,-trisphosphate production from the membrane can be summarised overleaf in figure I4;
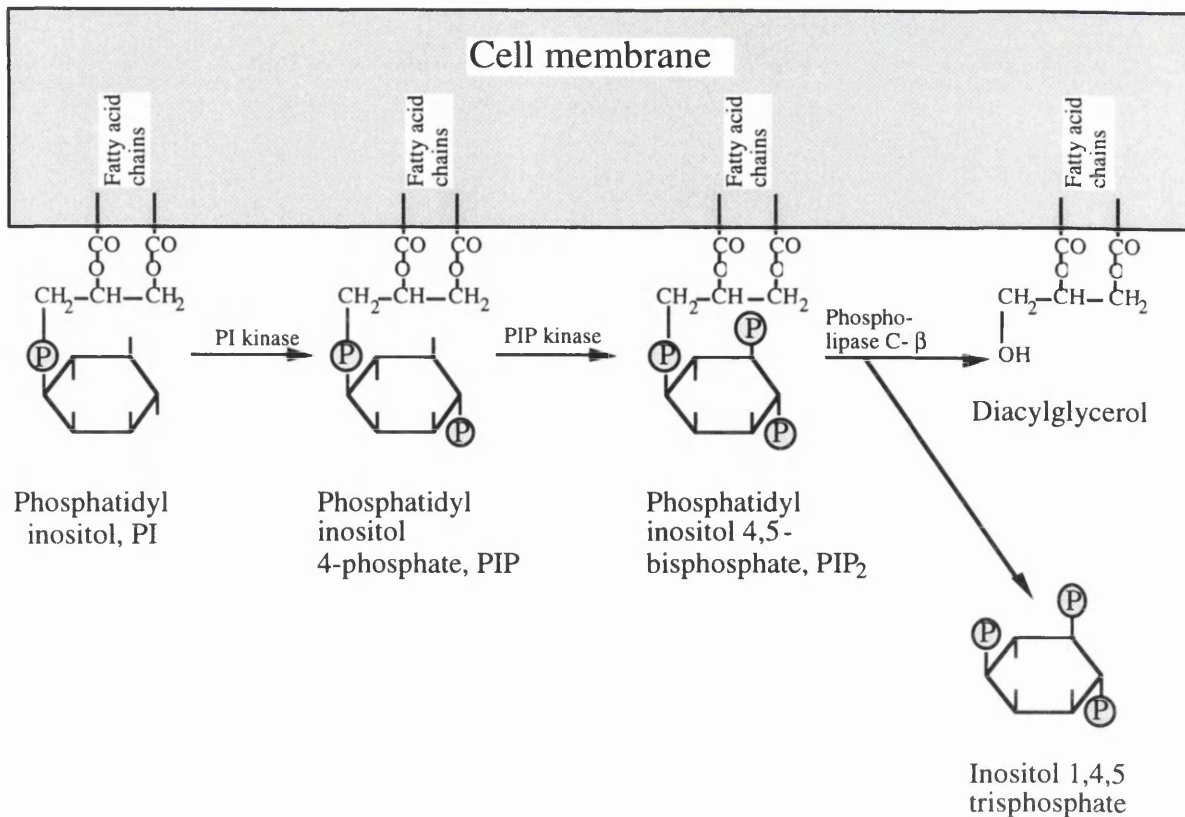
Figure I.4 The biochemical steps resulting in the production of the soluble second messenger inositol triphosphate from the membrane phospholipid phosphoinositol.

Evidence for such a diffusible second messenger mediated pathway has been found in detrusor smooth muscle in guinea-pigs, where muscarinic stimulation produced an accumulation of inositol phosphates (Iacovou *et al* 1989) determined by biochemical methods. Purinergic stimulation does not bring about such a rise in inositol phosphates (Iacovou *et al* 1990) and it was suggested that purinergic stimulation was more dependent on extracellular $[Ca^{2+}]$ than is muscarinic stimulation . Thus there is strong evidence for a role of the second messenger in tension generation mediated by the acetylcholine analogue, carbachol. Second messenger systems are also demonstrated in guinea-pig ureter, bladder and urethra (Wheeler *et al* 1995) by the measurement of inositol phosphates in these tissues in vitro following muscarinic and adrenergic stimulation. The quantity of inositol phosphates measured varied between the tissue types, possibly illustrating the specificity of

the cells and their different function enabling contraction-relaxation co-ordination in the lower urinary tract.

Recent measurements have determined that carbachol produces a half maximal stimulation of inositol phosphates at 8.3μM concentration in cultured human detrusor myocytes (Harriss 1995) and that the production of inositol phosphates in this preparation is linked to the stimulation of the muscarinic M_3 receptor, as M_3 receptor antagonists significantly antagonise the response generated by submaximal carbachol concentrations.

6.2. Detrusor electrophysiological events.

The mechanisms underlying action potential generation in all excitable tissues involve a sequence of voltage-dependent changes in membrane permeability, leading to cation influx down pre-existing electrochemical gradients. There is an initial ion flow leading to an inwardly directed current which is responsible for the depolarising phase of the action potential. This is normally followed by a switching off of the inward current and the activation of an outward repolarising current. There are two ionic species which are distributed so that there is a large electrochemical gradient which could generate inward current: Na^+ and Ca^{2+}. The equilibrium potentials for these ions is considerably positive to the membrane potential, so that an increase in their permeability will depolarise the cells. K^+ ions are usually involved in generating outward repolarising currents.

In fast conducting tissues such as nerve and striated muscle, the inward current is carried by Na^+, and since the concentration of Na^+ in the outside medium is large, the current flow per unit area of the membrane can be high. This leads to a rapid upstroke (up to 100V/s), and fast propagation of the signal. The Na^+ channels are blocked by tetrodotoxin (TTX), a highly specific blocker of these voltage sensitive Na^+ channels.

Smooth muscle does not need such rapid propagation of action potentials, and the upstroke of the action potential is caused by an inflow of Ca^{2+} ions through L-type Ca^{2+} channels. The Ca^{2+} based spikes have a much slower rising phase (less than 10V/s) because of the smaller current density flowing through the Ca^{2+} channels, are insensitive to lowering

extracellular Na^+, but reduced by lowering extracellular Ca^{2+}, and are blocked by the Ca^{2+} - antagonist drugs, e.g. nifedipine (Aidley 1989).

The precise role played by the detrusor action potential in the generation of detrusor tension remains uncertain despite the completion of many studies. Application of carbachol or acetylcholine to resting, isolated tissue does not always result in a membrane depolarisation large enough to reach action potential threshold (Wu *et al* 1995). There may be some involvement of L-type Ca^{2+} channels as nerve and muscle mediated phasic contractions can be partially inhibited by Ca^{2+} antagonists e.g. nicardipine (Fovaeus *et al* 1987). Hashtani has demonstrated an excitatory junction potential in guinea-pig detrusor preparations and this potential can be stimulated via the intramural nerves and blocked by ATP receptor desensitisation (Hashtani 1995). From these experiments a conclusion was drawn that nervous stimulation to the guinea-pig detrusor can release two neurotransmitters, ATP and acetylcholine, only the former of which has electrophysiological actions.

6.2. i) The detrusor action potential

The resting potential of isolated human detrusor smooth muscle is about -60mV and the cell is able to support a regenerative action potential when stimulated. The membrane potential value is near to the K^+ equilibrium potential and this supports the idea that at this resting potential the main permanent ion is K^+. The action potential has a depolarising phase of about 6V/s and an overall duration of the order of 10ms. Removal of extracellular Ca^{2+} immediately reduces the rate of depolarisation, whilst addition of K^+ channel blockers prolongs the duration without affecting significantly the depolarising phase (Montgomery *et al* 1992). These results from patch clamp experiments using isolated human detrusor myocytes, suggest that the depolarising phase is supported by Ca^{2+} influx and repolarisation is carried by an efflux of K^+ .

More direct evidence for the ionic nature of the action potential is available from voltage clamp experiments in isolated cells in guinea-pig where it is considered that Ca^{2+} influx through voltage operated Ca^{2+} channels is the key event in depolarisation- mediated

changes in $[Ca^{2+}]_i$ (Ganitkevich & Isenberg 1991). In human isolated detrusor smooth muscle cells Ca^{2+} influx is almost entirely mediated by L-type Ca^{2+} channels. This inward current is large enough to support the depolarising phase of the action potential. Extracellular $[Ca^{2+}]$ also affects the magnitude of the K^+, outward current. Decreasing extracellular $[Ca^{2+}]$ reduces the outward current magnitude and an increase in extracellular $[Ca^{2+}]$ increases this current. This suggests that the intracellular Ca^{2+} affects the K^+ current magnitude and could be interpreted as a protective effect on the cell as this modulation will prevent excessive rises in intracellular Ca^{2+}. An increase of Ca^{2+} influx through L -type channels will enhance repolarisation and thus limit the time that the Ca^{2+} channels are open.

6.2. ii) Electromechanical coupling

A relationship between the inward Ca^{2+} current and the activation of tension can be demonstrated in isolated detrusor smooth muscle cells. Several inotropic agents can modulate the magnitude of the L-type Ca^{2+} current and if the proportional increase or decrease of tension generated by these agents is compared to the same augmentation or depression of the L-type current a close linear dependence can be demonstrated (Fry, personal communication). Ganitevich has interpreted this as being evidence of Ca^{2+} induced Ca^{2+} release, whereby an influx of Ca^{2+} across the membrane through ionic channels can encourage a larger release from intracellular stores (Ganitkevich & Isenberg 1991).

The coupling of membrane events to intracellular Ca^{2+} release and the transient rise in intracellular $[Ca^{2+}]$ necessary to initiate contraction can be summarised in figure I5.
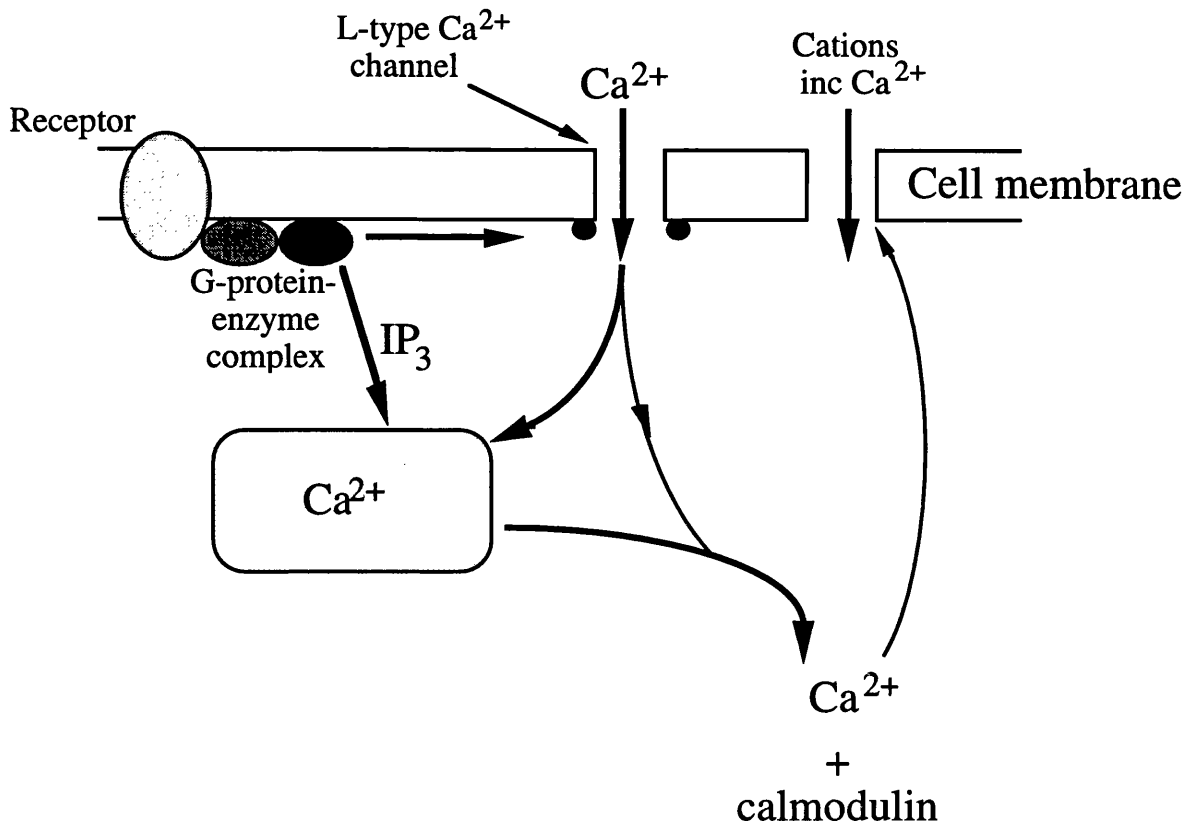
Figure I.5 A diagrammatic representation of the possible pathways in detrusor smooth muscle which determine the intracellular $[Ca^{2+}]$. The sarcoplasmic $[Ca^{2+}]$ can be raised by release of Ca^{2+} from intracellular stores. Ca^{2+} release can be stimulated either by production of soluble second messengers, such as IP_3, or by transmembrane Ca^{2+} influx through voltage operated L-type Ca^{2+} channels. The rise of intracellular Ca^{2+} may also secondarily open other ionic channels which show less specificity to cations but will also include a proportion of Ca^{2+} influx.

By discussing the initiation of smooth muscle contraction with particular reference to detrusor smooth muscle and realising the importance of Ca^{2+} ion metabolism within the cell and how $[Ca^{2+}]$ may be altered in the cell by the action of neurotransmitters we are able to consider various pathophysiological mechanisms that may underlie the aetiology of detrusor instability.

7. Overview of the current hypotheses regarding the aetiology of unstable detrusor contractions

As discussed, unstable bladder contractions detected by urodynamics in the absence of symptoms may be of no clinical significance. In clinical practice however the symptoms of an overactive bladder have profound affects on patients' lives (Jackson 1997). Unstable bladder contractions may occur in the presence of neurological disease, in the presence of obstruction or may be idiopathic; aetiological theories at present are not able to explain the pathophysiological processes for whole groups as discrete entities. Information has been collected in the study of the pathophysiology from clinical studies and many areas of basic science. An observation can be made from the clinical picture, an animal model or human model may be then used to test the hypothesis using whole animal, isolated multicellular or isolated single cell experimentation.

The core themes in the study of the pathophysiology include: a) an abnormality of the nervous control of the lower urinary tract, b) an obstruction to urinary flow c) a disease process which causes an alteration of the excitability or contractility of the detrusor smooth muscle and d) a change in the metabolism of the detrusor smooth muscle.

7. 1. Unstable detrusor contractions in the presence of neurological disease

The aetiology of uninhibited contractions in the presence of neurological disease, detrusor hyper-reflexia, depends on the anatomical position of the neurological defect. Cerebral cortex abnormalities lead to uninhibited bladder contractions, the micturition reflex is intact because the lesion is above the pontine micturition centre, (section 2.1.(iii) p31) and voiding even when completely involuntary is physiologically co-ordinated (Amis *et al* 1991). Other lesions in the suprasacral spinal cord disrupt the micturition reflex as the lesion is below the pons. Voiding in these patients is largely uncoordinated and may co-

exist with urethral sphincter dysynergia causing high pressure detrusor contractions against this closed sphincter which may lead to ureteric reflux and renal damage.

Other neurological disease processes are also included in this group despite similarities to idiopathic and obstructed detrusor instability, e.g. Multiple System Atrophy. In a study of 62 patients with the disease, 56% had been seen by a gynaecologist or urologist prior to the correct neurological diagnosis being made (Fowler 1998). The early development of incontinence and the severity of it is due to a number of factors including detrusor hyper-reflexia, loss of parasympathetic innervation of the detrusor muscle, leading to poor contractility, and incomplete emptying.

7. 2. Unstable bladder contractions in the absence of neurological disease.

7.2.(i) Detrusor instability in the presence of urethral obstruction.

Urethral obstruction is the only proven aetiological factor for the development of detrusor instability, based on clinical evaluation, but this is only accepted in relation to male prostatic obstruction. Studies have shown an incidence of detrusor instability in men with benign prostatic hypertrophy of 60% prior to transurethral resection of the prostate with this figure falling to 25% following treatment (Abrams *et al* 1979); similar results were published by others (Pope *et al* 1990). The same response to relief of outflow obstruction in the experimental pig model is also observed (Speakman *et al* 1991). Outflow obstruction occurs following endoscopic bladder neck procedures for female stress incontinence (Pope *et al* 1990) with reduced urinary flow rates and higher micturating detrusor pressures measured after a year. 15% of these previously urodynamically stable patients exhibited unstable detrusor contractions associated with symptoms a year later, requiring treatment.

Despite the acceptance that in men urethral obstruction is an aetiological factor in the development of detrusor instability the cellular mechanisms that occur to effect the excitability or contractility are unknown. Obstruction, as discussed, causes a largely reversible condition, the cause of the irreversible detrusor instability following

58

prostatectomy in some men or relief of urethral obstruction in some women is unexplained, and it is probable that the obstruction was accentuating a pre-existing idiopathic instability.

7.2. (ii.) Detrusor instability in the absence of urethral obstruction.

Detrusor instability not associated with outflow obstruction and in the absence of neurological disease is termed idiopathic. By definition it has no known proven aetiological factors. Devising animal models which accurately exhibit this condition is more difficult than models exhibiting obstruction. One model has been attempted using circumferential supratrigonal bladder transection in pigs (Sethia *et al* 1990). Such transection resulted in the development of detrusor instability in all pigs within the study. This is the only animal model and so much of the experimental work to determine the contractility and excitability of idiopathic instability has come from human sources and is scarce.

7.3 Excitability

Unstable detrusor contractions may result from an increase in the bladder's response to nervous stimulation or its excitability. The excitability of a smooth muscle preparation may be investigated by exposing an isolated smooth muscle strip to electrical field stimulation in an organ bath and measuring the force of any contraction generated. The response to increasing frequencies of stimulation, at a short pulse width, indicates the response to nerve mediated stimuli. Tetrotodoxin (a nerve blocking agent) would block any response if the stimulus affects the nerve only, but unable to block any direct muscle stimulation. The effect of disease on detrusor smooth muscle has been investigated in this way and conflicting reports suggest diverse opinion in this subject.

Human detrusor smooth muscle shows an increased sensitivity to field stimulation (i.e. greater response to lower frequencies of stimulation) using biopsy samples from patients with idiopathic detrusor instability and detrusor hyper-reflexia, as compared to control, stable bladder samples (Kinder & Mundy 1987; Eaton & Bates 1982). This greater degree

of excitability suggests that lower levels of parasympathetic activity are sufficient to cause contraction in the unstable detrusor.

In patients with urodynamically proven detrusor instability there is a rightward shift in force/frequency compared to control isolated detrusor smooth muscle (Harrison 1987). It was implied that such a decrease of excitability was a feature of detrusor instability alone. The presence of obstruction may be the only identifiable difference to account for these discrepancies.

Animal work shows appreciable species differences in relation to excitability. Animal models of obstruction have been described in the pig, guinea-pig, rat, rabbit and cat. The presence of obstruction is associated with urodynamic instability in the pig (Sibley 1985, Speakman *et al* 1987), in the rabbit (Harrison *et al* 1990) and the rat (Malmgren *et al* 1990), and with increased voiding pressures in the cat (Kato 1990) but no cystometric studies were reported in the rabbit and rat comparison (Levin *et al* 1993) or the guinea-pig studies (Seki *et al* 1992).

Decreased excitability in obstructed rabbit bladders was shown by a shift of the force/frequency relationship to the right (Levin *et al* 1993, Harrison *et al* 1990). The obstructed pig model exhibiting detrusor instability also showed such a decreased excitability (Sibley 1985) to nerve mediated stimulation. The rat (Levin *et al* 1993, Malmgren *et al* 1990), and cat studies (Kato *et al* 1990) all showed no alteration in the excitability of the isolated muscle strips from obstructed bladders compared to a control, unobstructed group. These results illustrate the species differences and although all groups were obstructed (in relation to their controls) it was impossible to determine if all were exhibiting unstable detrusor contractions.

7.4. Contractility

Various factors will alter the contractile function of detrusor smooth muscle.

i) The excitability of the muscle, this is discussed in the previous section.

ii) Hypertrophy.

iii) Denervation.

iv) Supersensitivity to neurotransmitters

v) Atropine resistance.

7.4.ii) Hypertrophy.

Cellular hypertrophy has been shown to exist in all types of human detrusor instability although to differing extents (Gallegos & Fry 1994). Samples from patients with partially obstructed bladders exhibit the greatest degree of hypertrophy but samples from patients with hyper-reflexic and idiopathic detrusor instability show a significant increase in cell surface area when compared to controls. In general obstruction causes hypertrophy as a compensatory response (Gosling *et al* 1986). However this response is different from the compensatory response of diabetic rats who exhibit hypertrophy as a direct consequence to cope with the fourfold increase in urine production (Uvelius *et al* 1986). This change in cell size in obstruction is associated with a change in cyto-skeletal make-up and an increase in the actin/myosin ratio in both human obstructed samples and rat models of hypertrophy secondary to urethral obstruction. There is also a decrease in the percentage volume of mitochondria in hypertrophied cells from obstructed rats (Gabella & Uvelius 1990). Connective tissue infiltration occurs in association with the hypertrophy and the amount of infiltration is proportional to the extent of the hypertrophy (Gilpin *et al* 1985).

7.4.iii) Denervation.

Autonomic denervation occurs in both animal obstructed models and human unstable bladders. There is a decrease in the cholinergic receptor density in hyper-reflexia (Restorick & Mundy 1989) in humans and this decrease in nerve receptor density is also apparent in the obstructed pig model. This decrease is inversely proportional to the length of time the obstruction was in place (Speakman *et al* 1987) and was in this case associated with an increase in sensitivity to muscarinic agonists. A denervated model of non- obstructed instability has been developed by transecting the autonomic nerve supply to the pig bladder and noting the development of unstable detrusor contractions again associated with a

61

supersensitivity to muscarinic agonist (Sethia *et al* 1990). This denervation in obstructed bladders is partly reversible after relief of the obstruction (Cumming & Chisholm 1992; Seki *et al* 1992). Such regeneration of nerves is also apparent following denervation of the bladder from rectal excision (Neal *et al* 1982).

7.4. iv) Supersensitivity to neurotransmitters

The magnitude of the isolated detrusor smooth muscle contraction to varying concentrations of muscarinic agonist quantifies the contractility of the preparation with respect to the agonist. Human studies have shown an increased sensitivity of obstructed unstable bladder samples to muscarinic agonists when compared to control bladders. There was a loss of nerve mediated contraction and the electrically stimulated tension / carbachol stimulated tension ratio decreased, implying denervation (Harrison *et al* 1987; Smith & Chapple 1994). These results were in conflict with previous work (Eaton and Bates 1982) which demonstrated no difference in the sensitivity of obstructed bladder samples to muscarinic agonists. Different animal species support each of the conflicting views. The obstructed pig model exhibited increased sensitivity to muscarinic agonists (Sibley 1987; Speakman *et al* 1987; Sethia *et al* 1990) as did the obstructed rabbit model (Harrison *et al* 1990). A moderate obstruction in the rat produced an increase in sensitivity but a severe obstruction was not different from control (Saito *et al* 1993). The obstructed bladder (but without an indication of the severity of obstruction), had the same sensitivity to muscarinic agonist as control, (Malmgren *et al* 1990) and these results were confirmed in the cat (Kato *et al* 1990).

Hyper-reflexic bladders are described by some authors to exhibit increased sensitivity (German *et al* 1993; Saito *et al* 1993), but others describe no difference with respect to control (Kinder & Mundy 1987; Nurse *et al* 1991 and Fry & Palfrey 1986). Detrusor from bladders with idiopathic instability showed no increase in muscarinic agonist sensitivity (Kinder & Mundy 1987).

7.4.v) Atropine resistance

Several authors have demonstrated the presence of atropine resistance (a small residual contraction of the field stimulated detrusor muscle strip in the presence of this anti-cholinergic drug). This observation has been described in detrusor samples from both normal and diseased bladders (Ruggieri *et al* 1990; Saito *et al* 1993). The presence of atropine resistance is of physiological significance in respect to the identification and role of other neurotransmitters in the detrusor (section 5.2.p48-49). Other authors have demonstrated no atropine sensitivity (Kinder & Mundy 1987; Palfrey *et al* 1984).

These differences in experimental results highlight that the underlying mechanism to detrusor instability is probably not simple. However attempts have been made to identify a common feature, denervation supersensitivity, present in cases of instability from whatever cause, (Brading & Turner 1994).

However detrusor instability is not completely explained by this "denervation supersensitivity" as it has been shown that supersensitivity to muscarinic stimuli is not demonstrated by non-obstructed human bladder samples. Hypertrophy and autonomic denervation appear to occur in all types of instability in both animals and humans, in the presence or absence of obstruction. Effective bladder and urethral function is not only dependent on the integrity of the autonomic innervation but also on the intracellular metabolism.

7.5. Detrusor smooth muscle metabolism

Detrusor smooth muscle in common with other smooth muscle relies heavily on aerobic metabolism. This is in contrast to fast skeletal muscle which requires short bursts of energy production and therefore requires most of its energy production to be derived from anaerobic metabolism (Haugaard *et al* 1987). This skeletal muscle anaerobic metabolism

requires a high level of glycogen, a substance that is scarce in normally functioning detrusor smooth muscle.

Detrusor smooth muscle relies on the breakdown of high energy phosphates derived from oxidative phosphorylation to contract and the initiation of contraction utilises more intracellular energy than the maintenance of contraction (Levin et al 1988). The substrate supplying the metabolism of high energy phosphates is predominately glucose (Levin et al 1981).

Specific cellular functions appear to utilise energy derived from selective sources such as glycolysis, oxidative phosphorylation or cytosolic ATP hydrolysis and this is referred to as functional compartmentation (Levin et al 1995). Bladder metabolism is characterised like that of most smooth muscle by substantial lactate production even under aerobic, resting conditions (Haugaard et al 1987). This observation provides evidence for the functional compartmentation of the metabolic control of contraction and other cellular processes in smooth muscle. Separating energy sources into these functional compartments can explain how specific contractile and cellular processes can be supported by aerobic, or adapt to anaerobic metabolism. This evidence has come from studies in which the inhibition of either glycolysis or oxidative phosphorylation specifically inhibits different intracellular energy utilisation processes (Levin et al 1983). These experiments showed

a) the contractile response to bethanecol (a muscarinic agonist) is biphasic in nature consisting of an initial rapid phasic rise in tension followed by a prolonged period of sustained tension;

b) the bladder's ability to empty is related to the sustained phase of the bladder body in response to muscarinic stimulation (Foveus et al 1987);

c) both the magnitude of the phasic contractile response to bethanecol and intracellular ATP concentration decrease gradually in the presence of hypoxia, the sustained phase of the contraction, and therefore its ability to empty is lost immediately on the initiation of hypoxia and reversed almost immediately following the removal of hypoxia (Levin et al 1983). These observations on in vitro preparations support the concept that sustained tension is supported by oxidative energy rather than by cytosolic ATP.

Cytosolic ATP levels are maintained by combined anaerobic metabolism of glucose to pyruvate and oxidative metabolism of pyruvate to CO_2 and H_2O within the mitochondria. Partial outlet obstruction has been shown to cause a shift from aerobic to anaerobic metabolism as demonstrated by the shift in glucose metabolism from CO_2 to lactic acid generation (Kato *et al* 1990). There is a marked decrease in the metabolism of pyruvate (mitochondrial substrate) to CO_2.

The cellular mechanism responsible for decreased oxidative metabolism involves decreased mitochondrial enzyme activity (Levin *et al* 1995). The decrease in enzyme activity due to partial outlet obstruction is either a result of a decrease in the number of mitochondria in the hypertrophied muscle, or a decrease in mitochondrial enzyme activity in each separate mitochondria

8. Ischaemia and detrusor function

It may be seen from the above that oxidative phosphorylation is reduced in partially obstructed bladders in animals. This decrease in activity is thought to be responsible for the decrease in contractile function during the sustained tension phase of bladder emptying, i.e. a decrease in aerobic activity will decrease the ability of the bladder to function properly. It is also known that unstable detrusor contractions are generated in obstructed bladders which have an associated poor ability to empty, although there is no clinical correlation between residual urine volumes and severity of the unstable contractions. Hypertrophy of detrusor cells may be enough to cause a relative lack of mitochondria but since different amounts of hypertrophy exist with non-related severity of symptoms, idiopathic detrusor instability and hyper-reflexia have a milder increase in cell size than obstructed bladders, so this does not explain the total effect. A lack of mitochondrial substrate must also be involved.

Ischaemia of the bladder prevents the bladder from receiving an adequate supply of oxygen (a tissue hypoxia) and also prevents adequate removal of waste products from the bladder.

Bladder blood flow decreases exponentially with increasing intravesical pressure (Dunn 1974). High detrusor pressures found in detrusor instability and detrusor hyper-reflexia may be enough to decrease blood flow (Azadzoi *et al* 1996) and that bladder filling is associated with a reduced blood flow as well as reduced PO_2 tension and pH in the bladder wall (Bellringer *et al* 1994; Batista *et al* 1996). Impaired arterial flow may also occur from systemic disease e.g. atherosclerosis, embolization, impaired venous outflow and thrombosis or by iatrogenic ligation (van Arsdalen *et al* 1983). More subtle mechanisms such as overdistension are probably more common. Overdistension and high intravesical pressures are not necessarily synonymous and overdistension accompanied by relatively low pressures have been shown to induce an impairment of blood flow.

Animal experiments investigating the effect of ischaemia on detrusor muscle function have been conducted in vivo by the ligation of one or both of the vesical arteries in rabbits and studying the effects of ischaemia. Complete ischaemia for one hour produced a 90% reduction in contractile force of the detrusor muscle and an inability of the rabbit bladders to empty more than 50% of their bladder volume and a decrease in the bladder compliance (Lin *et al* 1988). Long term effects suggest that although there is a recovery of function following this one ischaemic episode the is some cell damage which leads to fibrosis and a decrease in the functioning tissue mass.

Experiments show other smooth muscles are affected in a similar way to the presence of hypoxia. Guinea-pig taenia coli smooth muscle shows a close correlation between ATP production and tension generation and this tension is significantly reduced in the presence of reduced oxygen tension in the superfuste in experimental conditions (Ishida & Paul 1990). Exceptions are the pulmonary vascular smooth muscle that show an increase of force in the presence of hypoxia and is said to exhibit an oxygen sensing mechanism (Yaun *et al* 1993).

The aim of this thesis is to explore further the hypothesis that a derangement of detrusor smooth muscle metabolism, specifically cellular hypoxia, may be involved in the development of detrusor instability. In the normally functioning bladder, as it fills with urine, the blood flow to the bladder becomes reduced. This has been directly measured in the human bladder (Batista *et al* 1996) using laser Doppler flowmetry. This Doppler flowmetry demonstrated in 10 endoscopic cases that as the bladder was successively filled this resulted in a concomitant, successive reduction of mean blood flow. These findings were supported by experiments on anaesthetised dogs where bladder filling caused a significant decrease in bladder wall blood flow and oxygen tension with or without outlet obstruction. (Azadzoi *et al* 1996).

As the bladder fills the delivery of oxygen to the muscle tissue is reduced, so if the bladder overdistends it is reasonable to suggest that blood flow to the tissue further decreases.

Urethral outlet obstruction from hypertrophy of the prostate gland can lead to overdistension of the bladder, with or without high detrusor pressure generation. Neuropathic bladders from spinal cord injuries initially have a period of overdistension and patients with multiple sclerosis have great difficulty in adequately emptying the bladder and are frequently left with overdistended bladders. An episode of acute urinary retention prior to prostatectomy in obstructed bladders in men has been linked to poor relief from detrusor instability post operatively (Abrams 1979).

Bladder overdistension is a particular problem for women particularly in the antenatal period. Voiding difficulties have been shown to be common , 25% of an antenatal population complaining of a poor stream and 30% of incomplete emptying in early pregnancy (Cutner 1992). Urinary retention in pregnancy is rare and is usually due to an impacted retroverted uterus classically occurring at 16 weeks gestation (Cardozo & Cutner 1997), but in clinical practice occurs more commonly after delivery especially in connection with the use of epidural analgesia during labour. Post operative urinary retention can complicate most gynaecological surgery, occurring in 31% of women following total

abdominal hysterectomy (McCullough *et al* 1997). Functional damage is shown in bladders from women who have suffered a period of acute retention of urine (Mayo *et al* 1973) and this later resulted in the development of unstable detrusor contractions.

9. Hypoxia and the development of detrusor instability.

It has been discussed that ischaemia of the bladder may cause a lack of mitochondrial substrate and a subsequent decrease in the amount of energy supplied by oxidative phosphorylation for the initiation and maintenance of contraction. Ischaemia can be caused by overdistension of the bladder or by high detrusor pressure. If idiopathic instability produces similar symptoms to other types of instability it is reasonable to suppose a similar process i.e. hypoxia from reduced oxidative phosphorylation caused by bladder ischaemia or from some abnormality of mitochondrial activity may be involved.

Ischaemia of any organ prevents both the supply of substrate and the removal of waste products, any study of ischaemia on contractile function will therefore be complicated by many variables and may be imprecise. By blocking oxidative phosphorylation in the mitochondria specifically it is possible to study the effect of cellular hypoxia on detrusor smooth muscle function, minimising confounding variables. Thus investigating cellular hypoxia as an aetiological factor which may be responsible for the findings occurring in clinical ischaemia.

In this work patients from two patient groups have been selected, those with urodynamically stable or unstable bladders. The effect of cellular hypoxia was studied on each group of biopsies

68

10. Hypothesis

1. That cellular hypoxia exerts an effect on human detrusor contractile function.

2. Isolated human detrusor smooth muscle samples taken from patients with symptomatic detrusor instability (as defined by standardised conventional urodynamics) may show a difference in the effect of cellular hypoxia on contractile force to those isolated from patients with no such detrusor contractions.

3. Cellular hypoxia is associated with a derangement of intracellular Ca^{2+} regulation.

The Null hypothesis as applied to any of the above would be that hypoxia exerts no effect on the experimental variables.

11. Outline of the thesis

The aim is to study the effect of cellular hypoxia on the force of contraction of human isolated detrusor smooth muscle.

To determine if there is a difference between biopsies from stable and unstable bladders in this respect.

To determine the cellular mechanism that may be responsible for any change in function, in particular changes to intracellular Ca^{2+} regulation.

To demonstrate tissue hypoxia and anaerobic metabolism in the cell

To determine the pH of the cell in the presence of tissue hypoxia.

METHODS

The experiments described in this thesis were designed to investigate the effect of cellular hypoxia on human detrusor smooth muscle and investigate the possible mechanisms responsible for that effect with particular reference to intracellular Ca^{2+} metabolism. Experiments used either isolated detrusor muscle strips or isolated detrusor myocytes. All experiments used detrusor muscle samples from human bladders and were performed at the Institute of Urology and Nephrology, University College, London.

1. Urodynamic investigation, patient selection and sample collection.

1.1. Urodynamic Investigation

Urodynamic studies were performed in two centres, the United Elizabeth Garrett Anderson and Hospital for Women, Soho (the E.G.A.) and the Middlesex Hospital. Studies at the E.G.A. were performed by myself using a Lectromed 5 channel urodynamic recorder UIS 5000 (Lectromed, Letchworth, U.K.) and at the Middlesex by two research registrars using a computerised Lectromed Urodynamic Investigation System 6000 (Lectromed, Letchworth, U.K.). Both machines were serviced regularly by the same technician from the supplier and all of the investigators including myself had been taught to use the equipment by more than one consultant. We had each performed urodynamic studies in three hospitals using different machines and were regularly performing more than ten urodynamic studies a week at the time of this work. The method of performing the studies was the same in both centres. All of the urodynamic traces were reviewed by myself and two consultants, the urodynamic findings were considered in reference to the patients clinical picture and not in isolation.

A full history was taken from the patient and a clinical examination, including a neurological examination, performed prior to the urodynamic assessment. The patient was

asked to void into the flowmeter in private and then catheterised, the residual volume of urine noted, if any. Fluid filled pressure catheters were introduced, one into the bladder alongside the urethral catheter and another, to estimate abdominal pressure, into the rectum. All pressures were equilibrated to atmospheric pressure the transducers were positioned at the level of the symphisis pubis. The pressure recording was checked by asking the patient to cough initially and periodically throughout the study.

The bladder was filled with normal saline at room temperature at a rate of between 60 to 80mls per minute with the patient in the supine position. The patient was questioned regarding bladder sensation, pain with filling and urgency and the responses recorded on the trace. The bladder was filled until comfortably full, the maximum voided volume from the voiding diary being a guide, care was taken not to overdistend the bladder. The filling catheter was then removed and the patient asked to stand (the position of the transducers adjusted to remain at the level of the symphisis pubis) and cough. Then with the patient seated at the flowmeter the cough was repeated and voiding asked to commence.

At the end of the study the fluid filled pressure catheters were removed, the patient dressed and the findings were discussed with them both at that time and later in a follow-up appointment.

1.2. Patient Selection

Patients undergoing routine urological or urogynaecological surgery and who had previously undergone a urodynamic assessment were considered for inclusion in the study. The patients were grouped according to their urodynamic findings in accordance with the I.C.S. standardised terminology (Abrams *et al* 1988). All patients had unobstructed voiding and urodynamic evidence of detrusor activity, patients with no demonstrable detrusor activity were excluded. Patients who had undergone irradiation in the pelvis for the treatment of pelvic malignancy were also excluded in order to achieve a cohort of well matched samples. The ages of the patients ranged from 23 years to 64 years. Those patients receiving medication for the treatment of detrusor instability stopped consumption for the fortnight preceding the operation. In all cases the surgery was part of the normal

clinical management of their particular circumstances. All patients had an opportunity to read an information sheet and informed consent was obtained in accordance with U.C.L. Hospitals Ethical Committee guidelines.

The samples were either considered as a single group or were grouped according to the urodynamic findings of the patients as either stable or unstable. The stable group consisted of patients with no history of urgency and urge incontinence who had no phasic rise of detrusor pressure during the filling phase of conventional urodynamics, with normal compliance and who exhibited normal voiding detrusor pressures, these patients were diagnosed as having genuine stress incontinence. The unstable group were patients who exhibited phasic detrusor contractions associated with a sensation of urgency, during the filling phase of conventional urodynamics, who also had normal bladder compliance and unobstructed voiding. All patients were able to void completely and no patients had a co-existing urinary tract infection either at the time of urodynamic investigation or at operation.

1.3. Sample Collection

Biopsy specimens were taken endoscopically from the walls or dome of the bladder using Storz biopsy forceps, the trigone area of the bladder was avoided because of the known physiological differences of this region to the dome (Speakman *et al* 1988). Biopsies were also taken from the walls or dome of the bladder from patients undergoing open bladder surgery or suprapubic catheterisation following an abdominal bladder neck suspension.

2. Tissue preparation.

2.1. Isolated human detrusor smooth muscle strips.

Tissue samples were transported from the operating theatre in essentially Ca^{2+} free Tyrode's solution (HEPES-Tyrode's, a modified Tyrode's solution see Table 2, p77) at room temperature and taken to the laboratory as soon as was practicable for processing. The detrusor sample was then transferred to a dissection dish containing normal Tyrode's solution (Table 1, p76) and fixed to the base of the dish with fine syringe needles. This

ensured that there was little movement of the sample during dissection and thus minimised handling. The mucosa and serosa (in the case of full thickness biopsies) were carefully removed using fine dissecting scissors, or by blunt dissection which appeared to remove the submucosal connective tissue in addition to the mucosa, under the magnification of a microscope (Nikon AL5; Nikon Corporation, Tokyo, Japan). The appearance of detrusor smooth muscle is quite distinctive even to the naked eye as it is of a pearly white colour and has a translucent quality. It was possible in most cases to dissect along a longitudinal arrangement of muscle bundles to form strips of muscle <0.5mm diameter, <5mm length. If the biopsy was very small after the mucosa was removed the whole muscle content of the biopsy was tied to form a strip preparation (Palfrey *et al* 1984). These strips were used for tension recording.

2.2. Isolated human detrusor myocytes

Experiments with single cells were performed using human detrusor myocytes. The technique for the enzymatic digestion of human detrusor muscle to obtain viable single myocytes was described and established in this laboratory (Montgomery & Fry 1992; Gallegos & Fry 1994) and this protocol once mastered, was consistently successful in producing viable single myocytes.

Human detrusor was excised in the same way as preparing muscle strips save that Ca^{2+} free Tyrode's solution was placed in the dissection dish during the removal of serosa and mucosa instead of normal Tyrode's solution. Strips of muscle 5mm by 0.5mm were dissected and placed in 1ml centrifuge tubes (Sarstedt, Microfuge; Germany) containing a 1:4 dilution of an enzyme solution by Ca^{2+} free Tyrode's solution, (table 3 p77). A maximum of two strips were added to each tube. The tubes were refrigerated at 4° C overnight for incubation the following day. As many samples were not obtained before 4pm this part of the protocol was more for convenience rather than technical necessity. Samples stored in this way would produce viable cells up to 30 hours later.

The following morning the vials were placed in a prewarmed heating block (Grant Instruments Ltd., Cambridge, U.K.) at 37°C for 30 minutes. The supernatant was discarded, the strips washed in Ca^{2+} free Tyrode's solution and then placed in 1ml fresh Ca^{2+} free Tyrode's solution in a polypropylene vial (centrifuge tube as before). The digested tissue had the appearance of mucus and was slimy and difficult to transfer between vials. Dissecting the muscle into strips allowed greater ease of transferability and did not appear to reduce the cell yield.

The digested tissue was carefully chopped into pieces by repeated bisection and the tissue was then gently triturated using a glass pipette with a small volume rubber bulb. The, pipette had a short shank (10cm) and a fire polished tip, but was not silanised to render the inside water repellent, as is necessary for handling cardiac myocytes. After gentle trituration for about 5 minutes, taking care not to injure the cells by introducing air bubbles, a small volume of cell suspension (0.2ml) was placed onto the glass cover-slip which was the base of the superfusion bath and left to settle for 20-30 minutes. Viable cells settled and stuck to the cover-slip and many of these stayed adherent to the glass to allow superfusion and experimental investigation.

3. Solutions and chemicals

In all experiments a modified Tyrode's solution (Tyrode's) was used to superfuse the preparation, as a control and for equilibration before experiment. The composition is shown in table 1. Fresh Tyrode's solution was made up daily in deionized water from Milli-RO 10 plus (Millipore, Croxley Green, Watford, UK). The constituents were added in solid form or from 1M stock solutions (KCl, $MgCl_2$, $CaCl_2$, and NaH_2PO_4). The stock solutions had been made up in Analar grade water and stored at 4°C in a refrigerator for less than 4 weeks except $CaCl_2$ which was purchased as a 1M solution (BDH Chemicals Ltd.). The compounds in Tyrode's solution were Analar grade agents from BDH Chemicals Ltd. (Poole, Dorset, UK). Solids were weighed using the "Basic" balance (Sartorius Ltd. Epsom Surrey) and liquids were measured using calibrated, fixed or

variable volumetric pipettes. Changes to the Tyrode's solution formed the basis of the interventions performed in the experiments. These changes were made by the addition of relevant reagents.

Carbachol was added to form various concentrations from a stock solution (10mM). The stock solution was prepared using carbamylcholine chloride solid powder (Sigma Chemicals Poole, Dorset) and Analar grade water. The stock solution was refrigerated at $4^{o}C$ and discarded if not used within 4 weeks. The required concentrations of carbachol solution were created by dilution of aliquots of stock solution with freshly made Tyrode's. Potassium chloride solution was also prepared from a stock solution, the same used to prepare Tyrode's solution to yield the required final concentrations.

Other intervention solutions were prepared by the addition of Na cyanide dry granules (BDH Chemicals Ltd.) and caffeine (Sigma Chemicals) to Tyrode's solution. Na cyanide was handled with care, wearing gloves but by using granules the solutions did not need to be made up in a fume cupboard and was treated with the same respect as any other laboratory chemical. Any spills would have to have been washed with copious amounts of water and as an additional safety measure solutions were not stored.

An ungassed, nominally calcium free Tyrode's solution (HEPES-Tyrode's, table 2) was utilised for the collection and transportation of bladder biopsies from the operating theatre to the laboratory. If samples required storage for less than 24 hours before tension recording they were stored in this medium in a refrigerator at $4^{o}C$.

A wide variety of methods have been described to isolate smooth muscle cells from animals (Benham et al 1985; Klockner & Isenberg 1985a; Lang 1989) and a method based on certain aspects of these protocols was devised (Montgomery & Fry 1992) to produce viable isolated human detrusor myocytes. The enzyme mixture used in these experiments is the one described by Montgomery & Fry (1992) and produced consistent results with no alteration to the recipe. Collagenase-based digestion techniques have been widely used and these theoretically seem the most appropriate as the bladder contains a high proportion of collagen (Gilpin et al 1985). However the efficacy of the different batches of collagenases varies and so the collagenase used for the digestion mixture throughout these investigations

was kept the same, the constituents are shown in table 3. The collagenase had an activity of $239u.mg^{-1}$ (Batch number F9S4444, Worthington Biochem Corp, New Jersey, USA). All other compounds were obtained from Sigma Chemical Co Ltd. (Poole, Dorset, UK). In this work the batch number of the various enzymes remained the same throughout the experimental period.

The enzyme constituents were added (in solid form) to HEPES-Tyrode's and the mixture was prepared in 'bulk' to reduce errors in weighing and stored for up to 4 weeks in 1ml aliquots frozen at -4°C. Although the dissociating properties of the enzyme solution declines during storage there was no appreciable lack of efficacy over the 4 week period of frozen storage. Consecutive enzyme mixtures showed apparently equal efficacy in this experimental period

Collagenase activity is dependent on the presence of Ca^{2+} ions (Bond & van Wart 1984) and so the enzyme mixture was constituted in Ca^{2+} free HEPES-Tyrode's to avoid an excessive Ca^{2+} concentration and subsequent over-digestion of the sample which gave poor cell yields.

Table M1. Composition of normal Tyrode's solution. The solution was made up in deionised water and were continually gassed with 95% O_2 : 5% CO_2 maintaining a pH of 7.33±0.02

Chemicals	Concentration mM
NaCl	118.0
NaHCO$_3$	24.0
KCl	4.0
MgCl$_2$.6H$_2$O	1.0
NaH$_2$PO$_4$.2H$_2$O	0.4
CaCl$_2$.6H$_2$O	1.8
Glucose	6.1
Na pyruvate	5.0

Table M2 Composition of HEPES-Tyrode's solution. The solution was made up in deionised water and the pH adjusted to 7.1 with aliquots of NaOH

Chemicals	Concentration mM
NaCl	105.4
$NaHCO_3$	22.3
HEPES	19.5
KCl	3.6
$MgCl_2.6H_2O$	0.9
$NaH_2PO_4.2H_2O$	0.4
Glucose	5.4
Na pyruvate	4.5

Table M3

Composition of the digestion enzyme mixture. Constituents were added to HEPES Tyrode's solution.

Compound	Concentration mg/ml
Collagenase	2.0
Hyaluronidase type I-S	0.5
Hyaluronidase type III	0.5
Antitrypsin type II-S	0.9
Bovine albumin	5.0

4. Isometric tension recordings

4.1. Tissue mounting and equipment set up.

The effect of tissue hypoxia on the force of contraction generated by carbachol, was studied by recording isometric tension from isolated human detrusor smooth muscle strips. Strips of smooth muscle were dissected from specimens of human bladder tissue as previously described. The strips were tied with surgical suture thread (Persalls sutures, cornea silk, US 7/0) and transferred to the superfusion trough. This horizontal Perspex trough was 3mm wide with a static hook in its base. The sides of the trough contained parallel plate platinum electrodes. The muscle strips were mounted in the trough at the proximal end to the static hook, the distal end being tied to the hook from the isometric force displacement transducer (Model FT.03, Grass instrument Co. USA). Care was taken to ensure the transducer hook was only in contact with the muscle preparation and in no way touching the trough as this prevented accurate tension recording. The muscle strip was completely immersed in the flow of the superfusate to allow for maximum diffusion of agents and to prevent tissue damage from exposure to the air. Transfer of the tissue from the dissection dish to the trough was required to be as swift as possible, again to prevent tissue exposure to the air being in excess of a few seconds.

The force transducer was mounted on a micro-manipulator (Prior Instruments Ltd, Bishop's Stortford, Hertfordshire, U.K.) so that the resting length of the muscle strips could be adjusted. The apparatus was mounted on a heavy metal frame, standing in tubs of sand in order to minimise external vibration. The force transducer output was connected to a bridge amplifier with variable gain and high frequency cut-off at 5 Hz (ORMED Amplifier 5000 Series; ORMED Ltd. Welwyn Garden City, Hertfordshire, UK). The

output of the amplifier was connected to a chart recorder with a low pass filter of corner frequency 10Hz (Multitrace 2; ORMED Ltd. Hertfordshire , UK).

Stimulation pulses were generated by a stimulator (Stimulator Model 200, Palmer Bioscience, Sheerness, Kent) which was gated by a programmer (Model 150, Palmer Bioscience, Sheerness, Kent), and delivered to the preparation via platinum electrodes.

The superfusate solutions were equilibrated with 95% O_2 :5% CO_2, maintaining a pH of the superfusate of 7.33 ± 0.02, and warmed in a water tank at 37°C and delivered to the muscle preparation lying in the trough via a water jacketed, gravity-fed system warmed by a pumping thermostat (Thermoflow, MEDIC 471, Coniar Churchill Scientific supplies Ltd, Middlesex, UK). The superfusate had a flow rate of between 6-8 ml/minute, a fluid change took 2 minutes to reach the preparation.

4.2 Control experiments.

After mounting the muscle strips in the trough, they were equilibrated in Tyrode's solution gassed with 95% O_2 :5% CO_2 for an hour prior to interventions.

Nerve-mediated phasic contractions of detrusor were produced by applying 4 second trains of pulses at different frequencies each of 0.1ms duration. These conditions are designed to produce selective activation of the intrinsic nerves within the muscle (Brading 1987).

Tetrodotoxin (TTX) (Sigma Chemical Co. Ltd.) is a fast Na^+ channel blocker in nerve. Unlike nerve, in which the action potential is initiated by fast Na^+ channel activation, detrusor smooth muscle lacks such a Na^+ channel. Therefore TTX- sensitivity was used in 3 experiments to differentiate between the direct muscle stimulated response and indirect neurogenic activation via the embedded nervous network. Thus direct muscular action should be TTX-insensitive and the TTX- sensitive contraction should be due to indirect stimulation via the embedded nervous network (Palfrey et al 1984). The detrusor smooth muscle strip was field stimulated as above at 20 Hz and a 1µM solution of TTX superfused over the preparation. This intervention blocked the contraction (see results) and thus the contractions produced by these stimulation parameters were nerve mediated.

The nature of the innervation was determined by assessing the effects of cholinergic blockade with atropine in 4 preparations (3 from stable and 1 from unstable bladders). The electrically-induced phasic contraction was blocked in all preparations by the addition of $1\mu M$ atropine to the superfusate (see results). This suggested that this train pulse of electrical stimulation was neurogenic stimulation and the induced muscle contraction was mediated through muscarinic receptor stimulation.

To decide the frequency used in setting up these experiments, a series of pulse frequencies were given from 2 to 40 Hz to test the frequency response of human detrusor smooth muscle strips. Contraction magnitude was increased with increasing frequency and achieved a maximum at 40 Hz. Therefore 20 Hz, half maximum stimulation frequency was used to achieve control tension and to equilibrate the preparation prior to terminating electrical field stimulation and commencing the pharmacological interventions.

The concentration of carbachol used was determined by constructing dose response curves of this agonist. Carbachol in concentrations from 0.1, 0.3, 1.0, 3.0, 10.0 and 30.0 μM were used to stimulate human detrusor strips each for a duration of 60s. The contraction generated was recorded on a continuous trace and the preparation was allowed to rest for 15 minutes in Tyrode's solution between each carbachol concentration. The concentration of carbachol that elicited the half maximal response, $1\mu M$ was used (for details see results). The tension transducer was calibrated using fixed weights hanging vertically from the force transducer tip at each gain setting of the recording apparatus and the deflection plotted as a function of the mass of the weight.

The mass, m, is expressed as a force, F, in Newtons

$$F = mg \quad \text{where } g = 9.81 \text{ m.s}^{-2}$$
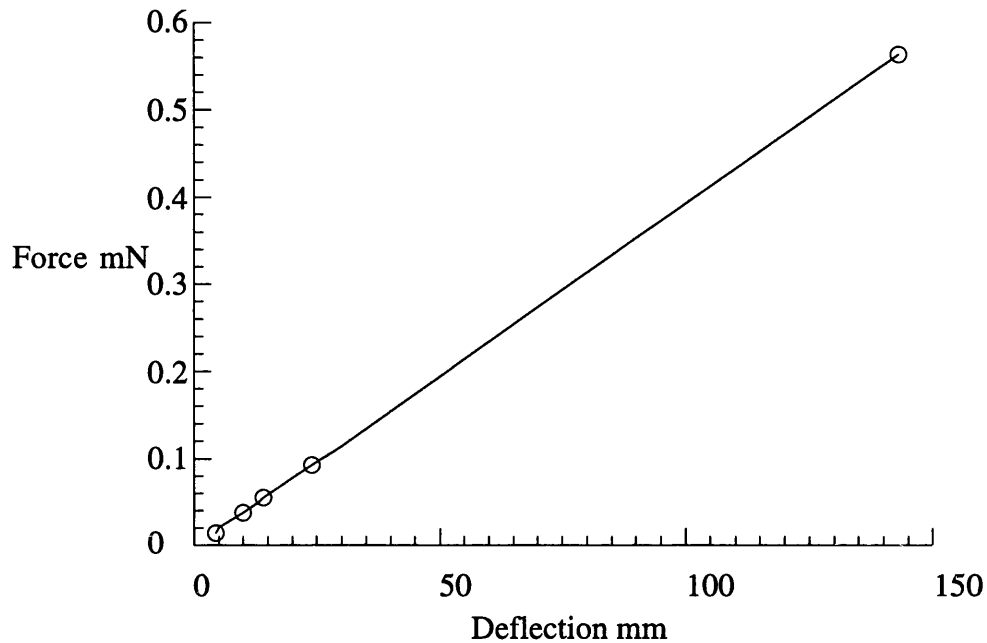
Figure M1. The relationship between deflection at fixed gain on the recording apparatus and the force applied to the force transducer.

A graph similar to the one above was plotted for each gain of the system. In all cases the plots were linear, passing through the origin. The deflection at a particular gain in the experiments could then be plotted as a force in mN for that mass of tissue and then converted to mN per g wet weight of tissue.

4.3. Experimental protocol.

Human detrusor muscle strips were fixed in the superfusion trough as described above. The resting length was adjusted to a point which caused a stable optimum contraction. The voltage of the stimulation pulse was set at least two-fold above the threshold of muscle activation. The strips were then equilibrated in normal Tyrode's without stimulation for one hour. Field stimulation was then commenced at a frequency of 20 Hz and the preparation

81

left once more to equilibrate at 20Hz in normal Tyrode's until a steady phasic tension was achieved.

The force frequency relationship of each preparation was first assessed. Force was initially recorded at 2Hz and then at 4,8,16,20 and a maximum of 40 Hz. This was to assess the nerve-mediated response of the tissue and to ascertain any differences between tissue in the nature or extent of nerve-mediated response. The preparation was then equilibrated to ensure consistent force at 20Hz stimulation.

The preparation was left unstimulated for 15 minutes in order to assess the level of spontaneous activity.

The superfusate was then changed by manual removal of the fluid feeder tube, from the normal Tyrode's reservoir to the intervention solution reservoir. This procedure allowed the introduction of a tiny air bubble into the tube so that the exact point of change of solutions could be monitored, as the progress of the intervention could be followed in the transparent fluid delivery system. The response of the muscle to these interventions was monitored continuously.

The response of human detrusor smooth muscle to the cholinergic agonist carbachol was assessed at increasing concentrations of agonist, from 0.1 μM to 10μM solutions. The muscle was exposed to intervention solution for 60 seconds in each case and the tonic response measured . The superfusate was then changed back to control and a fifteen minute interval was allowed before the introduction of a new intervention. The magnitude of the tonic response to half maximum concentration of agonist was calculated and used as a standard intervention for investigation of the action of hypoxic conditions.

The spontaneous activity of the muscle in the presence of metabolic hypoxia was measured for fifteen minutes after 5 minute equilibration in a 2 mM NaCN solution. The muscle was exposed to carbachol, in the continued presence of NaCN for 60 seconds, as in the control conditions. The preparation was then returned to NaCN containing Tyrode's until relaxation of the contraction was complete. The superfusate was then changed to control Tyrode's and a post- control stimulation with carbachol after 15 minutes in normal

Tyrode's was performed to ensure the preparation has not fatigued, and to determine if the effect of the hypoxic intervention was reversible.

Alternative agonists to carbachol were used in experiments to attempt to elucidate the site of action of extracellular 2mM NaCN. Potassium chloride, was added to Tyrode's to a final concentration of 100mM. This intervention was used with the same protocol as that for carbachol. Caffeine at a final concentration of 10mM was also used as an intervention in the presence and absence of 2mM NaCN. The protocol was also the same as for carbachol, but these experiments were limited as the results were not reproducible in all preparations tested.

The wet weight of the preparation was determined at the end of experimentation.

5. Measurement of intracellular Ca^{2+} by epifluoresence microscopy.

5.1. Intracellular Ca^{2+} fluorescent indicator.

In principle, an ion specific flourescent indicator was loaded into isolated single cells and the fluorescent output of a single cell monitored. The fluorescence output was obtained from two exciting wavelengths, chosen so that at one exciting wavelength the output changed little with variation of the concentration of intracellular Ca^{2+} whereas at the other, the change was large.

The fluorescent indicator Fura-2 (Grynkiewicz et al 1985) was used as an index of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Fura-2 has a high affinity and selectivity for Ca^{2+} and the Kd (224nM) lies within the physiological range. The fluorescence excitation spectrum of Fura-2 shifts progressively to a shorter wavelength as $[Ca^{2+}]_i$ increases; as a result the emission intensity at 340nm excitation is increased and at 380 nm excitation the emission is decreased the isofluoresence wavelength is about 360nm. The emission spectrum is unaltered by Ca^{2+} binding and has a maximum at about 510nm (Grynkiewicz et al 1985). Fura-2 was excited at 340 ± 10 and 380 ± 10 nm, the emitted light was collected between 400-510 nm see figure M2. Employing a ratio of these two outputs ensures that

the signal is independent of fluorochrome concentration which may arise from any unevenness in the thickness of the cell and eliminated artefacts due to loading or loss of the indicator from the cell.

5.2. Intracellular loading of indicator.

The loading of Ca^{2+} fluorescent indicators as their acetoxymethyl (AM) esters is the most commonly employed approach (Thomas & Delaville 1991). No specialist equipment is necessary and this method is applicable to single cells in addition to cell population measurements. Fura-2 AM was obtained from Molecular Probes Inc.(Eugene, Oregon, USA) These AM esters are strongly lipophilic and therefore freely penetrate the cell membrane. Once within the cell the ester is cleaved by non-specific intracellular esterases, releasing the hydrophilic free acid, which cannot cross the cell membrane and is thus trapped. Fura-2 was dissolved in the organic solvent dimethyl sulphoxide (DMSO; Sigma Chemical Co. Ltd.) to a concentration of 1mM and stored frozen at -20°C in 50μl aliquots. Isolated detrusor myocytes were loaded at room temperature by incubation for 30 mins in HEPES-Tyrode's containing 3μM of the AM form. Loading proceeds more rapidly at 37°C but has the disadvantage of some additional loading of subcellular organelles (Thomas & Delaville 1991). Therefore loading was performed at room temperature which minimises such compartmentation. As the cells remained in suspension following trituration, removal of the supernatant at the end of the incubation period, to prevent further loading, was not practicable. However no adverse effects from the prolonged incubation time were noted. Cells were used up to 14 hours after initial exposure to Fura-2.

5.3. The experimental set-up.
A schematic diagram of the experimental set-up for epifluorescence microscopy is shown in figure M2.
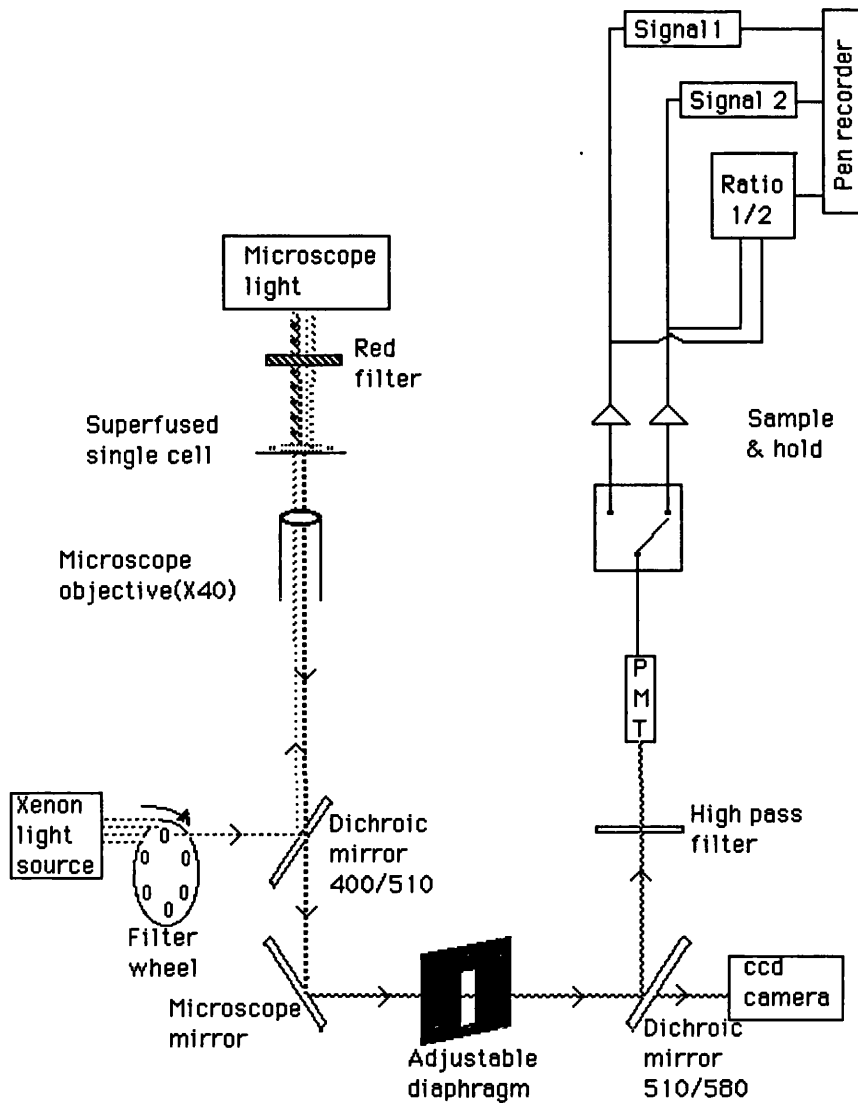
Figure M2 A schematic diagram of the epifluorescence microscopy system used for the measurement of intracellular Ca^{2+} with Fura-2.

5.3. i) The microscope

A Perspex superfusion trough with a heated water jacket was constructed and situated on an inverted stage microscope (Diaphot-TMD, Nikon Corporation, Tokyo, Japan), which was mounted on an air table (Ealing optics Ltd., Watford, UK) and enclosed in a Faraday cage covered with a black blanket. Solutions flowed into the trough at a rate of 3.5 ml/minute through a system, which was similar to the water jacket system previously described, temperature was maintained at $37 \pm 0.5°$ C . The solution in the trough was drawn to waste by suction to ensure a constant level of fluid.

5.3.ii). Light source transmission and collection

The epifluoresence system was supplied by Cairn Research Ltd., (Sittingbourne, Kent, UK). A Xenon short arc light (75W XBO; Osram Ltd., Berlin, Germany) was used to provide a light source, which provided a focused, high intensity, wide band width, white light source. This was directed via six band width ($\pm$ 10nm) interference filters of differing wavelength (340,360,380, 400,420 & 500nm) housed in a spinning wheel, to provide light of a specific wavelengths. The rate of rotation of the wheel was variable and was typically 20-30 rps. For excitation at two wavelengths 340 and 380nm, the other four filters were redundant and were blacked out. The excitation light was transmitted via a quartz fibre optic cable into the substage of the microscope and was reflected onto the cells in the perfusion trough by a dichroic mirror (400nm). Emitted light was focused in the light tube before a variable rectangular diaphragm which was closed around the cell chosen for experiment to limit the extracellular light signal, and passed down through a dichroic mirror (510nm). The microscope light source was used to position the cell and adjust the diaphragm before the experiment. The second dichroic mirror acted as a beam splitter. The higher wavelengths were directed to a CCD camera (Heiman CCD; Alrad Instruments Ltd., Newbury, Berks, UK.) and the image displayed on a monitor to facilitate adjustment of the

diaphragm prior to recording. The lower wavelengths 400-510nm were transmitted to the photomultiplier (PMT).

5.3.iii). Signal recording

The intensities of the emitted light at the two exciting wavelengths were recorded by two sample-and-hold amplifiers incorporated into the spectrophotometer system. The frequency of switch between these two amplifiers was synchronised to the rotation of the spinning filter wheel by an internal high frequency time clock so that sampling occurred only during the passage of the specific filter. The magnitude of the output could be adjusted by changing the voltage applied to the PMT. An analogue division circuit was also incorporated into the spectrophotometer system to produce a simultaneous ratio of the two signals which was displayed onto an oscilloscope (Model DSO 1604; Gould Inc., Essex, UK). This was facilitated by signals of similar size and therefore a ten fold gain was applied to the signal at 340nm excitation. The emitted light intensities and ratio from the two excitation wavelengths were recorded onto a chart recorder (EasyGraf TA240; Gould Electronics Ltd., Hainault, Essex, UK) or displayed and printed out on a storage oscilloscope for subsequent analysis.

5.4. Experimental procedure

A small drop (0.2ml) of Fura-2 loaded cell suspension was taken and placed in the superfusion trough. This drop was left unperfused for between 20-30 mins to allow the viable healthy cells to settle to the bottom of the trough and become adherent to the cover-slip which constituted the base of the superfusing chamber. Some of these cells became dislodged once superfusion had commenced but enough generally remained to allow experimentation. Intact cells were recognisable under light microscopy as appearing to be surrounded by a bright halo and had clear cytoplasm, whereas the damaged cells seemed to

possess more granular casts and appeared dull in comparison. Once superfusion with normal Tyrode's had started, viable cells were selected from their appearance under phase contrast and positioned using the monochrome monitor image, the diaphragm was then adjusted to reduce background fluorescence. The microscope cage was then blacked out and the room lights extinguished leaving only limited light from the corridor to enable the operation of the equipment and variation of the superfusate. The PMT voltage was increased until an adequate signal was obtained, The PMT output was monitored continuously using the oscilloscope (Gould Inc.).

Experiments were designed principally to assess the effect of metabolic cellular hypoxia on the cholinergic activation of human smooth muscle at a cellular level. All solutions were continuously gassed with 95% O_2: 5% CO_2 and were stored in 4 chambers and were delivered to the water-jacketed tubing via a specially designed tap allowing rapid switching between solutions.

Cholinergic stimulation was initially assessed using 10μM carbachol, for a 20 second exposure, the solutions were identical to those used in the tension experiments, and the transient rise of intracellular Ca^{2+} was taken as the control. The relationship between the concentration of agonist (carbachol) and magnitude of the Ca^{2+} transient was then assessed.

Hypoxia was achieved by changing the superfusate to Tyrode's solution containing 2mM NaCN, and 10μM carbachol was applied as before. This carbachol solution was prepared in a 2mM solution of NaCN in Tyrode's to maintain conditions during the intervention.

The investigation of the effect of hypoxia on the resting $[Ca^{2+}]$ was achieved by superfusing the cell with NaCN Tyrode's for several minutes and then returning the preparation to control Tyrode's .

The source of Ca^{2+} during the carbachol transient was investigated by stimulating the release of intracellular Ca^{2+} stores by the application of a 20 second pulse of 10mM

caffeine, The effect of hypoxia on these stores was also investigated by repeating this protocol in the presence of 2mM NaCN.

Finally, the effect of hypoxia on the Ca^{2+} transient induced by membrane depolarisation was tested by exposure of the preparation to Tyrode's containing 100mM KCl.

All experiments consisted of equilibration, control agonist exposure, intervention agonist exposure and post control agonist exposure, to assess viability of the preparation and reversibility of the intervention.

6. Calibration

Calibration of the system to establish accurately the relationship between the 340/380nm ratio signal and the absolute concentration of Ca^{2+} was performed in the absence of tissue at pH 7.0 with the assistance of Dr C. Wu. This procedure calculated the dissociation constant K_d for Fura-2 AM for Ca^{2+} at a constant pH. In addition the effects of 2mM NaCN and the effect of altering pH on the K_d value were separately determined. NaCN may exert its own effect on the Ca^{2+}- sensitivity of Fura-2 or change the value of intracellular pH. Therefore these two possible factors on the interpretation of the fluorescent signal could be determined independently.

In vitro determination of the Fura-2 dissociation constant

A solution was made to mimic the intracellular environment and used EGTA as a Ca^{2+} buffer.

Chemical	Concentration
KCl	120mM
NaCl	10mM
$MgCl_2$	1mM
HEPES	20mM
EGTA	5mM
Fura 2 acid	10µM

Table M4. Composition of solution used to mimic the intracellular environment.

Various free $[Ca^{2+}]$ were obtained by varying the amount of added $CaCl_2$ and calculated using an apparent EGTA dissociation constant (pK_{Ca}) of 6.44 at pH 7.0 according to the equation :

(1) $pCa = pK_{Ca} + \log ([EGTA] / [Ca\,EGTA])$

In this case [EGTA] + [CaEGTA] is the total added EGTA (in mM) and the [CaEGTA] is equivalent to the concentration of added $CaCl_2$.

Fura-2 fluorescence was measured at 37°C for each concentration of free Ca^{2+}. The determination of the apparent dissociation constant K_d of Fura-2 for Ca^{2+} used the following equation (Grynkiewicz et al 1985).

(2)　　　　　　$[Ca^{2+}] = K_d [(R - R_{min}) / (R_{max} - R)]\partial$

where R is the ratio of 340/380 nm for a given $[Ca^{2+}]$, R_{min} the ratio in the absence of Ca^{2+}

R_{max} the ratio in the presence of saturating Ca^{2+}. $\partial = F380_{min} / F380_{max}$,

i.e. the ratio of fluorescence intensities at 380nm in the absence of Ca^{2+} ($F380_{min}$) and in the

presence of saturating Ca^{2+} ($F380_{max}$). 0 Ca^{2+} solution and saturating solution were

obtained by using the intracellular mock solution containing 5mM EGTA with no added

$CaCl_2$ and with 10mM added $CaCl_2$ respectively.

A plot of R as a function of $[Ca^{2+}]$ was obtained and the value of K_d estimated from a least

squares fit of equation 2 to the data, see Results.

Having determined the K_d for control conditions the value was then also calculated by

repeating the calibration in the presence of 2mM NaCN. NaCN was made up to this

concentration in the intracellular mock solution. Additional calibration was performed

varying the pH of the control solution. In these solution the pCa was calculated from

equation 1 with the proviso that pK_{Ca} value is pH-dependent. The value was assumed to

change by 0.2 units for an increase of pH by 0.1 unit (Fry *et al* 1984).

All values of $[Ca^{2+}]$ quoted in the results were obtained from this in vitro calibration.

7. Measurement of intracellular NADH by autofluoresence microscopy

Work on isolated guinea-pig ventricular myocytes has shown that inhibiting the electron

transport chain at cytochrome a_3 increases NADH autofluorescence (Minezaki *et al* 1994).

In the presence of tissue hypoxia, cells respire anaerobically leading to an increase in the

ratio of NADH/NAD.

The experiments in this study were designed to determine whether 2mM NaCN causes cellular hypoxia in human isolated detrusor cells. Cells were not divided into patient groups for this series.

Single cells were placed in the superfusion trough without previous exposure to fluorescent intracellular dyes. NADH fluorescence was measured during excitation at 380nm excitation and emission measured between 400 and 510nm (Duchen *et al* 1992). In order to assess the level of background fluorescence, the fluorescence of the superfusate was measured in the absence of cells. This background fluorescence was negligible and so the level of autofluoresence was assumed to originate from the cell.

A suitable viable cell bathed in gassed Tyrode's solution, was selected from the view and the diaphragm closed around it to eliminate the background fluorescence. The cell was excited by 380nm light and the level of autofluoresence measured continuously on the oscilloscope. After equilibration of the cell for 5 minutes in Tyrode's the level of autofluoresence was measured. The superfusate was then changed to a superfusate containing 2mM NaCN and the cell allowed to equilibrate to these conditions. Minezaki *et al* (1994) described an effect on ventricular myocytes after 2 minutes exposure to NaCN which remained constant for up to 10 minutes. 5 minutes was therefore taken as a suitable length of exposure before the measurements. The superfusate was then returned to Tyrode's and the post control measurement recorded after another period of 5 minutes.

8. Measurement of pH

Epifluorescence microscopy was used to measure the intracellular pH of the cells in control conditions and in 2mM NaCN Tyrode's solution, using the specific fluorescent indicator BCECF (2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein) (Rink *et al* 1982).

BCECF was supplied as its acetoxymethyl ester (Molecular Probes Ltd). The loading properties of BCECF-AM being lipophilic until cleaved inside the cell are similar to that of

Fura-2 previously described, BCECF was also dissolved in DMSO and kept frozen in 1mM aliquots. Isolated detrusor smooth muscle cells were loaded at room temperature by incubation for 30 min in HEPES-Tyrode's solution containing 5µM BCECF-AM.

In these experiments, designed to assess the effect of 2mM NaCN on the intracellular pH of the cell excitation wavelengths of 500 and 430nm were used. 450nm is the isosbestic wavelength (where fluorescence output is not a function of pH). Emitted light was collected between 530 and 580nm using 500 and 580 nm dichroic mirrors I the fluorescence microscope in figure M2 and a high pass Wratten filter , 530nm, (Kodak Ltd) on the 580 dichroic mirror. A ratio (R) of fluorescence outputs at 500/430nm excitation gave an index of pH_i .

The cells were initially exposed to Tyrode's solution as a control and then the superfusate was changed to that containing 2mM NaCN. The superfusate was then returned to Tyrode's solution after a period of 5 minutes.

At the end of the experiment the signal was calibrated by superfusing the cell with a solution containing (in mM) KCl (140), $MgSO_4$ (1.2), KH_2PO_4 (1.2), HEPES (10) and 10µM nigericin (an H^+/K^+ exchanger). The pH of this solution was titrated to pH 4.0, 7.0 and 9.0 by the addition of aliquots of 1M KOH or HCl. The relationship between pH_i and R is given by the following equation

$$(3) \qquad pH_i = pK + \log(R - R_{min} / R_{max} - R) + \log (F430_{min} / F430_{max})$$

Where R_{min} and R_{max} are the ratios at the extreme acid and alkaline pH respectively. As pH_i is known during the calibration for each experiment the pK in control Tyrode's and 2mM NaCN Tyrode's solution can be calculated.

9. Data Handling and statistics

Data on isometric tension and the Ca^{2+} transient were recorded on chart paper and were measured manually; values obtained were put into a Statworks data sheet for analysis.
Data~was~expressed as mean ± one standard deviation (mean ± SD).
was normally distributed and

The Student's 't' test was performed using the Statworks™ programme on an Apple Macintosh computer and P<0.05 was accepted as a significant level.

Dose-response curves and force-frequency curves were fitted to equation (4) using a least squares iterative procedure, Kaleidagraph™ on Machintosh computer.

(4) $T = T_{max} \, S^n / \, K_{1/2} + S^n$

Where T is the contractile response, T_{max} the maximum response at high concentration of the agonist, or high frequency of stimulation, S the agonist concentration or stimulation frequency. $K_{1/2}$ is the concentration of agonist (EC_{50}) or frequency at which $T = T_{max}/2$ and n is a constant (generally set at n=1).

To test the significance of a regression coefficient, r, as a non-random description of n data points a "t" value was calculated from the equation:

(5) $t = r \, (n-2) / (1-r^2)$

The "t" value was expressed as a probability, p, that the line was significant from tables of the "t" distribution with n-2 degrees of freedom (Sokal & Rohlf 1969).

RESULTS

1. Patient selection

Urodynamic studies were performed by myself at The United Elizabeth Garrett Anderson Hospital & The Hospital for Women Soho, 144 Euston Road for 27 of the patients, the remaining 9 were performed at The Middlesex Hospital, Mortimer Street. The urodynamic records were available for study for all patients and the diagnosis of stable and unstable detrusor activity was made according to the International Continence Society standardisation of terminology of lower urinary tract function (Abrams *et al* 1988). All patients had urodynamic findings which were consistent with their symptoms. The unstable group consisted of 18 patients, 14 women and 4 men (mean age 42yrs, range 23-64). The stable group also consisted of 18 patients, all women (mean age 54yrs, range 40-60). None of the patients exhibited obstructed voiding and no patient had concurrent transitional cell carcinoma. All the patients in the "stable" group demonstrated genuine stress incontinence and underwent bladder neck surgery. For 9 of the patients in the "unstable" group the diagnosis of detrusor instability was new and the biopsies taken endoscopically , and for the remaining 9 the disease had proved unresponsive to medical treatment necessitating surgical augmentation of the bladder, a "clam enterocystoplasty".

Experiments were performed to determine the effects of hypoxia, generated by the addition of Na cyanide to the superfusate, on the contractility of detrusor strips and the regulation of the intracellular Ca^{2+} concentration in isolated human detrusor smooth muscle cells. Comparisons between muscle samples from unstable and stable bladders were made. Initial experiments quantified the effects using smooth muscle strips in vitro and subsequently

isolated smooth muscle cells were used to investigate the cellular bases of the phenomena in greater depth.

2. Tension recording in isolated human detrusor smooth muscle strips.

The contractile properties of samples of smooth muscle from stable and unstable bladders were determined carrying out standard interventions; the response to extracellular agonists such as carbachol, the relationship of the force of muscle contraction to the frequency of electrical field stimulation and the measurement of absolute tension and incidence of spontaneous activity in each group.

2.1. The response of detrusor smooth muscle to extracellular carbachol.

Strips of detrusor smooth muscle isolated from samples from patients with unstable and stable bladders were prepared as described in the Methods, superfused with Tyrode's solution and isometric tension recorded. Brief (60 second) applications of carbachol, an acetylcholine analogue, in increasing concentrations between 0.1 and 10μM were performed. This resulted in the development of a contraction which attained a steady state value.

Each application of carbachol was followed by a return to Tyrode's solution and the preparation allowed to relax fully before application of further carbachol-containing solutions. A 30μM carbachol concentration was used in 5 experiments and did not generate a larger contraction than that obtained at 10μM carbachol and was not routinely used as recovery from the exposure was slower and sometimes incomplete (see figure R1 p97) Exposure to carbachol for longer than 60s resulted in the same magnitude of steady state tension during the intervention but recovery was again slowed. The relationship between the concentration of carbachol and isometric tension in a typical experiment is shown in figure R1, the cumulative results for 19 muscle strips is shown in figure R2 p98.
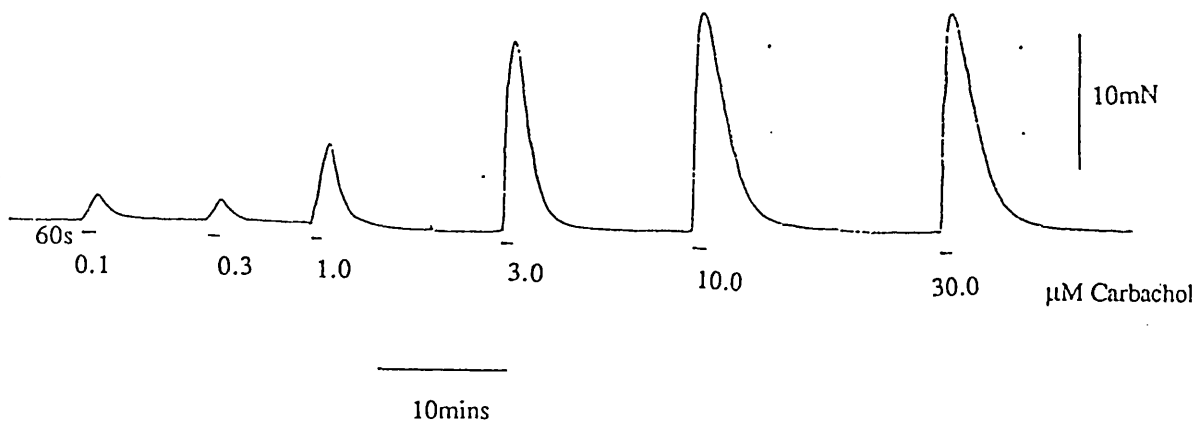
Figure R1. A continuous recording of isometric tension from an isolated strip of human detrusor taken from a stable bladder. The effect of a 60 second exposure to increasing concentrations of carbachol is shown.
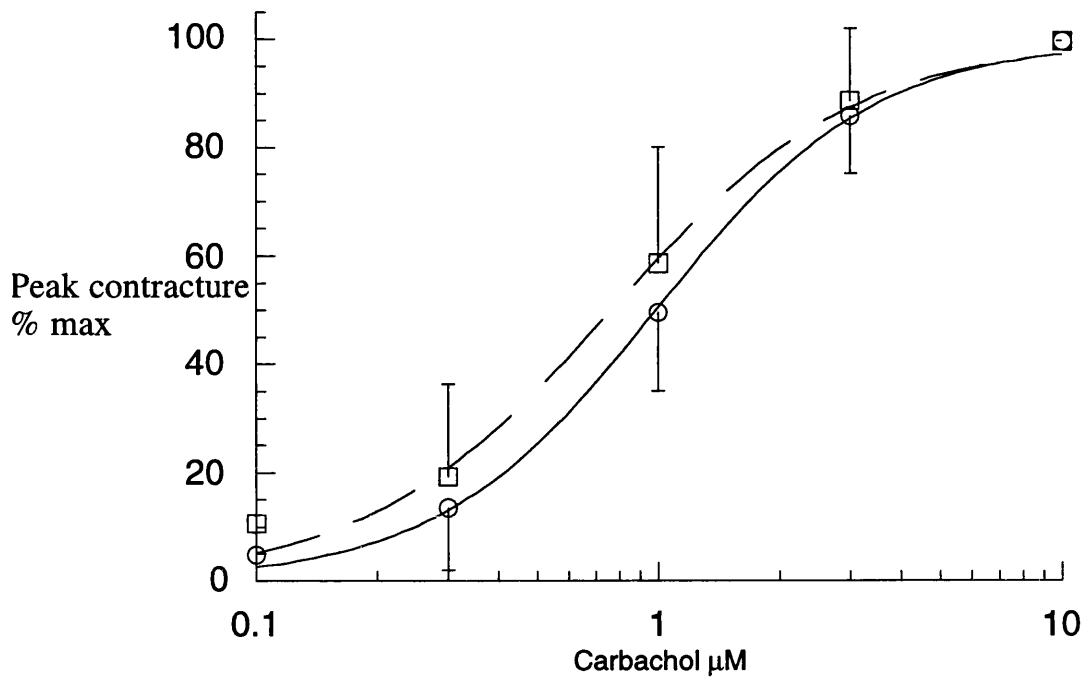
Figure R2. The relationship between concentration of carbachol (μM) and force of contraction of isolated human detrusor strips taken from 10 stable (circles) and 9 unstable (squares) bladders. Contraction is expressed as % of maximum. Mean data ± sd. The lines are fitted according to equation 4 p94.

Figure R2 shows the accumulated data from 10 strips from stable bladders and 9 strips taken from unstable bladders as dose response curves to carbachol. Data are plotted as a percentage of the maximum contraction obtained in each strip at high (10μM) carbachol concentrations. The EC_{50} (the half maximal concentration of carbachol (μM)) values were determined separately for each strip yielding mean values of 1.3 (range 0.78-2.0) μM and 1.3 (range 0.91-1.7) μM for samples from stable and unstable groups respectively, see table R1 overleaf. To calculate the mean values the individual EC_{50} values were converted to pEC_{50} values (-log EC_{50}) as the latter will be the normally distributed set as the ordinate on fig R2 is expressed in logarithmic co-ordinates. The mean EC_{50} values are quoted from the mean pEC_{50} and the ranges are calculated from the s.d. of the pEC_{50} sets.

Table R1 The mean EC_{50} values of samples from stable and unstable bladders. The mean pEC_{50} ($-logEC_{50}$) values are also shown for each group.

	Stable	Unstable
Mean $pEC_{50} \pm$ s.d.	5.9 ± 0.21	5.9 ± 0.14
Mean EC_{50} μM	1.3 (range 0.78-2.0)	1.3 (range 0.91-1.7)

These mean values of either EC_{50} or pEC_{50} for samples from stable and unstable bladders were not significantly different ($p>0.05$) when analysed by an unpaired Student's "t" test.

2.2. The contractility of detrusor smooth muscle.

A force / frequency relationship was established for each isolated smooth muscle strip and this was performed after the preparation had equilibrated in Tyrode's solution and prior to any pharmacological intervention. The preparation was stimulated every 90 seconds, with a 4s tetanic train, with pulses of 0.1ms duration and constant stimulator voltage (20V). The frequency of stimulation was varied between 2Hz and 40Hz. The stimulation protocol resulted in stimulation of the muscle via the attendant nerve network as contractions were abolished by the application of 1μM tetrodotoxin (TTX). This intervention was performed in 4 control experiments using 2 samples from stable and 2 from unstable bladders. 1μM atropine also abolished the contractions with no atropine resistant component in 4 samples (3 from stable and 1 from unstable bladders). Figure R3 p100 shows a sample trace of phasic contractions elicited at 20 Hz and abolished by 1μM TTX. Figure R4 p100 shows a similar sample trace where the contractions were abolished by 1μM atropine.
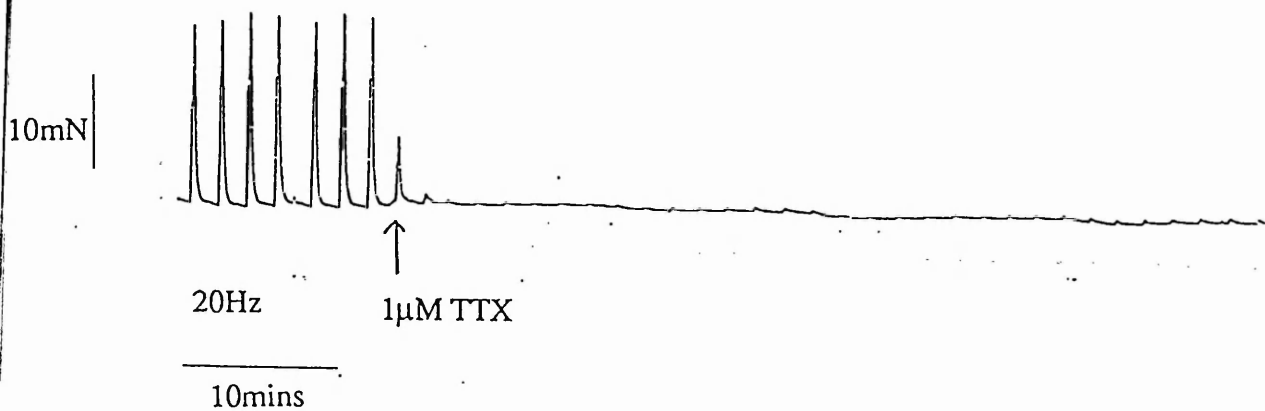
Figure R3 A continuous recording of force from an isolated strip of human detrusor muscle taken from a stable bladder. Field stimulation was applied at 90s intervals with a 4s tetanic train, and pulses of 0.1ms duration at 20 Hz. TTX was applied at the arrow.
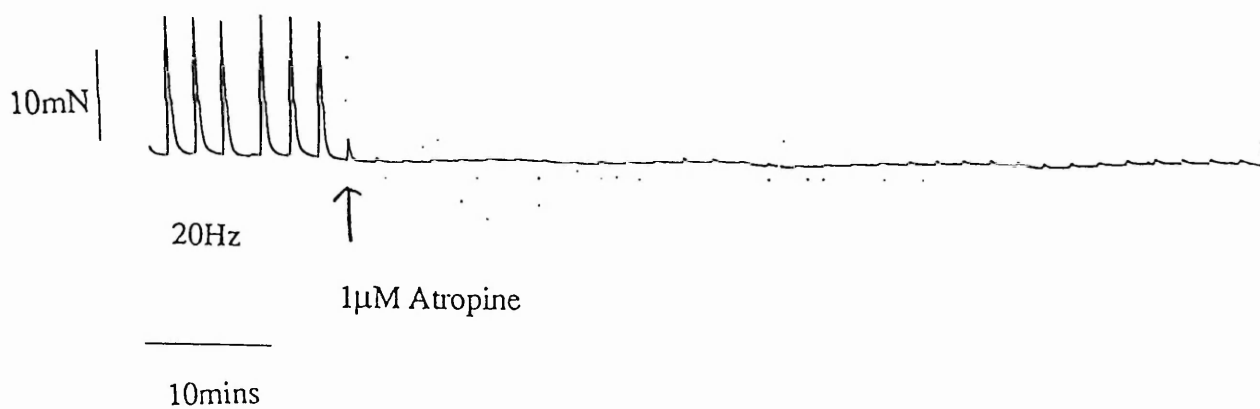
Figure R4 A continuous recording of an isolated strip of human detrusor muscle from a stable bladder. Field stimulation was applied at 90s intervals with a 4s tetanic train, and pulses of 0.1ms duration at 20 Hz. 1μM atropine was applied at the arrow.
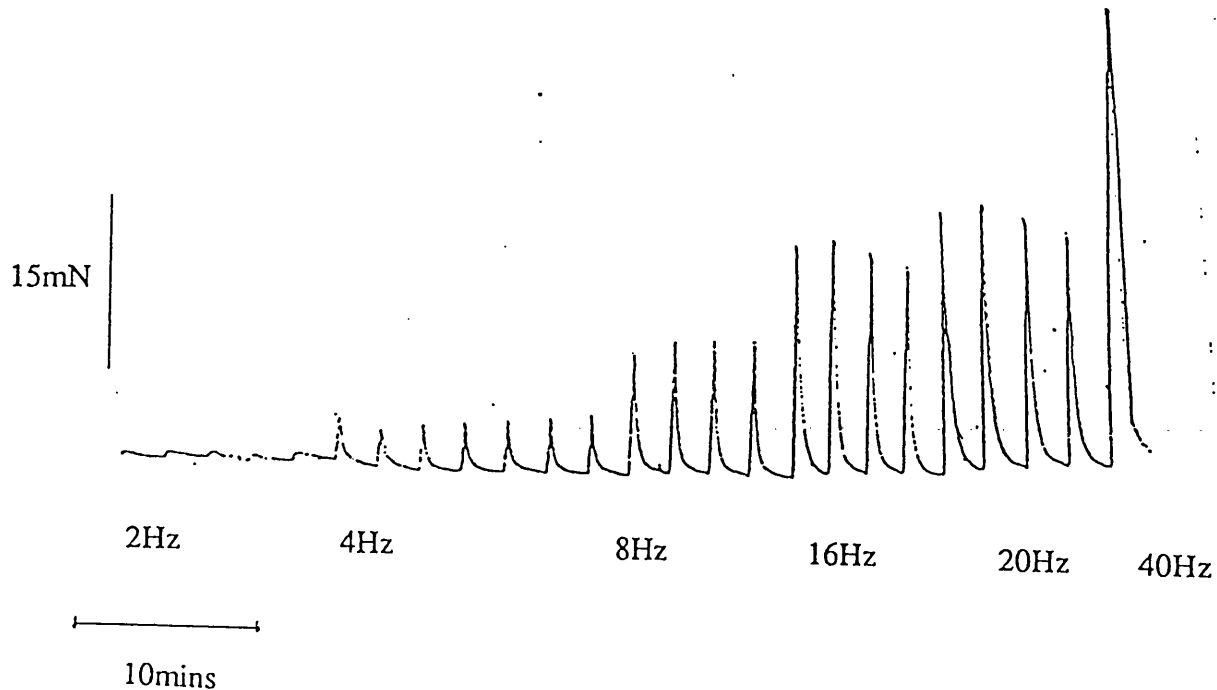
Figure R5 A continuous recording of phasic tension in an isolated detrusor strip from a stable bladder. Stimulation frequency is indicated, other stimulation variables are as given in the figure legend to figures R3 and R4.

Figure R5 illustrates the effect of increasing stimulation frequency on the phasic contraction magnitude. In general four contractions at each stimulation frequency were recorded. However the first one at each frequency was measured as in some preparations there was a subsequent decline.
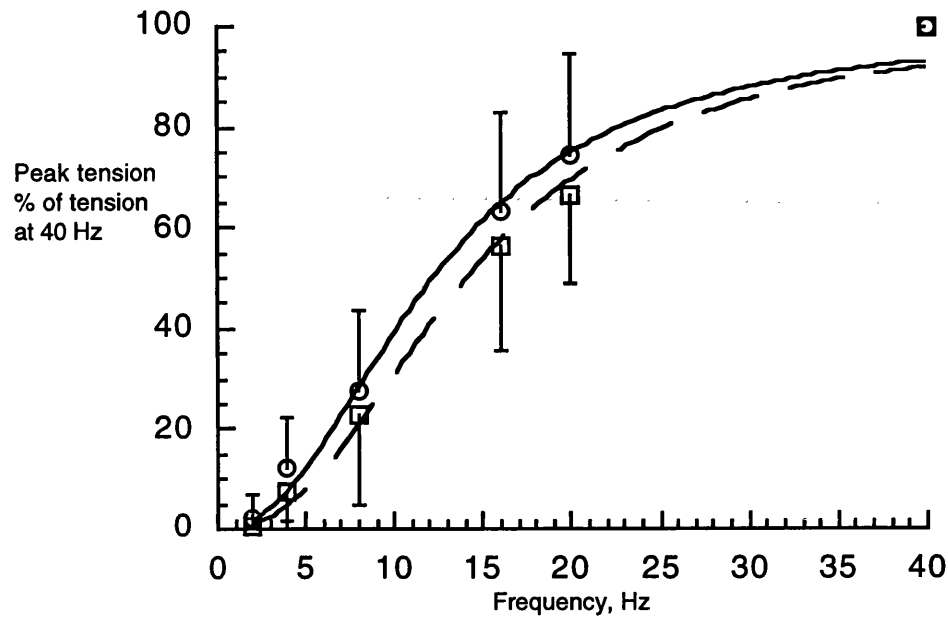
Figure R6. Mean force-frequency relationship for isolated detrusor smooth muscle strips from stable (circles n=14)) and unstable (squares n=16) bladders. The force is expressed as a percentage of the maximum tension generated at 40 Hz, mean values ± s.d. Data are given as mean ± s.d and a percentage of the maximum contraction, obtained at 40 Hz. Lines were obtained from equation 4 (p94).

Figure R6 summarises the effect of increasing the frequency of electrical stimulation on the contractile response of isolated smooth muscle strips. Half maximal tension was achieved at a stimulation frequency of 9.0 ± 5.5Hz (n=14) in the stable group and 11.2± 5.1 Hz (n=16) in the unstable group. These mean values were not significantly different between the two groups,(Student's "t" test p>0.05).

2.3. Measurement of absolute tension

The isolated strips used for experiments were of approximately equal length (4-5mm) following dissection from the biopsy of human bladder muscle. The peak force (mN)

generated would be expected to be proportional to the cross sectional area of the muscle, which in a fixed length preparation is therefore proportional to the weight of the strip. The tension generated by each preparation was normalised to a unit weight of tissue (see Methods for details of calibration) At 20Hz stimulation samples from stable bladders generated 15.2 ± 9.7 mN/mg wet weight and from unstable bladders 21.4 ± 10.6 mN/mg wet weight. There was no statistically significant difference between these two groups. (Student's "t" test, $p > 0.05$).

2.4. Spontaneous activity

The isolated smooth muscle strips were initially stimulated for at least an hour at a frequency of 20Hz in Tyrode's solution to equilibrate after dissection and mounting . Following this period the preparation was not electrically stimulated for 15 minutes so that any spontaneous activity could be observed.

No spontaneous activity was recorded in any of the preparations in aerobic conditions using the control superfusate.

Furthermore no spontaneous activity was recorded in the initial 15 minute period exposure to hypoxic conditions by the addition of 2mM NaCN to the superfusate, during which time the muscle was unstimulated.

2.5. The effect of cellular hypoxia on the force of contraction.

The effect of cellular hypoxia on the force of contraction of isolated smooth muscle following stimulation by carbachol was investigated. Cellular hypoxia was generated by the addition of 2mM NaCN to the superfusate. The strip was exposed to this NaCN containing solution for at least 5 minutes to equilibrate prior to carbachol interventions. Longer exposure to 2mM NaCN solution did not influence the tissue contractility or viability as the post-intervention control carbachol contraction was not significantly

different to the pre-intervention control. Therefore the effect of extracellular 2mM NaCN was shown to be fully reversible. Repeated applications of carbachol (after recovery from the previous intervention) in either normal Tyrode's or in Tyrode's + 2mM NaCN did not result in further significant changes to peak force, compared to the first response in the respective conditions.

10mN

60s 1μM Carbachol   60s 1μM Carbachol   60s 1μM Carbachol
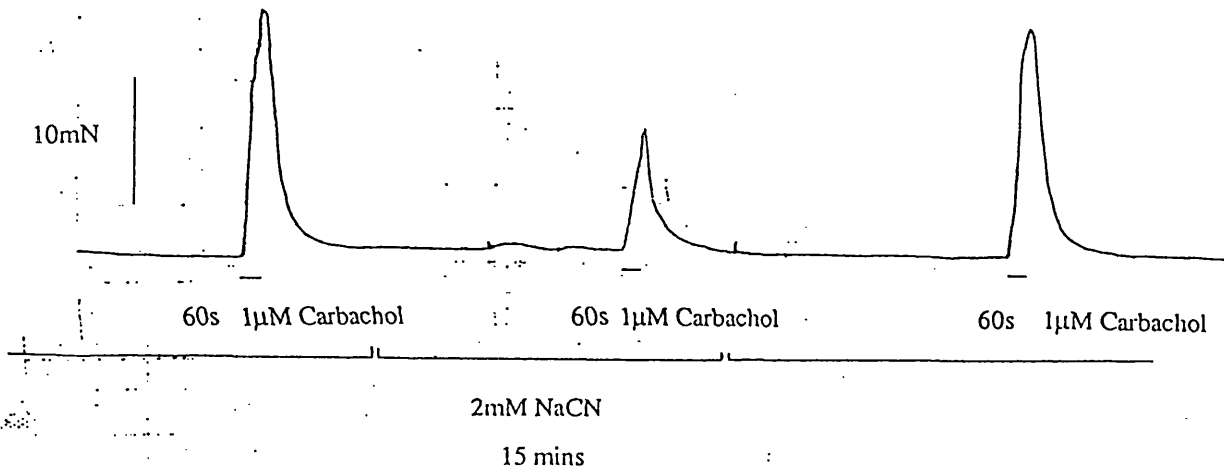
2mM NaCN
15 mins

Figure R7 The effect of 2mM NaCN on the force of contraction elicited by 1μM carbachol.

Figure R7 is a continuous trace of an isolated smooth muscle strip from an unstable bladder. The baseline represents the resting tension of the preparation. The preparation was exposed to 1μM carbachol solution for 60s and the contraction recorded. Following the removal of extracellular carbachol the preparation relaxed and the tension returned to the baseline. The preparation was then exposed to Tyrode's + 2mM NaCN and equilibrated for 5 minutes after which it was exposed to another stimulus of 1μM carbachol for 60s. The magnitude of the contraction in the presence of 2mM NaCN was less than that in control solution. Once force had returned to the resting tension in the presence of 2mM NaCN the control conditions were restored and equilibrated for 5 minutes before the application of carbachol. The effect of 2mM NaCN was fully reversible as shown by the similar magnitude of pre- and post-control intervention.

The decrease in the magnitude of the force of carbachol contraction of detrusor smooth muscle in the presence of 2mM NaCN occurred in all preparations, using samples from both stable and unstable bladders. However figure R8 shows that the proportional reduction was significantly greater with samples from stable bladders (p< 0.02)

100

80

**Contraction
in 1 μM
carbachol
% control**

60

40

20

0

control 2mM NaCN   control 2mM NaCN

Stable n=14   **Unstable n=16**
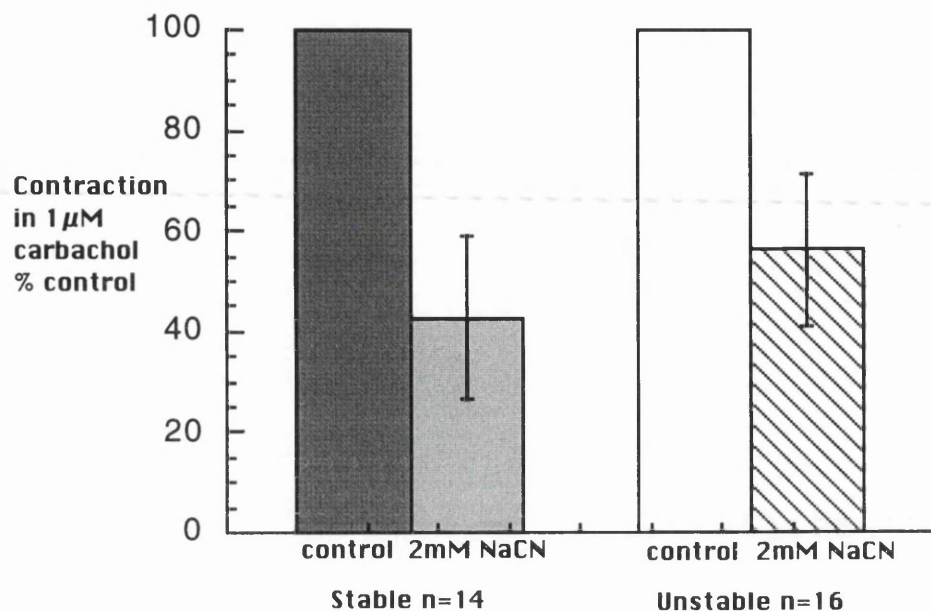
Figure R8 The mean reduction by 2mM NaCN of contraction force of isolated human detrusor smooth muscle strips from stable and unstable bladders in response to 1μM carbachol. Contractile force in 2mM NaCN is represented as a percentage of that in control solution. Mean data ± s.d, n= number of preparations.

Biopsy samples from stable bladders generated only $43 \pm 16\%$ of the control force in the presence of 2mM NaCN, whilst samples from unstable bladders generated of $56 \pm 15 \%$ control. This difference was significant ($p<0.02$).

2.6. The dependence of the negative inotropic effect of NaCN on the absolute force of contraction.

It is possible that the smaller percentage attenuation of the carbachol contracture by 2mM NaCN in the unstable group was a function of the different control contracture, compared to the stable, control group. Any possible relationship was therefore investigated. There was no significant difference in the absolute tension generated per g wet weight of tissue by

1μM carbachol in control superfusate between the two experimental groups (33.3 ± 31.7 mN/mg and 44.0 ± 32.0 mN/mg in biopsies from stable and unstable bladders respectively, p>0.05 unpaired Students "t" test). The proportional reduction of the carbachol contraction in the presence of 2mM NaCN was plotted as a function of the control carbachol contraction magnitude. The results are plotted in figure R9 for biopsy samples from stable (circles) and unstable (squares) bladders.
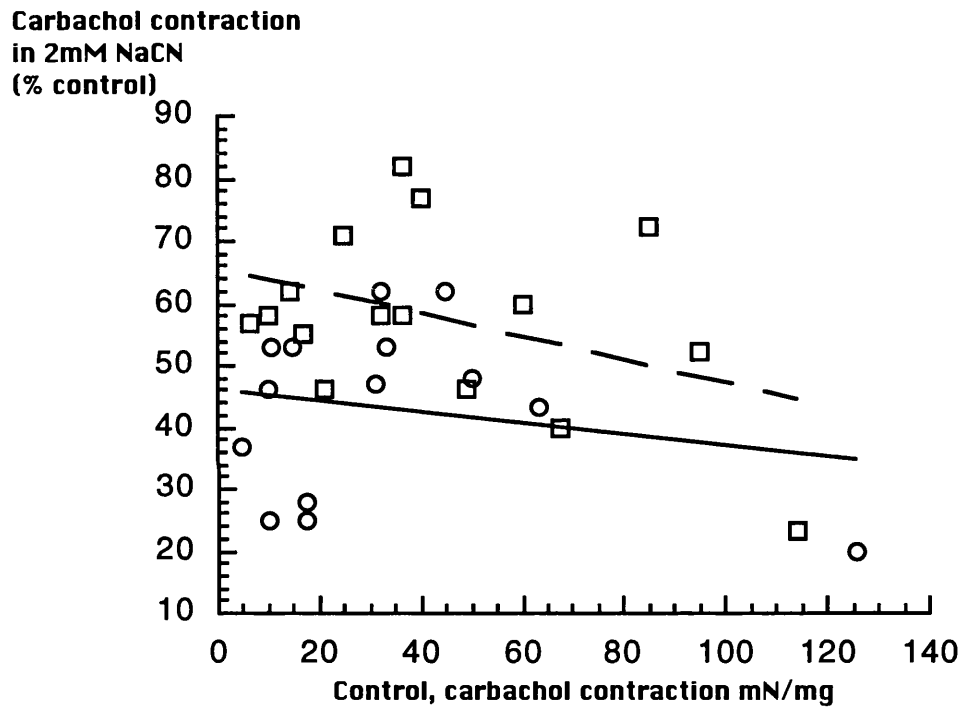
Figure R9 The relationship between the proportional reduction of the 1 μM carbachol contracture by 2mM NaCN and the absolute magnitude of the carbachol contracture in control conditions. Bladder biopsies are from stable bladders (circles) and from unstable bladders (squares). The lines are linear best fits through the two data sets (solid line stable: dotted line unstable)

Straight lines of best fit were drawn through the data, with values for the correlation co-efficients of r = 0.21 for data from stable bladder biopsies and r = 0.4 from unstable

bladder biopsies. These small values of r indicated there was no relationship between reduction of force and the initial contractility of the preparation. (p>0.05, see p 94 eq 5).

The same analysis was performed for the whole data set which also demonstrated a lack of relationship regardless of whether the samples came from stable or unstable bladders, in figure R10.
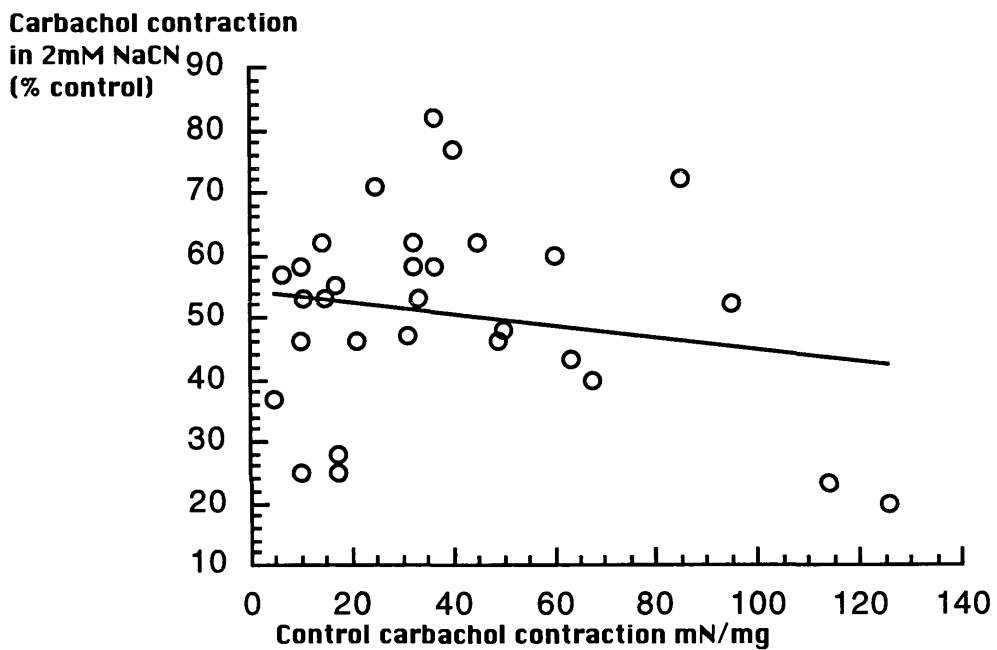
Figure R10. The relationship between the proportional reduction of the $1\mu M$ carbachol contracture by 2mM NaCN and the absolute magnitude of the carbachol contracture in control conditions.

A straight line of best fit was drawn through the whole data set with a correlation coefficient r = 0.16, (p >0.05).

It has been shown thus far that isolated smooth muscle samples from stable and unstable bladders were similar in several respects. They contracted with the same absolute tension when excited by electrical field stimulation and were not significantly different in their sensitivity to varying frequencies of field stimulation. Both muscle groups had the same sensitivity to carbachol stimulation and neither exhibited spontaneous activity. However there was a statistically significant difference between the two groups with respect to their response to extracellular 2mM NaCN. Samples from unstable bladders were less affected when exposed to extracellular NaCN and subsequently stimulated with carbachol, than those samples from stable bladders. This response was not dependent on the magnitude of the initial contraction in control circumstances.

In order to establish the cellular basis for the difference in contractile sensitivity to NaCN in the two groups Ca^{2+} metabolism in isolated detrusor muscle cells was investigated in more detail . Contraction was elicited by a rise of sarcoplasmic $[Ca^{2+}]$, which originates either from intracellular stores or ultimately as a result of Ca^{2+} crossing the cell membrane. The following sections examine both processes in turn. Firstly the effect of extracellular NaCN on transmembrane Ca^{2+} movement was measured by depolarising the muscle, by addition of 100mM KCl to the superfusate, in control conditions and in the presence of 2mM NaCN. This would open the L-type Ca^{2+} channels and allow Ca^{2+} influx. Secondly the effect of extracellular NaCN was studied on the contraction elicited by stimulating the release of Ca^{2+} from the intracellular stores with caffeine.

2.7. Effect of 2mM NaCN on the KCl contracture

Membrane depolarisation occurs if a high K^+ (100mM) Tyrode's solution is applied to an isolated detrusor smooth muscle strip (Fujii *et al* 1990).
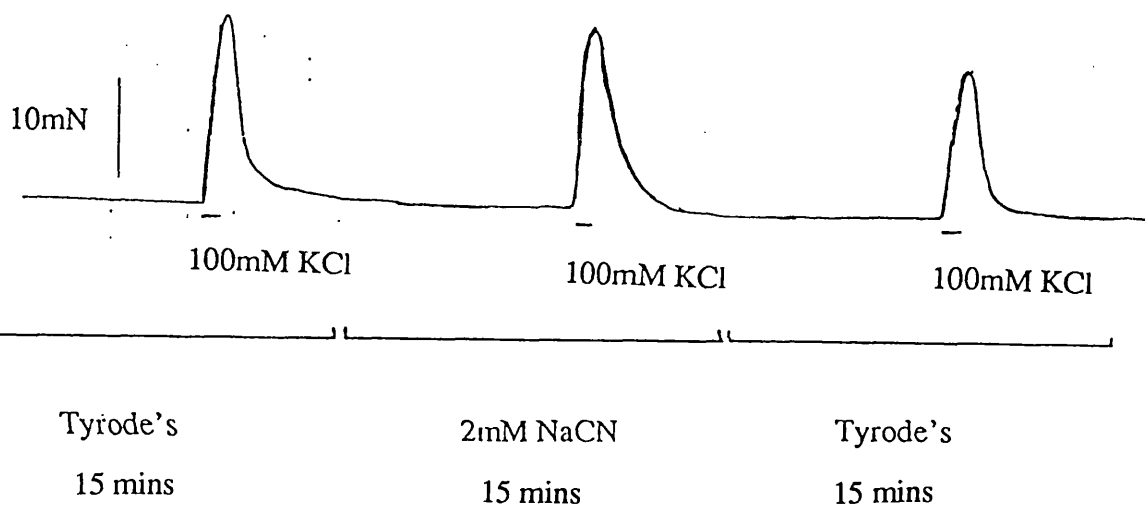
Figure R11

A continuous recording obtained from an isolated human detrusor smooth muscle strip (from a stable bladder) showing the force generated by the 60s application of 100mM KCl in control, hypoxic and control conditions.

The preparations in these experiments were exposed to 100mM KCl superfusate for 60s and then returned to Tyrode's solution. Following relaxation, 2mM NaCN was added to the superfusate and the muscle allowed to equilibrate for at least 5 minutes. The intervention was then repeated in these conditions and the preparation returned to control conditions as soon as the resting tension was restored. A post-intervention control was then performed.
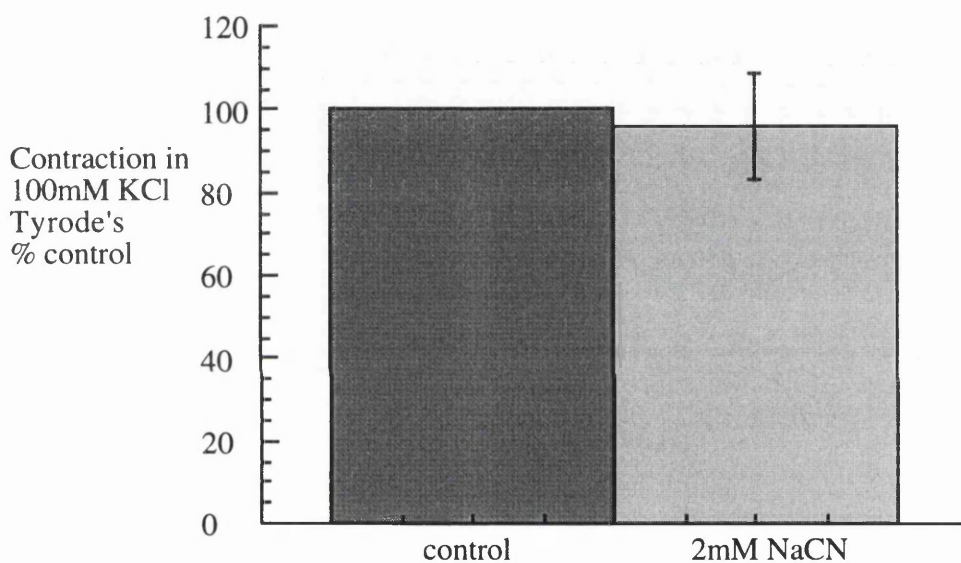
Figure R12

The mean effect of 2mM NaCN on the force of a 100mM KCl-induced contraction. Mean data ± s.d., n=7 samples taken from stable and unstable bladders and pooled as one group.

The mean force of the 100mM KCl induced contraction in the presence of 2mM NaCN was 96% ± 13% of the control contraction. The muscle samples have not been divided into stable and unstable patient groups for this series of experiments, 7 samples (4 from stable and 3 from unstable bladders) were investigated. There was no significant difference

(p>0.05, paired Students's "t" test) found between the tension generated in either the presence or absence of 2mM NaCN following membrane depolarisation therefore the groups were considered together.

2.8. The effect of 2mM NaCN on the caffeine induced contracture

10mM caffeine was used as the stimulus for contraction in 10 experiments on isolated detrusor smooth muscle strips from both stable and unstable bladders. This produced a measurable response in only 4 preparations, all from stable bladders. Increasing the concentration of caffeine did not increase the contraction magnitude in those preparations which responded to a concentration of 10mM, or induce a contraction in those which failed to produce a response in 10mM caffeine. The lack of response to caffeine was not matched by a similar unresponsiveness to electrical stimulation or the addition of carbachol. (see Discussion section 5.2 p173).

Muscle strips were exposed to a 10mM caffeine solution for 60s and the tension generated was recorded. The exposure was repeated after the muscle had been exposed to a 2mM NaCN solution for at least 5 minutes. Following this procedure the strip was returned to the Tyrode's solution, allowed to equilibrate for at least 5 minutes, and the caffeine stimulus repeated once more to provide a post-intervention control.
A typical trace is shown overleaf.

10mN

10mM Caffeine   10mM Caffeine   10mM Caffeine

Tyrode's   2mM NaCN   Tyrode's

15 mins
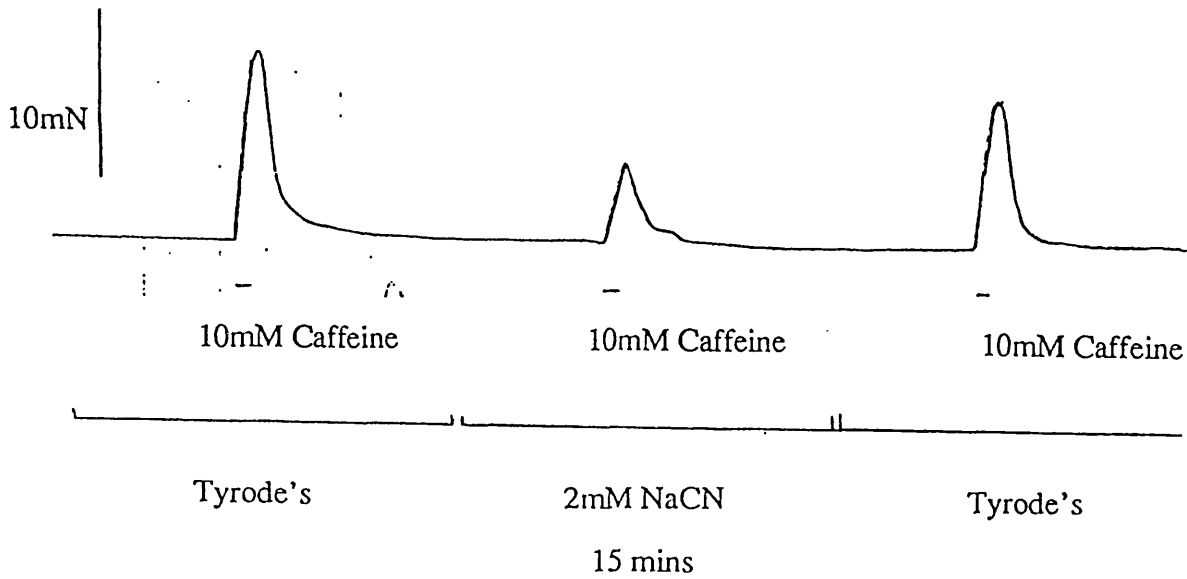
Figure R13. A trace of an isolated human detrusor muscle strip taken from a stable bladder. The effect of a 60s application of 10mM caffeine is shown in the presence and absence of 2mM NaCN.

The caffeine-induced contracture was significantly reduced in the presence of 2mM NaCN. The contracture in these circumstances achieved only 42%± 16% of the mean control as

illustrated in figure R13. This effect was fully reversible as there was no significant difference in the force of contraction between the pre- and post-intervention control.
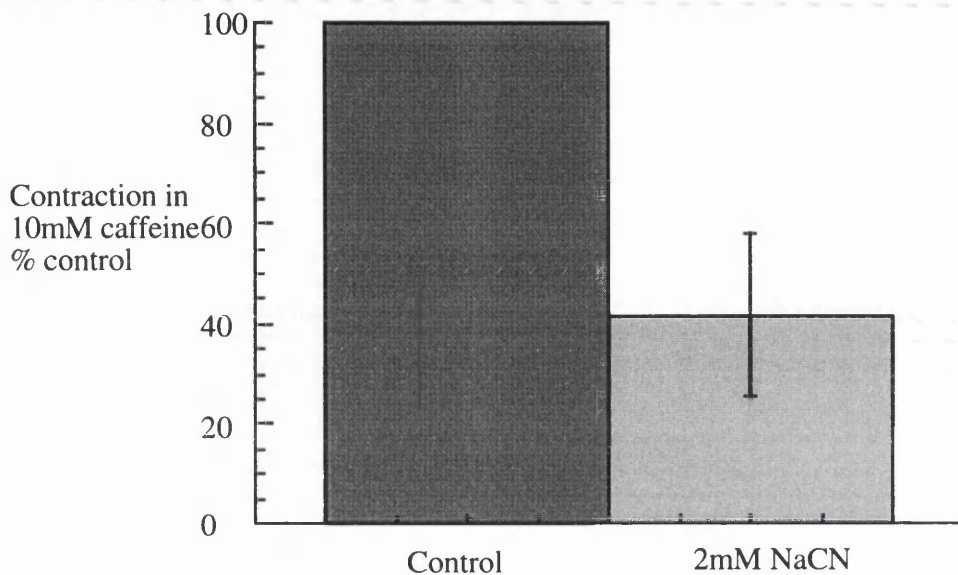
Figure R14. The reduction of the caffeine contracture in the presence of 2mM NaCN. Data, mean ± s.d., n=4.

2.9. Conclusion of isolated human detrusor muscle strip experiments.

Cellular hypoxia, produced by the addition of 2mM NaCN to the superfusate of an isolated detrusor smooth muscle strip, produced a negative inotropic effect. It reduced the force of contraction when stimulated by carbachol in all muscle preparations. This effect was fully reversible. Muscle from unstable bladders when stimulated by carbachol was shown to be significantly less affected by the hypoxic insult than muscle samples from stable bladders.

This difference cannot be explained by variability of the two groups to sensitivity towards the agonist or in their absolute tension generated by carbachol addition as these variables have been shown to be similar.

2mM NaCN did not affect the contracture generated by 100mM KCl. However in the proportion of strips generating a caffeine contracture, 2mM NaCN reduced their magnitude.

In principle, carbachol could generate force by releasing Ca^{2+} from an internal site (caffeine was used to study this), or by increasing influx of Ca^{2+} via the cell membrane (KCl was used to study this). The transmembrane route did not appear to be affected by NaCN but the Ca^{2+} released from the internal store could have been affected. However the caffeine experiments were equivocal, as caffeine did not always generate a contracture. Therefore intracellular Ca^{2+} metabolism was studied in detail by measuring $[Ca^{2+}]_i$ in isolated cells under similar conditions, to the above tension experiments.

3. The measurement of intracellular calcium in isolated human detrusor smooth muscle cells.

Isolated smooth muscle cells were prepared by the digestion of human detrusor smooth muscle samples from stable and unstable bladders. The method is described in detail in the methods chapter. The rise of the intracellular $[Ca^{2+}]$, $[Ca^{2+}]_i$, responsible for the initiation of the contractile machinery was measured using the fluorescent indicator Fura-2 and recording the ratio of signals at 340/380nm. Figure R15 shows the signal changes during

the application of 10μM carbachol for 20 seconds; the bottom two traces show the fluorescence intensity from the Fura-2 when illuminated at 340 (lower) and 380(middle) nm. The top trace is the ratio of the two signals, 340/380 ratio and is a function of the $[Ca^{2+}]_i$. An upward deflection results from an increase in $[Ca^{2+}]_i$. The rise of $[Ca^{2+}]_i$ was not sustained and henceforth will be referred to as the Ca^{2+} -transient.

The carbachol- induced Ca^{2+}-transient was characterised by a rise of the $[Ca^{2+}]_i$, a decline and an undershoot of the baseline value with an eventual return to the baseline. A typical trace measured following a 20s application of 10μM carbachol is shown. Longer applications did not produce any significant change in either the magnitude or duration of the Ca^{2+}- transient.
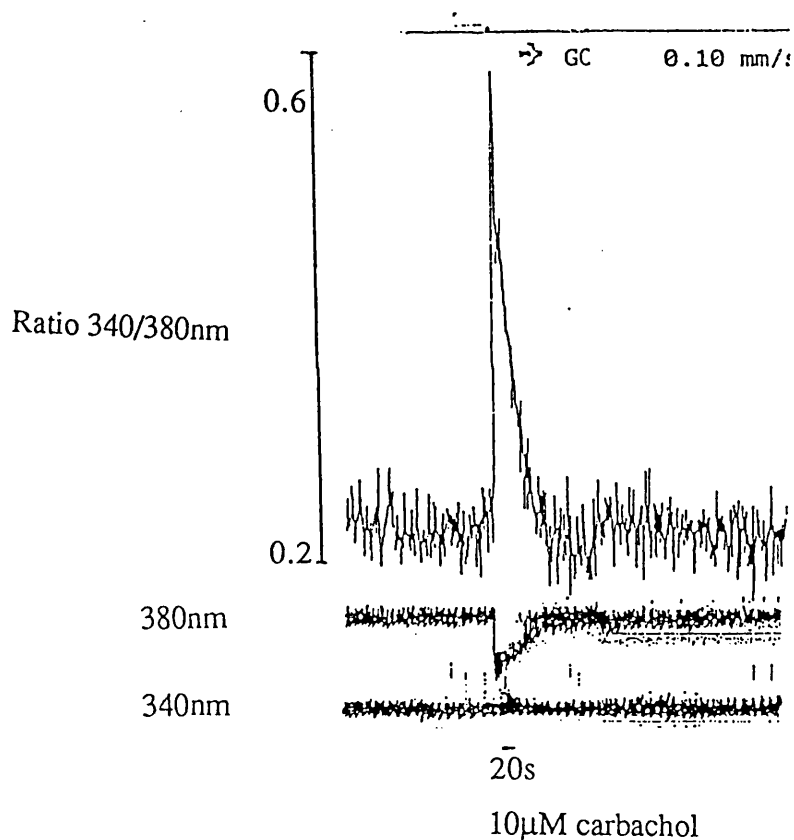
Figure R15. A Ca^{2+} -transient in response to 10μM carbachol for 20s. The transient rise of the ratio of fluorescence intensity from Fura-2 at 340/380nm represents a rise of intracellular Ca^{2+} in response to 10μM carbachol.

116

Experiments were carried out to determine the sensitivity of the Ca^{2+}-transient to the carbachol concentration, and whether there was a difference between cells obtained from stable and unstable bladder biopsies.

The effect of hypoxia on Ca^{2+} metabolism in the cell was also investigated. This included the effect of cellular hypoxia on:

i) stimulation of the muscarinic receptors by carbachol.

ii) stimulation of the intracellular Ca^{2+} stores by caffeine to release Ca^{2+}

iii) Ca^{2+} influx initiated by membrane depolarisation.

The Fura-2 fluorescence ratio was calibrated in control and hypoxic conditions, in the absence of cells, so these effects could be translated into absolute $[Ca^{2+}]$ values. Experiments were also performed to determine whether cellular hypoxia was sufficient to generate an intracellular acidosis and an accumulation of NADH, both indices of increased anaerobic metabolism.

3.1. Calibration of fluorescence ratio

An in vitro calibration was performed. This determined the fluorescence ratio at increasing concentrations of Ca^{2+} in the absence of cells. This calibration was performed in control solution and with 2mM NaCN added. This was to establish the effect of 2mM NaCN on the fluorescent properties of Fura-2 and therefore provide accurate calibration of the $[Ca^{2+}]_i$ in signals .

An intracellular mock solution was used with varying amounts of added $CaCl_2$, to give various known $[Ca^{2+}]$. Fura-2 fluorescence was measured at 37°C for each concentration of free $[Ca^{2+}]$ (see Methods). The determination of the Fura-2 dissociation constant K_d with free $[Ca^{2+}]$ was achieved using the following equation;

$$[Ca^{2+}] = K_d [(R - Rmin) / (Rmax - R)]. \partial$$

where R is the ratio of 340/380nm for a given $[Ca^{2+}]$, Rmin the ratio in the absence of

Ca^{2+} , Rmax the ratio in the presence of saturating Ca^{2+} and ∂=F380min / F380max i.e.

fluorescence intensities at 380nm in the absence of Ca^{2+} and presence of saturating Ca^{2+}

respectively.

Figure R16 shows the relationship between the ratio, R, and $[Ca^{2+}]$ of the calibration

solution.
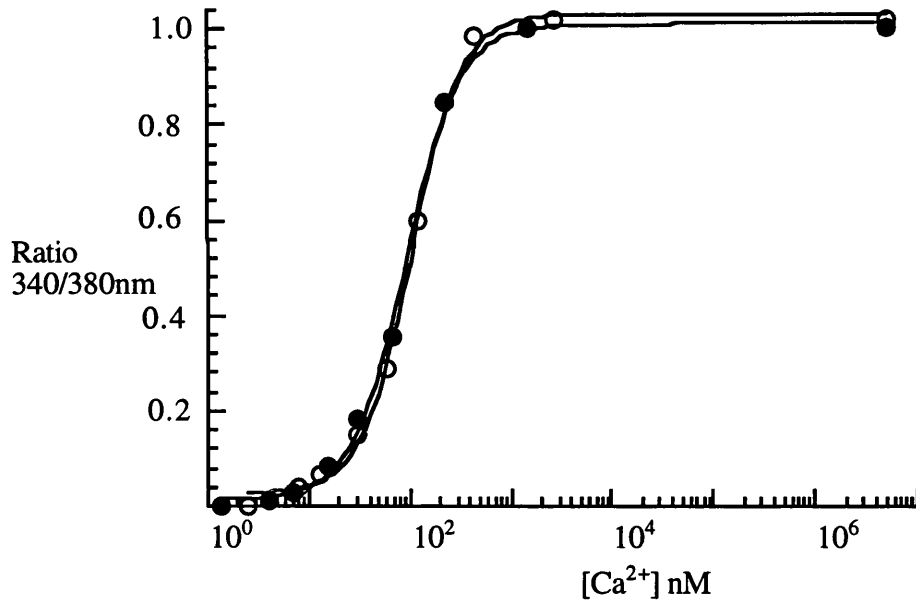
Figure R16. The relationship between the 340/380nm Fura-2 ratio and the $[Ca^{2+}]$ in control

conditions (clear circles) and in the presence of 2mMNaCN (filled circles). The max value

of the ratio =1.0.

From this equation and the data shown in fig R16 the value for the Fura-2 K_d in control

conditions was 283nM and in 2mMNaCN was 353nM. This higher K_d in 2mM NaCN

means that in these conditions Fura-2 has less affinity for Ca^{2+}, shifting the curve to the right.

In 20 experiments it was possible to measure the absolute magnitudes of the individual 340nm and 380nm signals as well as the 340/380nm ratio and then calculate the absolute $[Ca^{2+}]$. The individual raw 340 and 380nm signals were present on these traces, the other experiments, performed before the calibration had been attempted had not had the raw signals displayed on the printed traces and therefore could not be considered for these calculations. The above equation was used with K_d values of 283nM for control conditions and 353nM for 2mM NaCN conditions. Results in later sections will be considered first as ratios then expressed as absolute $[Ca^{2+}]$ values where possible. As the calibration was in vitro, the calculated $[Ca^{2+}]$ is assumed to be equivalent to the intracellular $[Ca^{2+}]$ (see Discussion section 2.5 p157).

It was important initially to determine the relationship between the measured 340/380 ratios and the calculated $[Ca^{2+}]$ in both normoxic and hypoxic conditions. This permitted calculation of the proportional change of $[Ca^{2+}]_i$ from the change of the ratio signal and established if there was any relationship between the initial value of $[Ca^{2+}]$ and the magnitude of subsequent interventions.

Figure R17 shows expanded regions of the calibration curves in figure R16 in the range of $[Ca^{2+}]$ 10-1000nM. During the experimental interventions described here the range of 340/380nm ratio values was within the approximately linear part of the dissociation curve. Thus a change of the 340/380 ratio is approximately equivalent to a proportional change of $[Ca^{2+}]$.
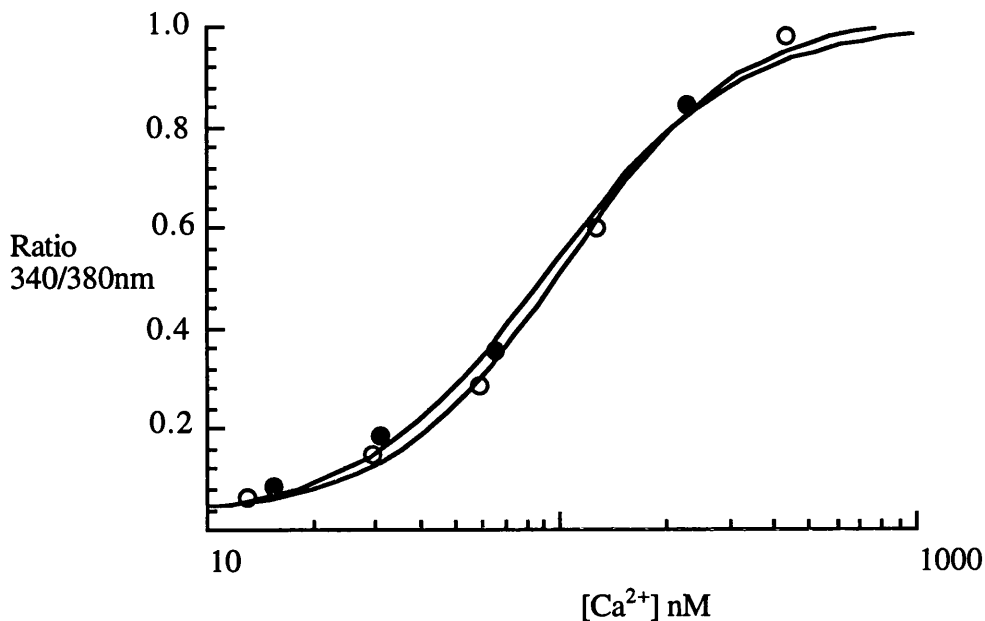
Figure R17. The relationship between the in vitro 340/380nm Fura-2 ratio and the $[Ca^{2+}]$ (range 10-1000 nM) in control conditions (filled circles) and in the presence of 2mM NaCN (clear circles).

Figure R18, overleaf, plots the magnitude of the 340/380 nm ratio change generated by 10μM carbachol in 2mM NaCN. These values on the ordinate are expressed as a proportion of a similar carbachol intervention under control conditions. These proportional values are plotted as a function of the calculated proportional increase of the $[Ca^{2+}]_i$ in carbachol generated in 2mM NaCN compared to control.

$[Ca^{2+}]_i$ (nM) in 2mM NaCN % control

Ca^{2+}-transient ratio in 2mM NaCN, % of control
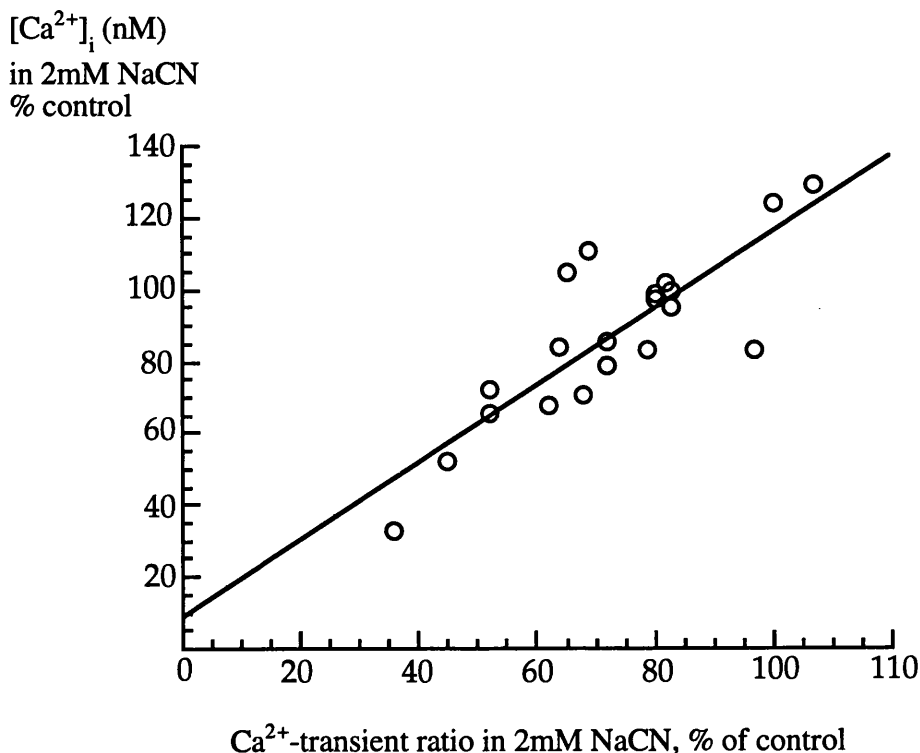
Figure R18. The relationship between the relative magnitude of Ca^{2+}-transients in hypoxic conditions when measured as a 340/380 ratio and when calculated as an absolute $[Ca^{2+}]$. The values are expressed as a fraction of the control transient .

The slope of line was 0.83, with a value of r =0.84, p< 0.05, this further illustrates that changes to the $[Ca^{2+}]$ occurred over the approximately linear region of the calibration curve.

The percentage reduction of the 10μM carbachol Ca^{2+}-transient in the presence of 2mM NaCN was not a function of the magnitude of the control Ca^{2+}-transient. Figure R19 shows a plot of these two variables and a least-squares linear fit showed no significant relationship (r =0.54, p>0.05, n=20).

121

% of control
in 2mM
NaCN

10µM Carbachol Ca-transient, change in $[Ca^{2+}]_i nM$
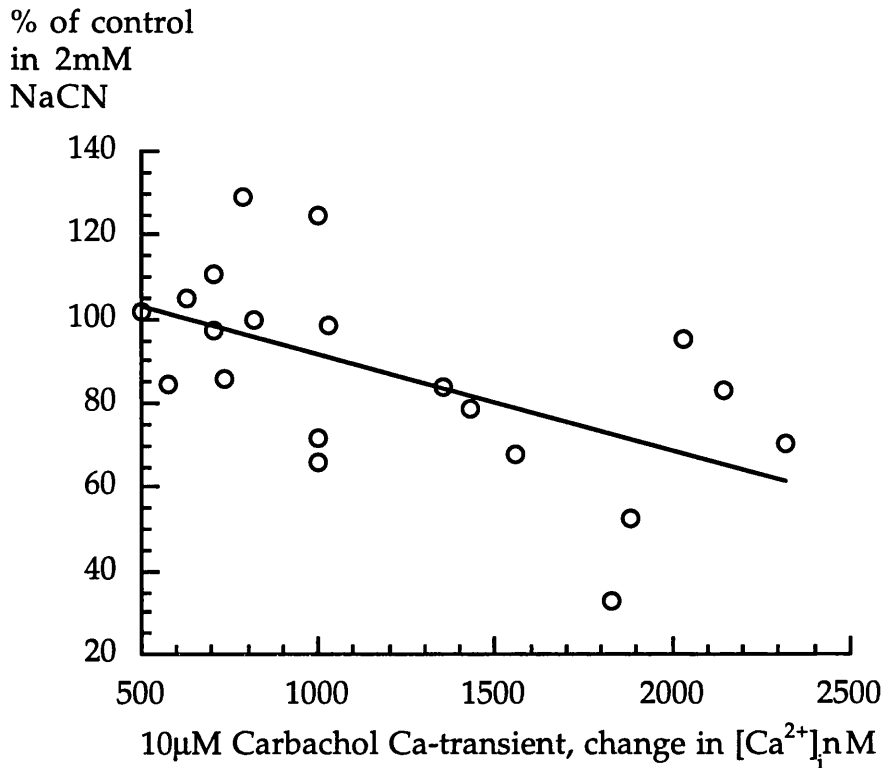
Figure R19. A scatter plot showing the relationship between the percentage change of the Ca^{2+} transient in 2mM NaCN and the absolute magnitude of the carbachol-induced transient. The least-squares best fit straight line is included but there was no significant relationship between the variables (see text).

Thus the effect of 2mM NaCN on the cell is independent of the magnitude of the control carbachol Ca^{2+}-transient.

3.2. The measurement of intracellular $[H^+]$

The action of 2mM NaCN of the intracellular pH of the cell was investigated to determine whether the cellular hypoxia was sufficient to generate an intracellular acidosis.

Human detrusor cells were prepared by the methods earlier described and loaded with the intracellular fluorescent indicator BCECF, which binds to H^+, thereby changing its fluorescence spectrum, and can thus be used to measure the intracellular $[H^+]$.

Isolated human detrusor smooth muscle cells were placed on the cover-slip chamber. The chamber was superfused with gassed Tyrode's solution at 37°C and a viable cell was chosen. The cell was excited by light at wavelengths of 430 and 500nm and the fluorescent output was collected between 530 and 580nm.

The cell was equilibrated in Tyrode's solution and then exposed to Tyrode's solution containing 2mM NaCN and any fluorescence change recorded, as shown in figure R20. The cell remained in the 2mM NaCN solution for 5 minutes. These experiments (performed in 4 cells), showed an initial rise in the ratio, representing an increase of pH_i on introduction of the 2mM NaCN solution which then fell to a steady state pH_i after 2 minutes and remained stable thereafter for the duration of the intervention. The pH changes were fully reversible even after 10 minutes exposure to NaCN solution. 5 minutes was chosen as a standard intervention time, when pH_i was recorded. After the 5 minute exposure to 2mM NaCN the superfusate was changed to normal Tyrode's solution once more and left for 5 minutes before pH_i was again recorded.

Following these interventions each cell was calibrated to allow calculation of absolute values of pH_i. The cell was superfused with a solution containing 10µM nigericin, a H^+/K^+ exchanger and 20mM HEPES. This solution had been titrated to values of pH 7, pH 9 and pH 4. The emission ratio at each of these pH values was recorded on the same continuous trace as seen in figure R20.

Ratio500/430nm

nigericin →

Tyrode's

2mM NaCN

Tyrode's

pH 7

pH 4

pH 9

5mins
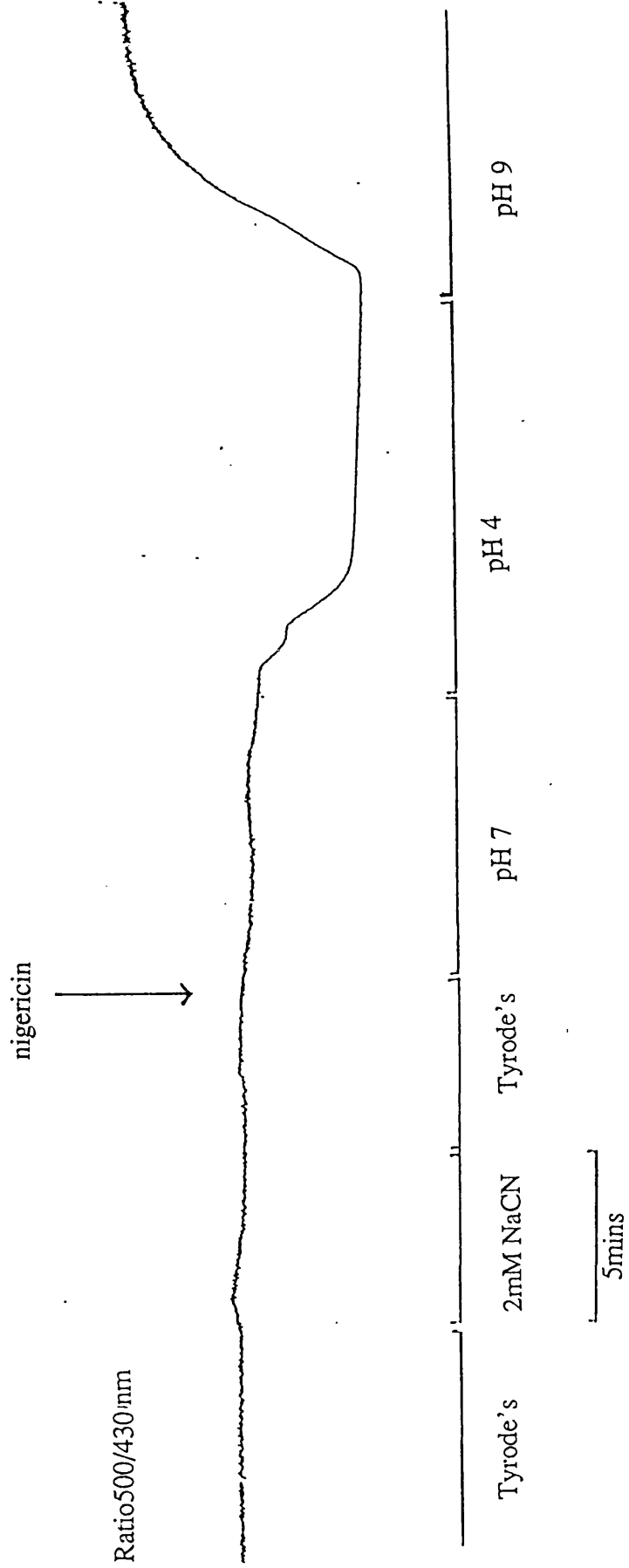
Figure R20. A trace of the ratio of light emitted by the intracellular fluorescent indicator BCECF in an isolated human detrusor myocyte taken from a stable bladder. pH_i is calculated from the ratio of emitted light when excited at 500 and 430nm, 500/430 ratio.

124

The relationship between the 500/430nm ratio and the pH of the intracellular environment is given by the following equation;

$$pH_i = pK + \log (R - R_{min}) / (R_{max} - R) + \log (F430_{min} / F430_{max})$$

where R is the ratio at pH 7, R_{min} the ratio at pH 4, and R_{max} the ratio at pH 9. $F430_{min}$ and $F430_{max}$ are the absolute values of emission during excitation at 430nm at pH 4.0 and 9.0 respectively.

The pK was calculated from the rearrangement of the above equation using the ratio value obtained at pH 7, $R_{7.0}$ i.e.

$$pK = 7.0 - \log [(R_{7.0} - R_{min}) / (R_{max} - R_{7.0})] - \log(F430_{min} / F430_{max})$$

The pK was then used with the corresponding R values to calculate pH_i immediately after exposure to 2mM NaCN and then after the cell had equilibrated at 5 minutes. The results are shown in table R2 as mean values ± s.d. (n=4).

Table R2 The mean pH_i (±s.d.) recorded in control conditions, peak value and steady state value in 2mM NaCN.

Tyrode's	Peak pH_i in CN⁻	Steady state pH_i
7.01 ± 0.01	7.05 ± 0.05 (p<0.05)	7.02 ± 0.02

These results show there was a significant increase of pH_i at the peak of the change, (Student's paired "t" test, p>0.05) in 2mM NaCN, but at steady state the value was not significantly different from control.

3.3. The measurement of cell autofluoresence.

Cyanide ions affect the mitochondria by inhibiting the terminal cytochrome and hence oxidative phosphorylation. This leads to an increase in cellular anaerobic respiration and an increase of the NADH/NAD ratio. In order to assess that the concentration of NaCN used in these experiments exerted its expected effect on the mitochondria it was necessary to estimate changes to the NADH/NAD ratio and hence confirm that 2mM NaCN caused cellular hypoxia.

The autofluoresence (i.e. in the absence of fluorochrome) of isolated single human detrusor smooth muscle cells was measured at 380nm. This has been shown in other cells (Minezaki et al 1994) to be a function of the NADH/NAD ratio such that an increase in NADH concentration enhances cell autofluorescence. The level of autofluorescence was measured in control conditions and in the presence of 2mM NaCN. The measurements at 5 minutes exposure to 2mM NaCN were compared with the mean of the pre- and post-controls.

126
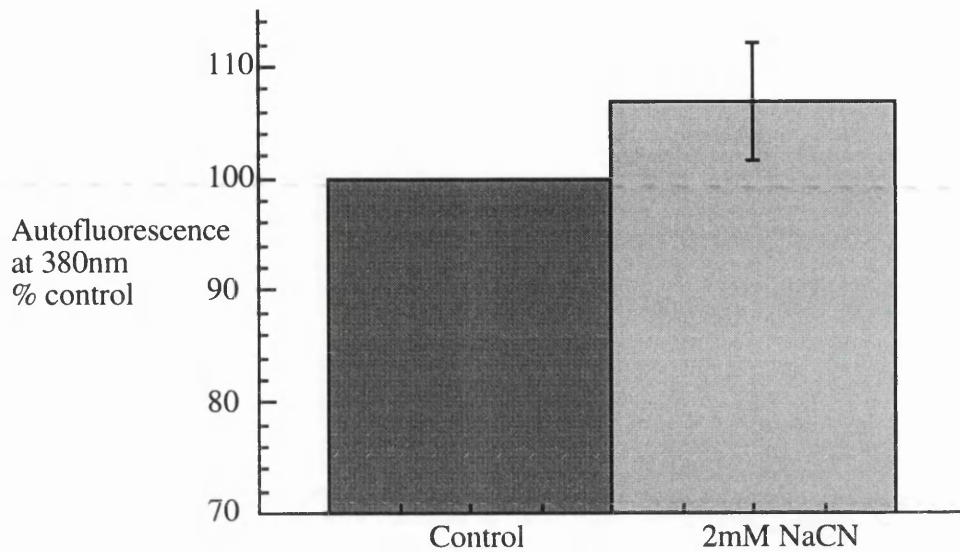
Figure R21. The mean autoflouresence observed in control conditions and in the presence of 2mM NaCN of 18 isolated myocytes from stable bladders. The autoflourescence is expressed as a percentage of the control. Mean data ±s.d.

The mean autoflouresence in the presence of 2mM NaCN was 107% ± 5% of the autofluoresence in control conditions and represented a significant increase (p=0.01 paired Student's "t" test). This supports the hypothesis that NaCN is acting by attenuating mitochondrial oxidative phosphorylation.

The level of background fluorescence, measured in the absence of cells, from the superfusate alone was less than 5% of that when a cell was present and did not alter when the superfusate was changed from Tyrode's to one containing 2mM NaCN. Therefore the level of autofluoresence was assumed to originate from the cell.

3.4. The effect of 2mM NaCN on the resting sarcoplasmic [Ca^{2+}]

The addition of 2mM NaCN to the superfusate elevated the resting Fura-2 fluorescence ratio in 29 of 32 cells in this study. This rise, is shown in figure R22 as the increased baseline between the dotted lines. It was rapid following addition of 2mM NaCN, and reached a steady state unaltered by increasing the duration of exposure. It was shown to be reversible on return to the control Tyrode's solution. The relative magnitude of the rise of the fluorescence signal in figure R22 is shown in comparison to a 10μM carbachol evoked Ca^{2+}-transient.

10μM carbachol

20s

Resting level in 2mM NaCN

Resting level in Tyrode's

Tyrode's

2mM NaCN
5 mins
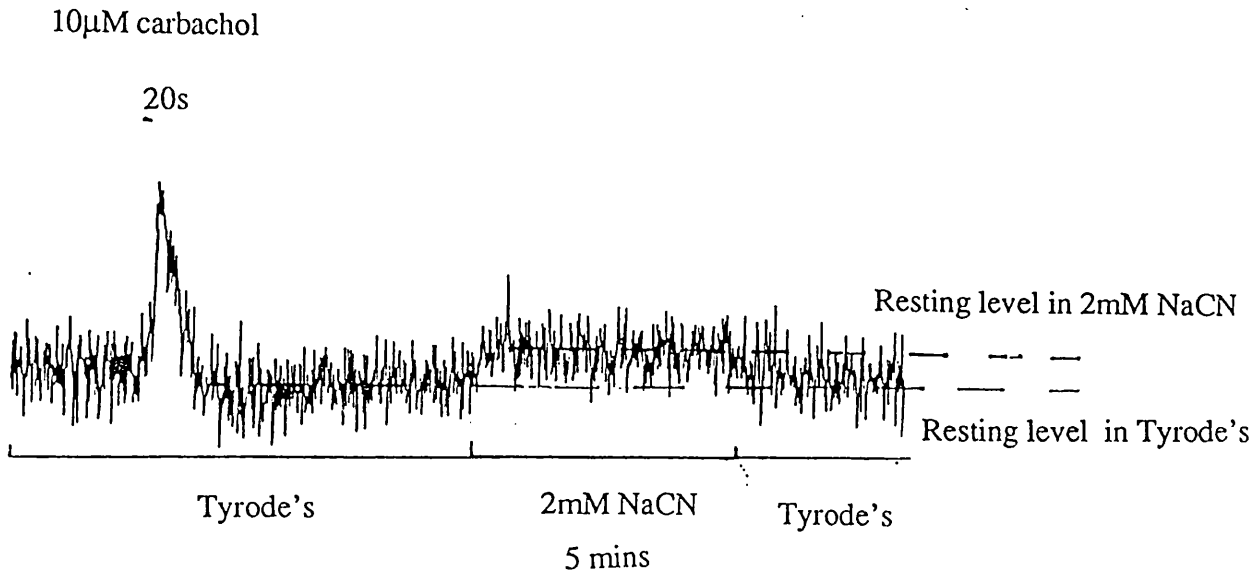
Tyrode's

Figure R22. A trace showing the elevation of the Fura-2 340/380nm fluorescence ratio in the presence of 2mM NaCN, and on its return to control superfusate. A control 10μM carbachol transient in normal Tyrode's solution is shown for comparison.

128

The resting $[Ca^{2+}]_i$ of each cell which is represented by this baseline was calculated in the presence of 2mM NaCN and in control conditions for 9 cells from unstable bladders and 11 cells from stable bladders using the appropriate calibration curves. These values are illustrated in figure R23.
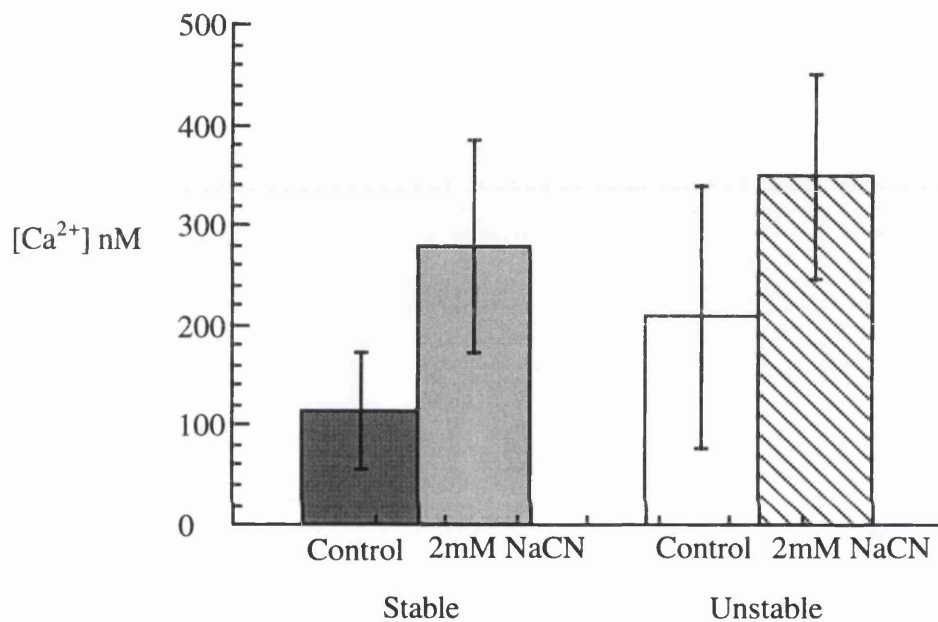
Figure R23. The resting values of the $[Ca^{2+}]_i$ in control and in the presence of 2mM NaCN using cells from stable (n=11) and unstable (n=9) bladders. Mean ± s.d.

The mean control resting value of the $[Ca^{2+}]_i$ was 114 ± 59nM and 209 ±131nM in cells from stable and unstable bladders respectively, there was no significant difference between these two groups (p>0.05 unpaired "t" test). The $[Ca^{2+}]_i$ rose in the presence of 2mM NaCN to 279 ± 106nM and 350 ± 103 nM (stable and unstable respectively). The rise in the sarcoplasmic $[Ca^{2+}]$ in the presence of 2mM NaCN from the control resting $[Ca^{2+}]_i$ was significant (p<0.001) in both groups. The mean change of sarcoplasmic $[Ca^{2+}]$ from control to intervention conditions was 165 ± 104nM and 152 ± 100nM (stable and unstable

respectively). There was no significant difference in the magnitude of this rise in the groups (p>0.05 unpaired "t" test) and so all cells were considered as a single set giving a mean rise in resting $[Ca^{2+}]_i$ of $154 \pm 24nM$.

These data show that the addition of 2mM NaCN to the superfusate resulted in a similar increase of $[Ca^{2+}]_i$ in cells from both diseased and normal bladders.

In the following experiments results are given in absolute values of $[Ca^{2+}]_i$ and the change of $[Ca^{2+}]_i$ from resting values to peak values during an intervention, such as carbachol addition.

3.5. The response of isolated detrusor smooth muscle cell to extracellular carbachol

The typical Ca^{2+}-transient in response to a brief exposure to extracellular carbachol has been described. In the following experiments the relationship between the concentration of carbachol and the magnitude of the evoked Ca^{2+}-transient was measured. Figure R24 shows a typical experiment in which a single cell was exposed briefly to increasing concentrations of carbachol. The Ca^{2+}-transient increased in magnitude until a maximum was achieved at between 3 and 10μM.
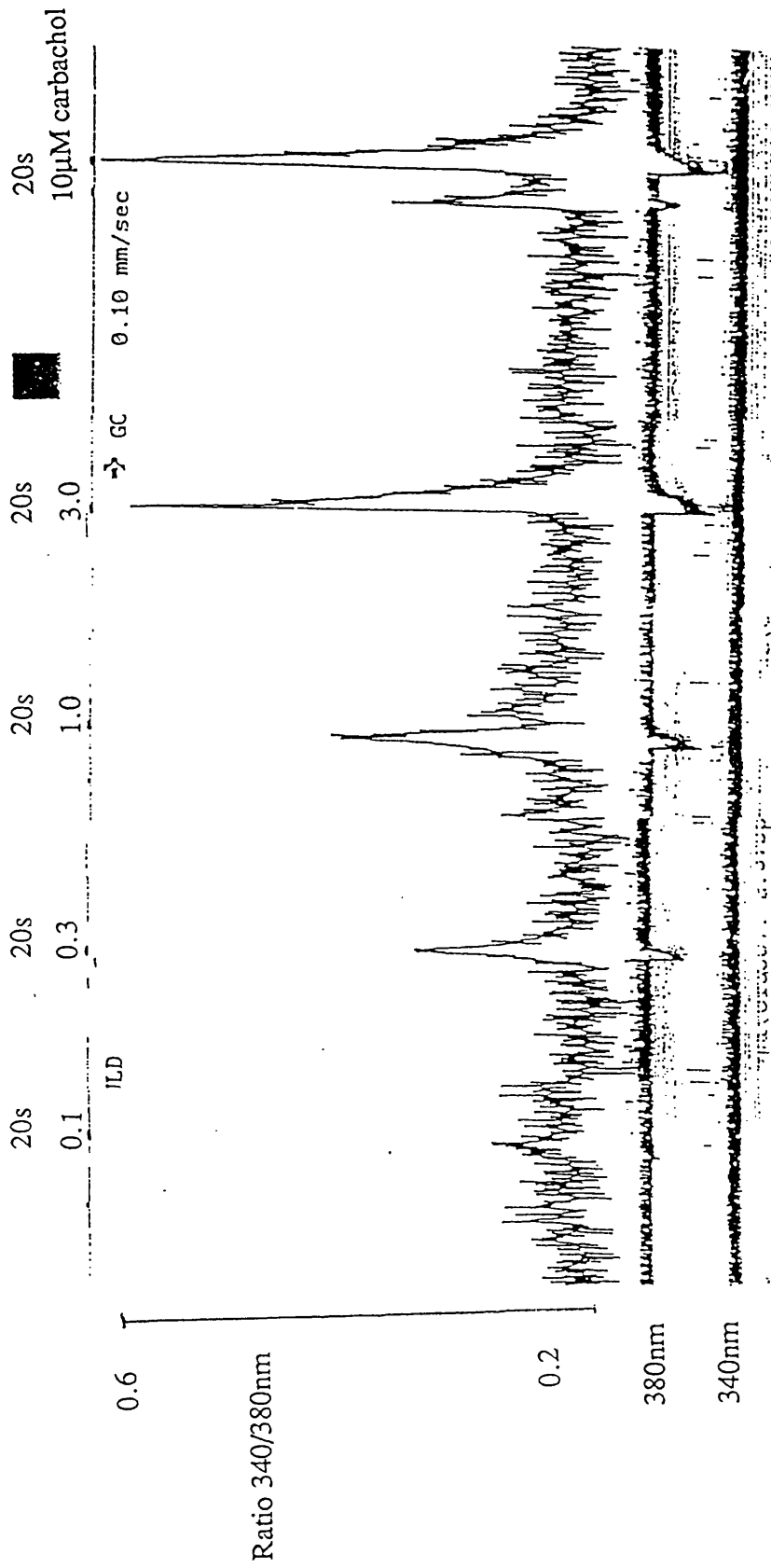
Figure R24. A typical trace demonstrating the response of a single detrusor smooth muscle cell to increasing concentrations of carbachol (0.1, 0.3, 1.0, 3.0, 10μM). The transient rise of the $[Ca^{2+}]_i$ (illustrated as an upward deflection of the trace from the baseline) increases in magnitude with larger concentrations of carbachol. A spontaneous transient, occurring prior to that evoked by 10μM carbachol is also shown.

131

Transient rises of the $[Ca^{2+}]_i$ that occurred without prior stimulation by agonist, spontaneous Ca^{2+}-transients, were common in the majority of cells. They were similar in character to transients evoked by carbachol or caffeine and occurred in both control and hypoxic conditions, in samples from both stable and unstable bladders with equal frequency.

The dose-response relationship between the concentration of carbachol and the height of the evoked Ca^{2+}-transient is plotted in figure R25. The Ca^{2+}-transient is expressed as a percentage of the maximum Ca^{2+}-transient evoked by $10\mu M$ carbachol. Data are shown as mean ± s.d. from 8 cells from stable and 14 from unstable bladders
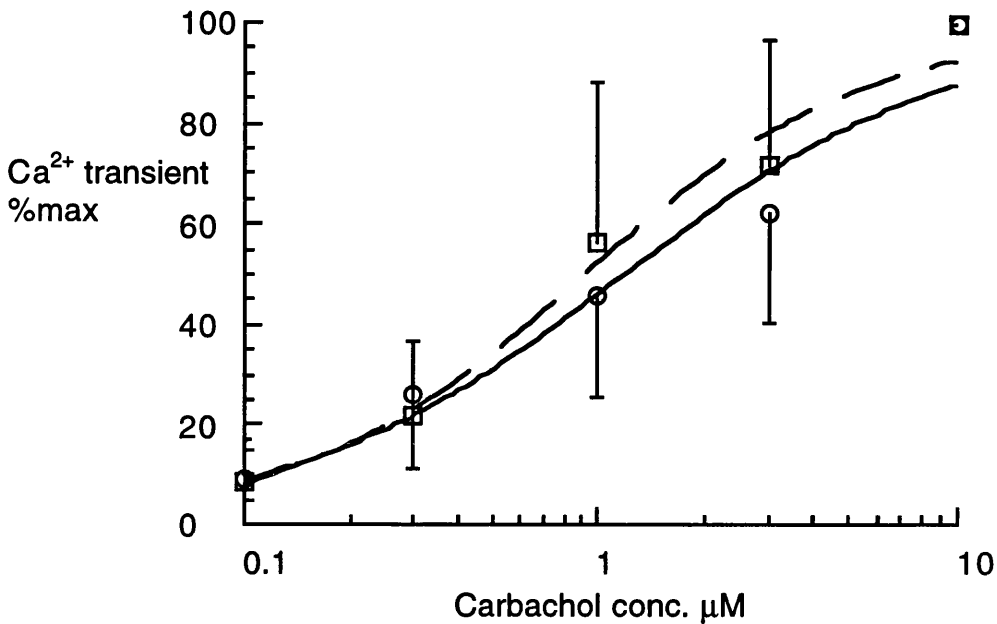
Figure R25. The dose-response relationship between carbachol concentration and the peak value of the Ca^{2+}-transient. The Ca^{2+}-transient is expressed as a percentage of the maximum response. Mean ± s.d.; (n=8 in the stable group (circles) and n=14 in the unstable group (squares)). The lines are best fits to the data using the equation on page 86.

Dose-response relationships were obtained for each cell. This enabled the EC_{50} value (carbachol concentration yielding a half maximum response) to be calculated for each cell in both the unstable and stable bladder groups.

The mean EC_{50} value was calculated for both the stable and unstable groups. The EC_{50} value for the stable group was 1.5 ± 1.3 µM carbachol (mean ± s.d. n=8) and for the unstable group was 1.3 ± 1.1µM carbachol (mean ± s.d. n=14).

These values for the half maximal concentration show no significant difference between the two experimental groups (p> 0.05 unpaired "t" test).

3.6. The effect of 2mM NaCN on the carbachol-induced-Ca^{2+}-transient.

It has been shown previously that 2mM NaCN reduced the force of the carbachol contracture in isolated detrusor smooth muscle strips. This series of experiments was designed to investigate the cellular mechanism underlying this phenomenon. The extent to which hypoxia reduced force in muscle strips differed between detrusor muscle samples from stable and unstable bladders. This series of experiments investigated the effect of 2mM NaCN on the Ca^{2+} transient using isolated detrusor myocytes from stable and unstable bladders.

Cells were superfused with normal Tyrode's solution and a control Ca^{2+} transient evoked by a 20s exposure to 10μM carbachol obtained. The cell was then exposed to 2mM NaCN Tyrode's solution for 3-5 minutes to equilibrate. After this time 10μM carbachol stimulation was again applied for 20s and a Ca^{2+} transient obtained before removing the 2mM NaCN Tyrode's solution and returning the cell to control conditions. A post-control carbachol induced Ca^{2+} transient was obtained in each case and the mean of these two controls is stated as the average "control".

Figure R26 shows that the Ca^{2+} transient was reduced in magnitude by 2mM NaCN, note also the initial increase of resting $[Ca^{2+}]_i$ on the initial exposure to 2mM NaCN.
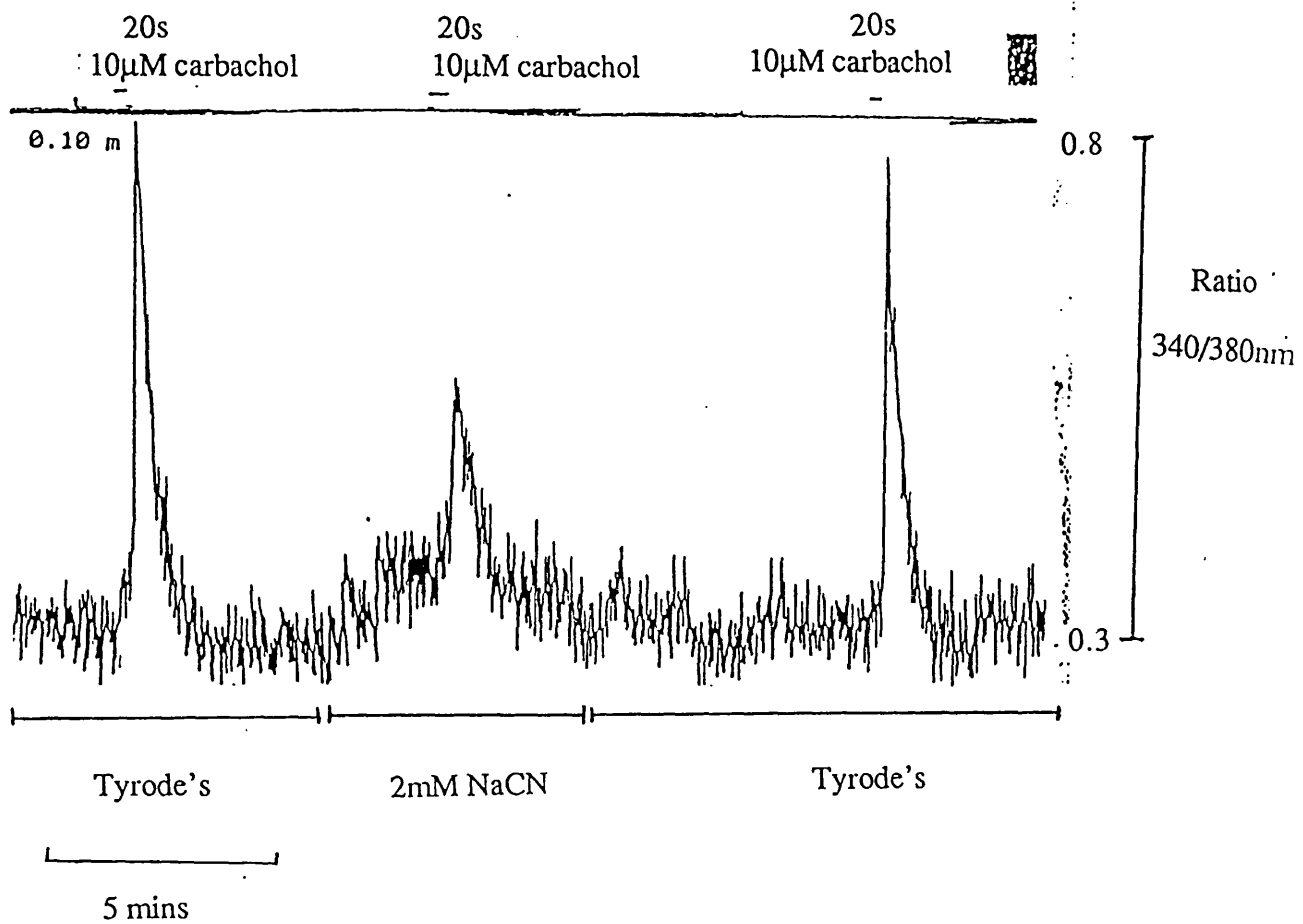
Figure R26. A typical trace showing the Ca^{2+}-transient evoked by $10\mu M$ carbachol in the presence and absence of 2mM NaCN. The cell was isolated from a sample of detrusor smooth muscle from an unstable bladder.

Figure R27 shows the mean data of the effect of 2mM NaCN on the 10μM carbachol Ca^{2+}-transient obtained from 12 cells from stable bladders and 20 cells from unstable bladders. The mean magnitude of the Ca^{2+}-transient in 2mM NaCN from the stable group was 63% ± 18% of the control and from the unstable group was 77% ± 18% of control.
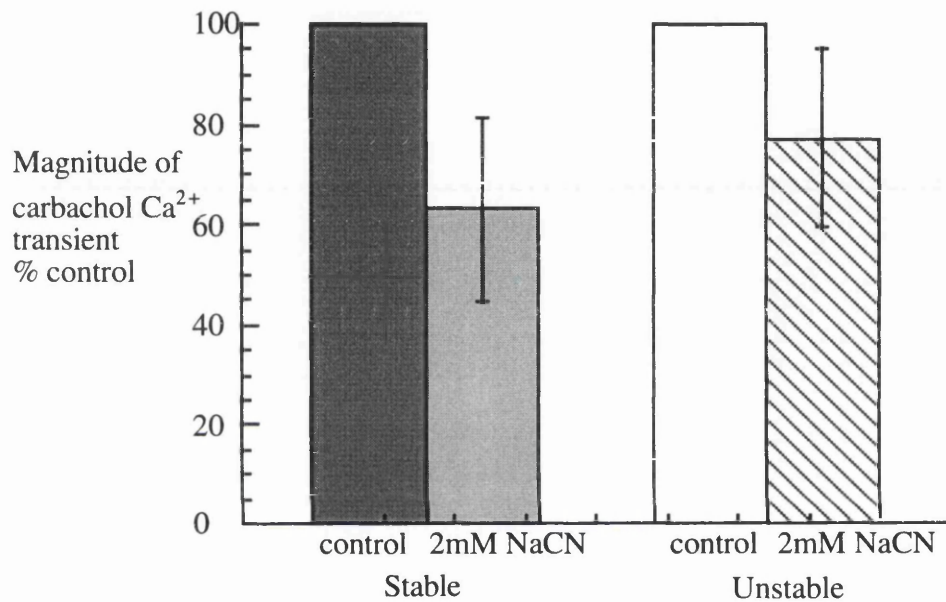
Figure R27. The effect of 2mM NaCN on the Ca^{2+}-transient evoked by 10μM carbachol. Data are shown (mean ± s.d.) for control conditions in the absence of NaCN (control) and in the presence of 2mM NaCN for cells from stable (n=12) and unstable (n=20) bladders.

However, the mean magnitude of the Ca^{2+}-transient evoked by 10μM carbachol generated in the presence of 2mM NaCN was significantly reduced in both groups, when compared to the control (p<0.001). The magnitude of the transient was significantly less affected in the unstable group compared to the stable group (p< 0.05).

It was possible to calculate the absolute $[Ca^{2+}]_i$ in 11 cells from stable and 9 cells from unstable bladders. The values of the mean peak $[Ca^{2+}]_i$ and the change of $[Ca^{2+}]_i$ is shown

in table R3 under control conditions and in the presence of 2mM NaCN, both as absolute values and percentage change from control.

Table R3.   The carbachol induced transient rise of $[Ca^{2+}]_i$ in control conditions and in the presence of 2mM NaCN in cells from stable (n=11) and unstable (n=9) bladders. *p< 0.05 2mM NaCN compared to control.

	Stable		Unstable	
	Control	**2mM NaCN**	**Control**	**2mM NaCN**
Mean peak $[Ca^{2+}]_i$ nM ± s.d.	**1096 ± 497**	**778 ± 231 ***	**1329 ± 665**	**1212 ± 477**
% control peak s.d.	**100**	**75 ± 23 ***	**100**	**97 ± 21**
Change in $[Ca^{2+}]_i$ from resting value nM ± sd.	**981 ± 487**	**498 ± 180 ***	**1119 ± 717**	**861 ± 527 ***
% control change $[Ca^{2+}]_i$ from resting value nM ± s.d.	**100**	**57 ± 21 ***	**100**	**79 ± 22 ***

The following conclusions can be drawn from table R3.

1) There was a significant decrease in the carbachol evoked peak $[Ca^{2+}]_i$ in 2mM NaCN compared to that in control solutions in cells from stable bladders (paired Student's "t" test p<0.001)

2) There was no significant difference (paired Student's "t" test p>0.07) in the carbachol evoked peak $[Ca^{2+}]_i$ in 2mM NaCN compared to that in control solutions in cells from unstable bladders when calculated using absolute values or percentage of control, (col 1 & 2 shown by the absence of "*").

These observations do not take into account the different resting levels of $[Ca^{2+}]_i$ in individual cells, or the change of resting $[Ca^{2+}]_i$ in 2mM NaCN compared to control, Tyrode's solution.

Therefore the absolute change in $[Ca^{2+}]_i$ evoked by 10 μM carbachol was calculated by subtracting the resting $[Ca^{2+}]$ from the peak $[Ca^{2+}]$ in control and intervention solutions.

3) There was a significant attenuation (paired Student's "t" test p<0.02) of the change of $[Ca^{2+}]$ by carbachol in 2mM NaCN, compared to control Tyrode's solution, of both groups, (col 3 & 4).

4) Moreover this attenuation was significantly greater in cells from stable bladders (unpaired Student's "t" test p<0.02) than those from unstable bladders, (col 3 & 4).

3.6.i) The relationship between NaCN concentration and attenuation of the 10μM carbachol evoked Ca^{2+} transient.

The concentration-dependence of the reduction of the carbachol Ca^{2+} transient with NaCN was measured to check that the concentration used, 2mM, was optimal. The height of the Ca^{2+} transient produced in increasing concentrations of NaCN was compared to the control in 5 cells. After exposure of the cell to a concentration of NaCN the cell was allowed to rest in control conditions before the exposure to a different concentration of NaCN. The mean attenuated expressed as a percentage of the control Ca^{2+}-transient was plotted as a function of the NaCN concentration and is shown in figure R28
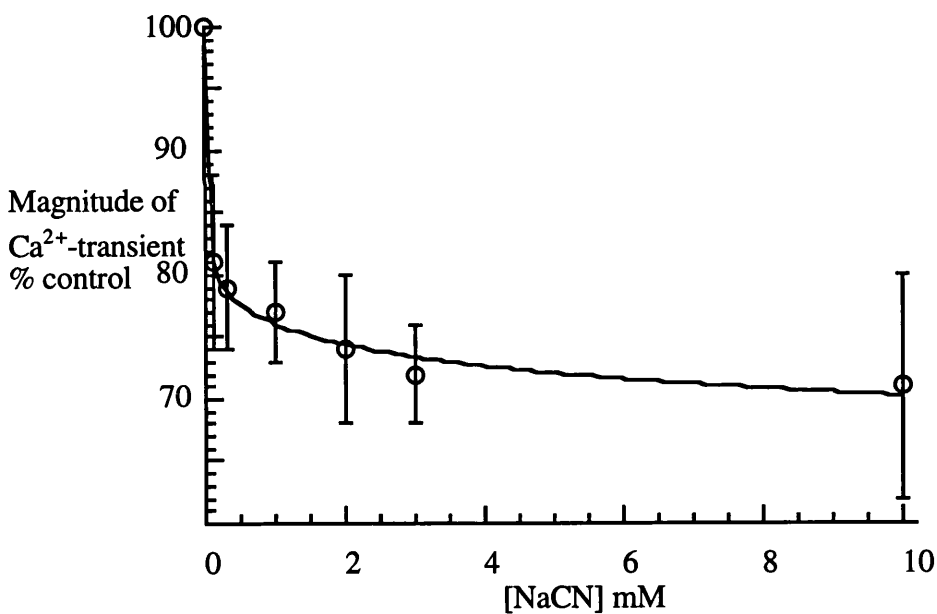
Figure R28. The reduction of the carbachol induced Ca^{2+}-transient as a function of [NaCN]. Ca^{2+}-transients were measured as the % magnitude evoked by the application of

10μM carbachol in control conditions. Mean ± s.d., n=5 cells for all concentrations except 2mM where n = 20.

The data in figure R28 were fit to the equation

$$C = [m-(C_{max} [s]/EC_{50}+[s])] + n$$

Where EC_{50} is the concentration of NaCN producing a half maximal effect, [s] the NaCN concentration, C_{max} is the difference between the magnitude of the transient C at zero and at very high NaCN concentrations and m, n, are constants. A value of 50μM was obtained for the EC_{50} value of NaCN.

In further experiments 2mM NaCN was used as it clearly elicited a maximal response.

It has been shown that 2mM NaCN affects the magnitude of the carbachol induced Ca^{2+}-transient in cells from both stable and unstable bladders. The possible site of action of cellular hypoxia was next investigated by assessing the effect of 2mM NaCN on the release of Ca^{2+} from the sarcoplasmic Ca^{2+} stores and on the transmembrane influx of Ca^{2+}. The protocols used were similar to those used to determine the cellular site of action of NaCN on the carbachol induced contracture.

3.7. The KCl -evoked Ca^{2+}-transient.

2mM NaCN has been shown previously to have no effect on the KCl contracture in isolated smooth muscle strips from stable and unstable bladders. Parallel experiments were carried out to investigate the effect of 2mM NaCN on the KCl-induced-Ca^{2+}-transient in isolated detrusor smooth muscle cells. The 100mM KCl evoked Ca^{2+} transient is shown following to one evoked by 10 μM carbachol from the same cell in figure R29.

10μM carbachol

20s

100mM KCl

20s

0.6

Ratio 340/380nm

0.2

5mins
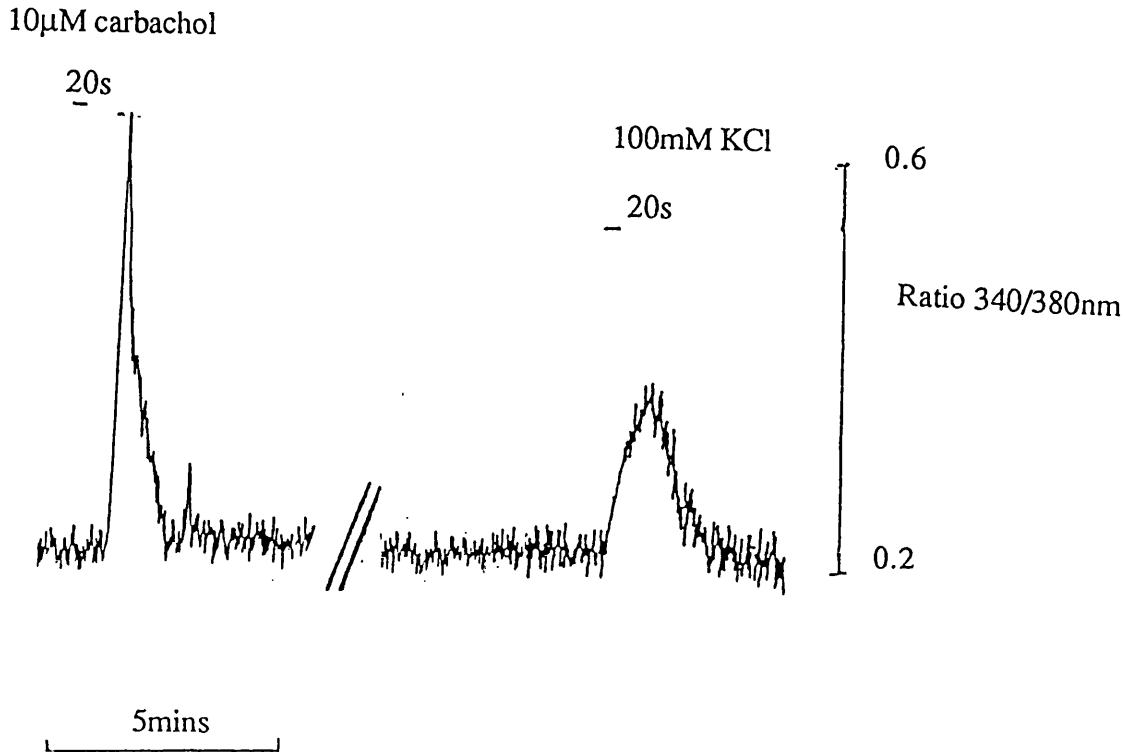
Figure R29. A typical trace illustrating both a 10μM carbachol and 100mM KCl evoked Ca^{2+}- transient in an isolated human detrusor myocyte from a stable bladder. The carbachol evoked transient is rapid with a prompt decline to baseline, no undershoot is demonstrated in this cell. The KCl evoked transient is smaller in magnitude with a slower return to baseline.

3.7.i) The response of the KCl-induced Ca^{2+}-transient to 2mM NaCN.

These experiments paralleled those performed investigating carbachol- and caffeine-induced-Ca^{2+}-transients. 15 cells were isolated from stable (n=5) and unstable (n=10) bladders and exposed to 100mM KCl for 20s in control and 2mM NaCN solutions; all cells

141

produced transients following KCl and carbachol exposure. Figure R30 shows the KCl-induced-Ca^{2+}-transient in a control solution and in the presence of 2mM NaCN. Both transients are similar in magnitude.
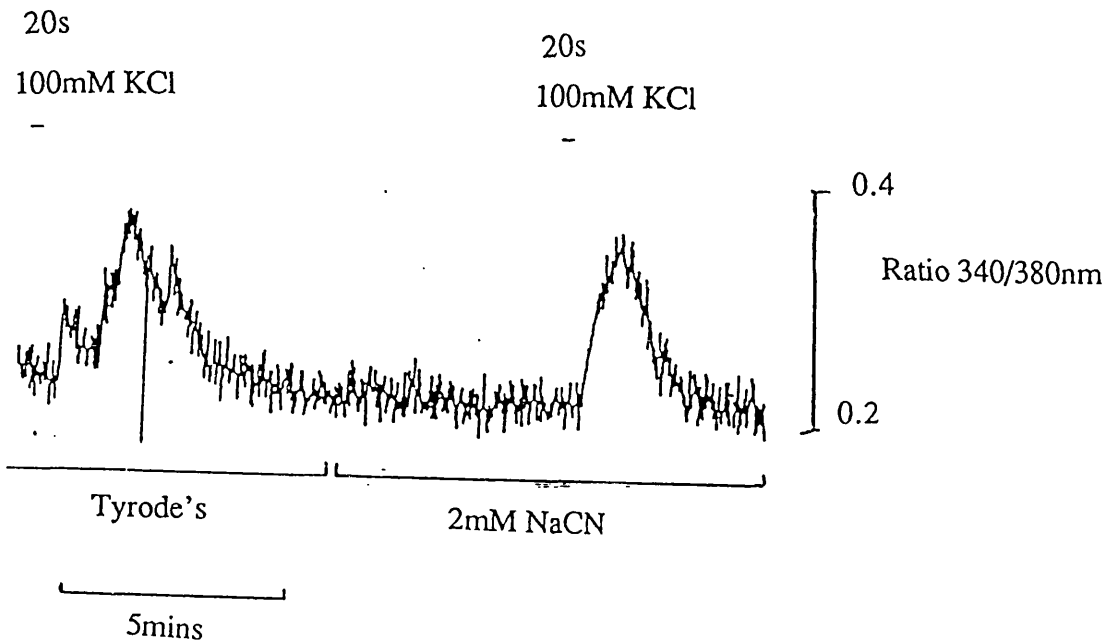
Figure R30. A continuous recording of the ratio of Fura-2 fluorescence at 340/380nm in an isolated human detrusor myocyte. The response to a 20s exposure to KCl in control and 2mM NaCN solutions is shown. There was no rise in baseline fluorescence in 2mM NaCN solution in this particular cell.

Figure R30 shows no significant difference between the KCl-induced Ca^{2+}-transients under control conditions and in the presence of 2mM NaCN (Student's paired "t" test p>0.05). As the addition of 2mM NaCN did not produce any significant difference in the magnitude of the KCl-induced-Ca^{2+}-transient cells from stable and unstable bladders were considered as a single set.
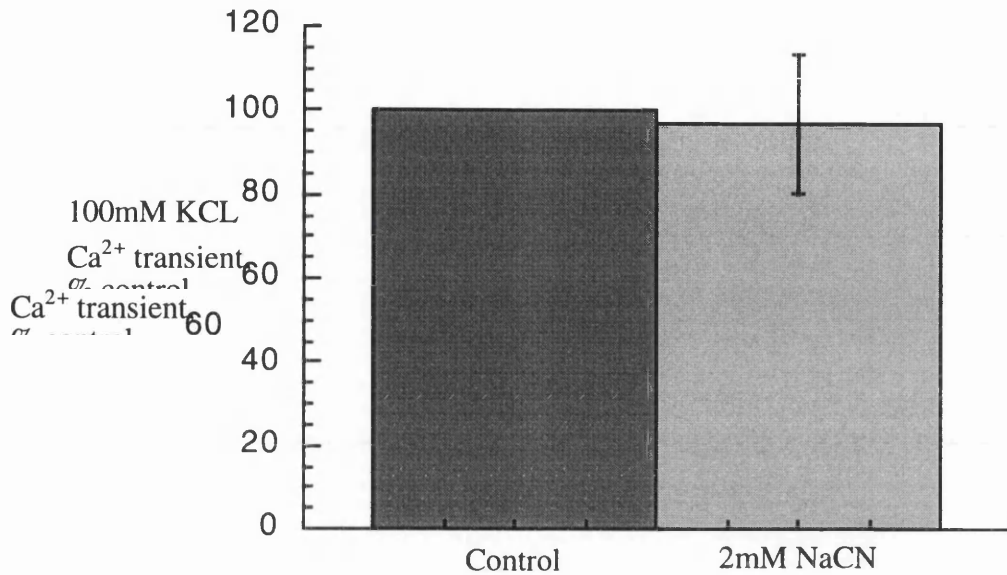
Figure R31. The effect of 2mM NaCN on the magnitude of the KCl-evoked Ca^{2+}-transient. Mean ± s.d. (n=15)

The mean KCl-evoked Ca^{2+}-transient in 2mM NaCN was 97% ±17% s.d. of the control transient, therefore 2mM NaCN exerted no effect on the KCl-evoked Ca^{2+}-transient.

3.8. Effect of caffeine on intracellular Ca^{2+}

Caffeine affects the release of intracellular Ca^{2+} by activating intracellular Ca^{2+} stores leading to a transient increase in the $[Ca^{2+}]_i$. The transient rise in $[Ca^{2+}]_i$ produced by 10mM caffeine was similar in magnitude to that evoked by 10μM carbachol and was also similar in exhibiting an undershoot. Figure R32 illustrates two consecutive Ca^{2+}-transients, the first evoked by 10μM carbachol and the second by 10mM caffeine. The configurations

of the two Ca^{2+}-transients are similar lending weight to the hypothesis that both originate from a similar mechanism, namely release from an intracellular store.
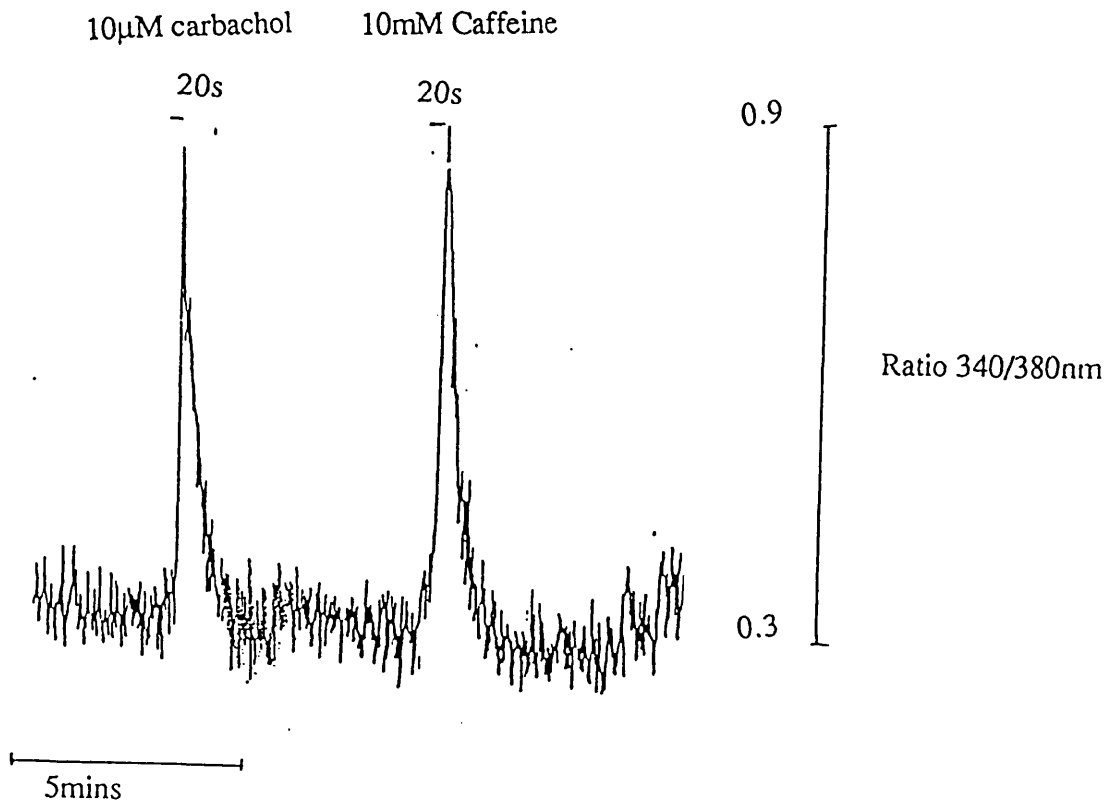
Figure R32

A continuous recording of the ratio of Fura-2 fluorescence at 340/380nm of an isolated human detrusor myocyte from an unstable bladder which demonstrates transients evoked by 10μM carbachol and 10mM caffeine in control conditions.

The mean peak $[Ca^{2+}]_i$ evoked by $10\mu M$ carbachol was $1200 \pm 468nM$ (n=20) and the peak $[Ca^{2+}]$ evoked by $10mM$ caffeine was $1343 \pm 561nM$ (n=9) in cells from both stable and unstable bladders.

The effect of $2mM$ NaCN on the caffeine-induced-Ca^{2+}-transient was next studied.

3.8.i). The effect of 2mM NaCN on the caffeine-induced-Ca^{2+} transient

It has been shown previously that $2mM$ NaCN reduced the magnitude of both the carbachol and caffeine contracture in isolated detrusor smooth muscle strips and also the magnitude of the carbachol-induced-Ca^{2+}-transient. This series of experiments was designed to investigate further the cellular mechanism underlying these phenomena. The extent to which the carbachol contracture and carbachol-induced-Ca^{2+}-transient were attenuated differed according to whether tissue from stable or unstable bladders was used. Therefore the data from cells obtained from both stable and unstable bladder biopsies were used in these experiments and considered separately.

Cells were superfused with normal Tyrode's solution and a control Ca^{2+}-transient in $10mM$ caffeine obtained. The cell was then exposed to a $2mM$ NaCN Tyrode's solution for 3-5 minutes and a $10mM$ caffeine Ca^{2+}-transient obtained before removing the $2mM$ NaCN solution and returning the cell to control conditions. A post-control caffeine-induced Ca^{2+} transient was obtained and the mean of the pre- and post intervention Ca^{2+}-transients is stated as the "control".

Figure R33 shows that the Ca^{2+}-transient in the presence of $2mM$ NaCN was significantly reduced compared to the pre- and post-controls. Note also the rise of the $[Ca^{2+}]_i$ immediately on addition of $2mM$ NaCN.
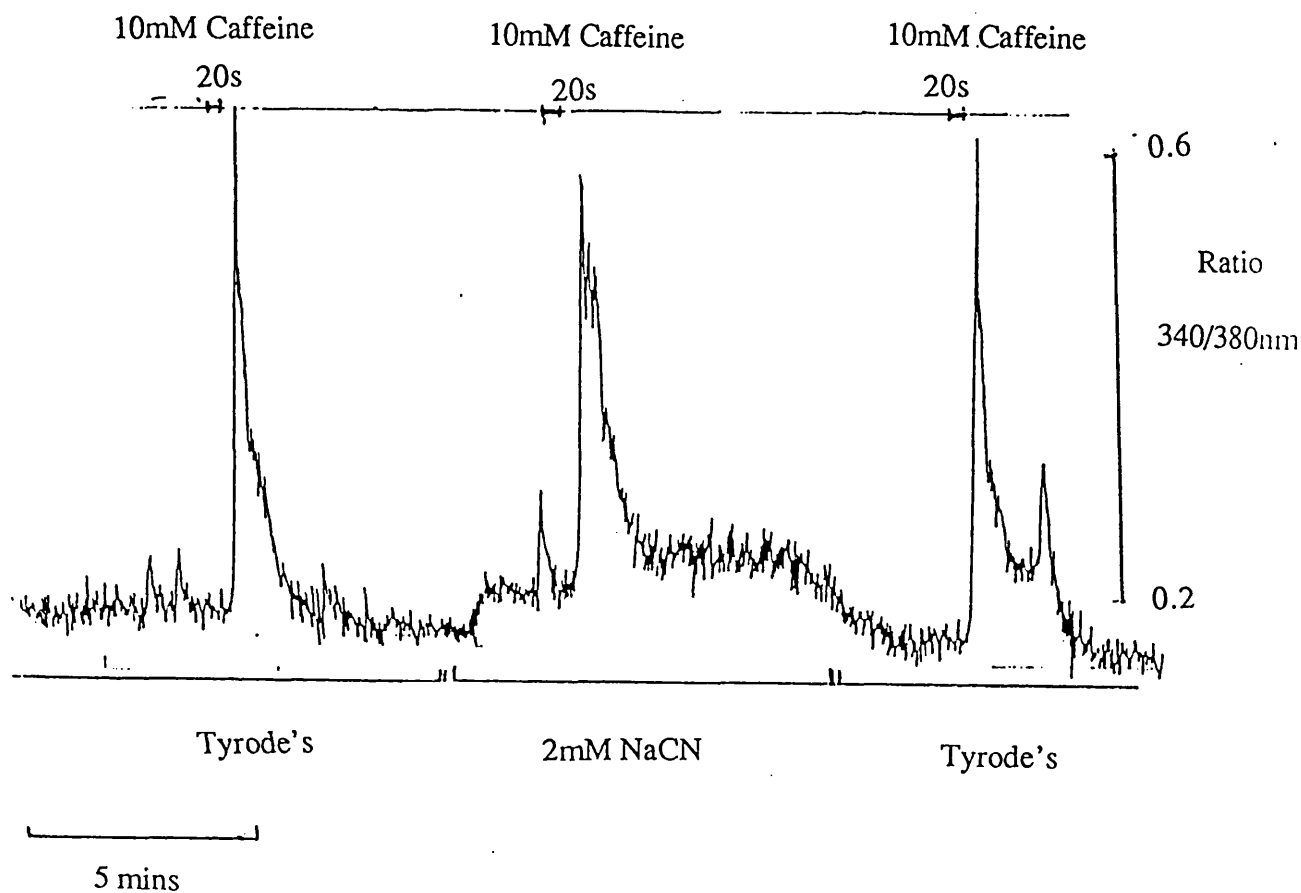
Figure R33. A typical continuous recording of the Fura-2 fluorescence ratio of an isolated human detrusor myocyte from a stable bladder showing the Ca^{2+}-transient evoked by a 20s application of 10 mM caffeine in control solution and in the presence of 2mM NaCN.

Figure R34 shows the mean proportional reduction of the caffeine-induced-Ca^{2+}-transient by 2mM NaCN from 10 cells from stable bladders and 14 cells from unstable bladders.
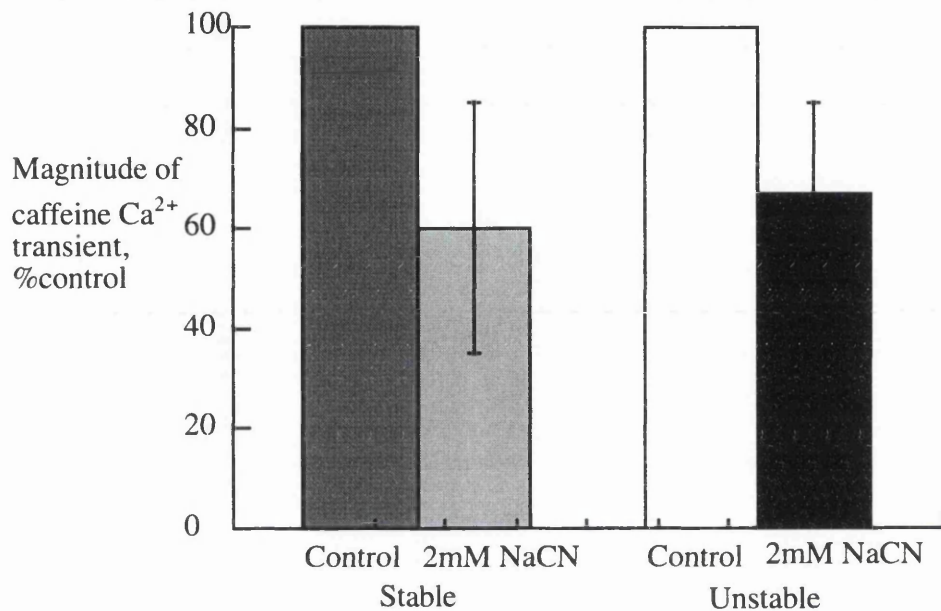
Figure R34. The magnitude of the caffeine-induced-Ca^{2+}-transients measured from the resting level following application of 10mM caffeine in the presence and absence of 2mM NaCN in cells from stable (n=10) and unstable (n=14) bladders. Mean ± s.d.

2mM NaCN significantly decreased the magnitude of the caffeine induced Ca^{2+} transient in both stable and unstable samples (p<0.05). However there was no significant difference between cells isolated from the two experimental groups in the degree of attenuation. The mean Ca^{2+} transient in the presence of 2mM NaCN in the stable group was 60 ± 26% (s.d.) and the unstable group mean was 67 ± 18% (s.d.) of the control(p>0.05).

It was possible to calculate the 10mM caffeine Ca^{2+}-transient in absolute values of $[Ca^{2+}]_i$ in 9 cells (4 from stable and 5 from unstable bladders). Since these numbers are small and

there was no difference observed between the cells from stable and unstable bladders, they are grouped together.

Table R4 shows the effect of 2mM NaCN on the 10mM caffeine-induced-Ca^{2+} -transient as either the peak value of $[Ca^{2+}]_i$ or the absolute increase of $[Ca^{2+}]_i$ from the resting to peak value; similar to the procedure shown with carbachol-induced-Ca^{2+}-transients.

Table R4. The reduction of the caffeine-induced-Ca^{2+}-transient shown as values of $[Ca^{2+}]_i$ in the presence of 2mM NaCN. Both the absolute $[Ca^{2+}]_i$ and the caffeine induced rise of $[Ca^{2+}]_i$ are significantly reduced by the presence of 2mM NaCN (paired "t" test p<0.02).

	Control	2mM NaCN
10mM caffeine evoked peak $[Ca^{2+}]$ nM Mean ± s.d.	1343 ± 561	1001 ± 530
10mM caffeine evoked rise $[Ca^{2+}]$ nM Mean ± s.d.	1176 ± 517	677 ± 321

2mM NaCN significantly reduced the 10 mM caffeine-induced rise in $[Ca^{2+}]$ (p<0.02) when compared to the control. Therefore cellular hypoxia must influence the release of Ca^{2+} from intracellular Ca^{2+} stores.

3.9 Summary of isolated human detrusor myocyte experiments

The calibration of the fluorescent Fura-2 signal to $[Ca^{2+}]$ values showed there was an approximately linear relationship in the range of these experiments between the ratio of fluorescent signals and the corresponding $[Ca^{2+}]_i$. A calibration constant was calculated for both control and intervention solutions which enabled the calculation of $[Ca^{2+}]_i$.

2mM NaCN elevated the resting $[Ca^{2+}]_i$ in isolated human detrusor myocytes. This rise occurred in cells from both stable and unstable bladders to an equal extent and was fully reversible on return to control solution.

2mM NaCN caused no significant change in the pH_i as measured by the fluorescent indicator BCECF.

2mM NaCN caused a rise in detrusor myocyte autofluorescence interpreted as an increase of the ratio of NADH to NAD, and indicating anaerobic respiration.

2mM NaCN reduced the magnitude of the carbachol-and caffeine-evoked-Ca^{2+} -transients. Cells from stable bladders were more sensitive to this intervention with respect to the carbachol-evoked-Ca^{2+} -transient, than those from unstable bladders.

2mM NaCN exerted no effect on the KCl-evoked Ca^{2+}-transient in cells from either stable or unstable bladders.

DISCUSSION

1. Patient Selection

All the work here described relates to experiments performed on human detrusor smooth muscle biopsies donated by patients attending UCL Hospitals. These biopsies were divided into two groups, those from stable or unstable bladders according to the findings on urodynamic testing using ICS standards. All patients had a full history and clinical examination to exclude other pathology.

1.1 Urodynamic assessment and patient groups

Idiopathic detrusor instability is by definition of unknown aetiology, it is classified by the International Continence Society as bladder overactivity in the absence of a clinically relevant neurological disorder (Abrams *et al* 1988) and in many cases it can be difficult to diagnose. The presence of phasic, or unstable detrusor contractions on a conventional urodynamic study, whilst the subject is attempting to inhibit micturition does not in itself constitute a diagnosis. 10% of asymptomatic patients demonstrated such contractions in a study by Turner-Warwick (Turner-Warwick 1979) and 18% of healthy volunteers in another study (Vereecken & Das 1985). Ambulatory monitoring is more sensitive in the detection of phasic detrusor contractions and has been reported in 31 to 69% of healthy volunteers (Heslington & Hilton 1995; Vereecken & Das 1985) without symptoms giving weight to the opinion that the phrase "detrusor instability" is merely a urodynamic observation, not a condition (Griffiths 1988). However the I.C.S. standardisation allows for such observations during urodynamics by stating that urodynamic diagnoses should correlate with the patient's symptoms and signs, e.g. the presence of an unstable contraction in an asymptomatic continent patient does not warrant a diagnosis of detrusor overactivity during storage (Abrams *et al* 1988).

The diagnosis of detrusor instability in this study was made only in the presence of clinical symptoms of instability. Provocative conventional urodynamics were performed routinely in both centres by myself (at the Elizabeth Garrett Anderson Hospital) and two other research registrars(at the Middlesex Hospital Institute of Urology) using the same techniques and applying I.C.S. standardisation. The results of the urodynamics were reviewed and discussed with experienced consultants and surgery planned depending on the clinical circumstances of each individual. The diagnosis of detrusor instability was made after each patient had a normal gross neurological examination which was performed at the time of the urodynamic study appointment. However there are problems with relying on a simple gross neurological examination alone, in clinical practice further tests may be employed if the diagnosis of detrusor instability and normal neurological function is in doubt.

Detrusor hyperreflexia is defined as overactivity due to disturbance of the nervous control mechanism. The term detrusor hyperreflexia should only be used when there is objective evidence of a relevant neurological disorder (Abrams *et al* 1988). Further tests have been reported as detecting neurological lesions even in patients with no symptoms and signs of neurological disease e.g. computerised tomography and MRI of the brain have been shown to pick up previously undetected lesions in elderly men (Kidata *et al* 1992) and the use of an ice water test in urodynamics may assist in the detection of neuropathic bladder (Artibani 1997). Lower urinary tract symptoms are seldom the presenting symptom in multiple sclerosis (Fowler, personal communication) and neurophysiological tests are usually reserved for those patients with neurological or unusual symptoms. The treatment options for the patients in relation to their clinical circumstances in this study had already been considered and further expensive studies in the absence of symptoms which would not have altered the management of these individuals was unrealistic. However considering the published evidence it is entirely possible that an asymptomatic neurological abnormality might have been undetected in this study if further elaborate tests had been performed.

The use of conventional urodynamics to diagnose a stable bladder i.e. one that does not demonstrate phasic detrusor contractions during the filling phase is long established. Its main use is in the assessment of an individual's appropriateness for corrective bladder neck surgery in the treatment of genuine stress incontinence. Failure rates of the Burch colposuspension have been shown to be lower in those patients with stable bladders (25%) than those with unstable bladder contractions on urodynamics (74%) (Jorgensen*et al* 1988) and it is considered that the presence of these contractions is a relative contraindication to surgery (Griffiths 1988). Conventional urodynamics are however less sensitive at the detection of unstable detrusor contractions in women with irritative symptoms and there is an advantage in combining ambulatory monitoring with conventional urodynamics to detect unstable contractions; a combined approach detecting unstable contractions in 84% of symptomatic women (Van Waalwijk-Van Doorn *et al* 1991). Patients in this study were only recruited as stable controls if they had no irritative symptoms and so further investigation using ambulatory monitoring, even if phasic detrusor contractions were discovered, would not have changed their diagnosis as such phasic contractions detected by any system must be considered in the light of the clinical picture (Abrams 1988).

The control group of patients in this study were suffering from genuine stress incontinence, or urethral sphincter incompetence. There was no evidence of unstable bladder contractions on the preoperative conventional urodynamic studies and no leakage during filling. In addition they did not complain of irritative symptoms. Stress incontinence was demonstrated on increasing abdominal pressure without concomitant detrusor pressure rise. All patients exhibited detrusor activity during voiding and most importantly no patient at short-term follow-up (3 and 6 months) had developed irritative symptoms or incomplete emptying. It is of course possible that following surgery in the long term a small percentage of these women may in time develop de novo detrusor instability, but the reports suggesting the incidence of de novo instability as 18% had developed unstable contractions

by 3 months post surgery (Cardozo 1979). Repeat urodynamics were not performed in our group as they were not clinically indicated at the follow-up visits.

Any group of patients by the nature of their presence in a urological clinic are not completely normal. It is unethical and expensive to operate on healthy volunteers therefore all human control groups should be as well matched and as suitable as possible. This group were considered well aged matched to the unstable group and had a normal filling phase of conventional urodynamics. As the study was designed to compare stable with unstable it is suitable to utilise this stable group as controls.

Recruitment into the study was made on the day of, or the day before operation to limit possible bias in the scheduling of each patients surgery and each patient had an opportunity to read an information leaflet, sign a consent form and discuss the research with me prior to the surgery.

I performed the experimental work described, with supervision as necessary, at the Institute of Urology and Nephrology, the in vitro calibration of Fura-2 fluorescence was carried out with the assistance of Dr C. Wu.

2. Methods

2.1. Usefulness of isolated preparations

Isolated smooth muscle preparations, strips or myocytes, superfused in organ baths permit controlled pharmacological interventions. The agent can be added to the superfusate, and then washed off again. This enables a record to be made of the action of that particular chemical on the contractility without reference to the bioavailability of the agent or its potential side-effects. This is of use in the preliminary investigation of possible therapeutic agents particularly if the isolated strip originates from human tissue. The preparation in this

153

in vitro form is also helpful in the study of pathophysiology, by enabling the use of potentially toxic chemicals to create an environment that may have occurred in the development of disease where in vivo studies are inappropriate, for example the effect of exposure of human detrusor strips to a low PO_2 solution (Thomas & Fry 1996), or attempting to correlate bladder dysfunction to tissue abnormality.

Despite the versatility of such preparations care must be taken when extrapolating the results of such experiments to whole organ preparations or in the vivo case. In the intact organ tissue-tissue interactions occur and the translation of tension to pressure changes will not be proportional.

However studies on the contractile properties of animal bladder both in vitro and in vivo have shown a correlation between both methods of experimentation (Levin *et al* 1981 &1983) and provides evidence that for the these models at least results from isolated preparations compare well to in vivo experiments.

2.2. Isolated detrusor smooth muscle strips.

Isolated detrusor smooth muscle strips superfused in organ baths are used extensively for in vitro experiments on both human and animal bladder biopsies and much of the current knowledge of detrusor smooth muscle physiology is derived from this mode of experimentation.

Initial studies with such preparations used biopsies obtained at open operation. This procedure results in a full thickness biopsy of the bladder which may be dissected into several isolated preparations (Eaton & Bates 1982). This has the advantage that one biopsy will provide several preparations but has disadvantages. These included: I) the tendency to dissect larger isolated strips which are more likely to become hypoxic as oxygen is unable to diffuse through greater than 0.5mm , ii) as open bladder surgery is largely reserved for those patients with severe detrusor abnormality or tumour, samples were not always representative of a wide clinical spectrum of detrusor dysfunction and iii) open operations

154

are much less frequent than endoscopic procedures making this an inefficient source of material.

Later endoscopic harvesting of detrusor biopsies was described, giving rise to smaller but equally viable muscle strips (Palfrey *et al* 1984). A comparison of these two techniques has shown there was no difference in the viability or experimental usefulness of tissue obtained from either method (Harrison *et al* 1988). Biopsies for the present study were taken either at open operation as a full thickness sample or endoscopically using Storz biopsy forceps. All biopsies were taken from the dome of the bladder, so that samples from the trigone or bladder neck were avoided. Great care was taken in the dissection of the full thickness biopsies to ensure uniformity in size and weight of the final preparation. There were no differences in the tension generated per wet weight of tissue or the yield of isolated myocytes between the biopsies taken endoscopically and from those at open operation. None of the patients who participated in the study suffered complications as a result of the biopsy, however some of the biopsies taken were very superficial and contained very little muscle and so were not used.

2.3. Measurement of tension generation in muscle strips

The muscle bath set-up enables a study to be made of the response of the muscle strip to pharmacological intervention by adding agents to the superfusate, and by exposing the strip to electrical field stimulation, thus testing nerve-mediated contractions. Field stimulation of detrusor smooth muscle strips has been used to characterise preparations, the contraction that the muscle strip produces when electrically stimulated, is nerve-mediated, and not from direct muscle stimulation, as it can be blocked by tetrodotoxin (TTX). It has been shown that even atropine resistant contractions found in diseased human muscle can be blocked by TTX (Bayliss *et al* 1998). Nerve-mediated contractions have been studied by measuring the tension generated by the strip in varying solutions. Field stimulation has been used to describe the electrically induced contractile properties of an animal (pig) model of non- obstructive detrusor instability (Sethia *et al* 1990), and others have described

similar properties of the rat detrusor (Uvelius 1986), obstructed rabbit bladder (Kato *et al* 1988), obstructed pig bladder (Speakman *et al* 1987) and also human detrusor obtained from patients with several clinical conditions such as, hyper-reflexia (Nurse *et al* 1991) idiopathic instability and patients with stable bladders (Eaton & Bates 1982 and Thomas & Fry 1996). Therefore it was appropriate to include in these studies a muscle characterisation so that the preparation could be compared to other studies.

2.4. Isolated detrusor myocytes

Individual myocytes isolated from guinea-pig detrusor are widely used as an experimental system. The isolation of single detrusor myocytes has been described for both guinea-pig (Ganitkevich *et al* 1987) and man (Montgomery & Fry 1992). The technique described by Montgomery & Fry has been adopted by other authors and the cells isolated have subsequently been cultured to provide cell lines (Harriss 1995, Baskin *et al* 1993). Using this isolation technique, once mastered, viable single human detrusor cells were produced in a relatively consistent manner. In order to minimise problems enzyme solutions were made up in batches and frozen in aliquots. It was found that the batches varied slightly in efficacy, in the number of viable cells isolated despite being made in the same way. The viability of the cells by the procedure was indicated by their appearance (of a spindle shaped cell) and the presence of reproducible agonist-induced intracellular Ca^{2+} release which indicates that the signalling system from the membrane receptor to intracellular Ca^{2+} store is functionally intact. Cells remained viable for several hours, the post-intervention controls towards the end of the experiments did not differ significantly from the pre-intervention control transient.

The cells used in the autofluoresence experiments were not loaded with fluorescent indicator and so their viability could not be checked by the response to agonist in the same way. The selection of viable cells depended much more on the appearance of the cell. These experiments were performed once I had gained sufficient experience in selecting viable

Fura-2 loaded cells. Each cell selected for the autofluoresence experiments exhibited a change in the autofluoresence in the presence of 2mM NaCN compared to control, a change of solutions in the absence of cells caused no variation in background fluorescence. This indicates the selected cells were responsible for the change and therefore viable.

2.5. Estimation of the ionized intracellular calcium concentration

Fluorescent intracellular dyes are commonly used to measure changes of the cytoplasmic concentration of intracellular ions. Fura-2, a Ca^{2+} indicator, was loaded into cells at room temperature rather than at 37 ^0C as this is thought to minimise compartmentalisation of the dye in subcellular organelles (Thomas & Delaville 1991). The fluorescent signal was calibrated in the present study using known concentrations of Ca^{2+} in mock intracellular solutions, in the absence of cells, and results are expressed as concentrations of Ca^{2+}. Since the calibration was performed in this way it does not take account of the compartmentalisation that may have occurred, such compartmentalisation in the organelles would lead to an overestimation of the actual intracellular $[Ca^{2+}]$. Therefore although reference is made to total estimations of intracellular $[Ca^{2+}]$ comparisons are confined to changes in $[Ca^{2+}]$, the influence of the compartmentalised portion of the indicator thus minimised. Many authors quote $[Ca^{2+}]$ the form of fluorescence ratios (Levin 1999) thus ignoring the above uncertainties.

The calibration of the Fura-2 fluorescence to calculate the estimated intracellular $[Ca^{2+}]$ was also useful in that it demonstrated the range of ratios in this work was over the linear part of the dissociation curve for both control and intervention solutions. This means that measuring a transient as a fraction of the control peak is proportional to the change of $[Ca^{2+}]$.

One concern in this study was that 2mM NaCN would itself alter the properties of the Fura-2 fluorescence and could give rise to misleading results. However the dissociation constant

K_d was calculated in the study for both a control mock intracellular solution and one containing 2mM NaCN thus eliminating changes in fluorescence caused by direct action of NaCN. Both dissociation constants were very similar, 283nM in control solution and 353nM in 2mM NaCN solution. For each of the cells where calculations were used to estimate the intracellular $[Ca^{2+}]$ the appropriate dissociation constant was used, thus minimising artefact. The main value of this separation was in the explanation of the rise in resting baseline observed in the presence of 2mM NaCN the calibration enabled us to be confident that it represented a real rise in intracellular $[Ca^{2+}]$.

2.6. Measurement of intracellular pH.

The pH of the sarcoplasm was measured as it has been shown that cellular hypoxia causes an initial extracellular alkalosis followed by extracellular acidosis (Thomas & Fry 1996). Each cell investigated in this part of the present study was exposed to nigericin, a H^+/K^+ exchanger, effectively making the cell permeable to H^+ so that the intracellular environment assumes the same pH as the extracellular solution in order to calibrate the signal and provide a control pH reading for each cell. BCECF has the same disadvantages as Fura-2 in potentially loading in an uneven manner throughout the cell, however by loading at room temperature these problems were again minimised (Thomas & Delaville 1991).

2.7. The measurement of cell autofluoresence.

This measurement uses the natural fluorescence of NADH to provide a qualitative measure of anaerobic activity. This was the first time that this technique had been applied to human detrusor muscle and so the value of the measurements was limited. However the consistency of the increase of fluorescence with every cell that was exposed to 2mM NaCN and the control experiments that were performed measuring background fluorescence (see section 3.3 in Results) increases the value of this technique and allows some initial

conclusions. The presence of 2mM NaCN caused the cells to respire anaerobically measured as an increase of the NADH fluorescence. This is useful to illustrate that the addition of the 2mM NaCN, the main intervention agent in this study, was responsible for causing cellular hypoxia.

3. Objectives of the experiments

It has been hypothesised that bladder ischaemia caused by overdistention, acute or chronic retention, hypertrophy and ageing or some other underlying process is an aetiological factor in the development of poor contractile function and unstable detrusor contractions (Levin *et al* 1999). By studying tissue that is taken from young patients with no history of obstruction it is possible to look at the aetiological agent specifically with no confounding variables. The effect of ischaemia has been suggested to be as a result of cellular hypoxia (Levin *et al* 1999) but this does not take into account the effect of other changes that are present in ischaemia e.g. accumulation of other waste products of metabolism.

The main aim of this work was to study the effect of cellular hypoxia, specifically, on the force of contraction of human isolated detrusor smooth muscle. This was to determine how significant this factor was when considering the clinical picture of ischaemia or as a part of a disease process altering normal detrusor metabolism. Thereafter it was attempted to determine the cellular mechanism that may be involved in any observed contractile effect, and determine if the magnitude of the effect is different between samples from stable or unstable bladders.

Once these aims were achieved it became necessary to determine if such results were compromised by artefacts and so facilitate correct interpretation of the data.

One of the aims of the thesis was to determine if any differences were present between samples from stable and unstable bladders with respect to their response to cellular hypoxia. In order that the experimental results from the two groups could be compared the basic characteristics of the preparations were determined. The absolute tension and electrical characteristics of the samples in this study are compared to similar experiments described by other authors, see Discussion section 4, p161.

There has been much work, from many centres, studying the differences between detrusor from stable, obstructed and unstable bladders. However, there has been relatively little work concerning idiopathic instability. The reasons for this are threefold; firstly animal models of obstruction have been developed showing good evidence of increased detrusor pressure in the filling phase of urodynamics following iatrogenic obstruction, and so tissue has been plentiful. Secondly, in clinical practice the prevalence of prostatic hypertrophy is high and sufferers are unable to ignore the symptoms of incomplete emptying and eventually, retention of urine, so more affected men come earlier to seek medical help. Thirdly, idiopathic instability comprises a more heterogeneous group of patients with some overlap with hyperreflexia, ageing and poor bladder neck support in women. In particular these patients often do not suffer an acute event and so seek medical help later and less commonly. Thus with more opportunities to glean samples from both animal and human sources, more work has been published using tissue from obstructed bladders.

There are however important differences to note between animal and human tissue. The urodynamic parameters are not standardised for animal models of instability, all urodynamics are (for technical and ethical reasons) performed under anaesthesia so no detrusor pressure rise can be associated with a sensation of urgency and also many studies are performed using animals that would never exhibit detrusor instability in their natural environment, as only domestic, trained pets and territorial animals demonstrate voluntary voiding.

Detrusor instability caused by outlet obstruction is reversible on removal of that obstruction and so may differ from the progressive nature of idiopathic detrusor instability. However, it is argued that there is a common underlying mechanism for the development (Turner &

Brading 1994) and so the results of the experiments will be discussed with reference to normal controls, obstructed cases, animal models and other studies of idiopathic disease where possible.

Results

4. The characteristics of the preparations

Various factors will alter the contractile function of a detrusor smooth muscle preparation, see Introduction, section 9.

1) The excitability of the muscle, to electrical field stimulation and spontaneous activity.

2) The absolute tension generated by the preparation in relation to the mass of tissue and the proportion of muscle. This is important in hypertrophic growth for example.

3) Denervation of the muscle.

4) Sensitivity to neurotransmitters

5) Atropine resistance and the manifestation of secondary transmitters

4.1. Excitability and spontaneous activity.

Electrical field stimulation of the muscle strips and the construction of force-frequency relationships gives an additional measure of the contractility of the preparation. Field stimulation assesses the response of the preparation to nervous stimulation. If a preparation contracts more forcefully to low frequencies of stimulation it could be said to be more excitable than one that requires a greater frequency of field stimulation to contract. In this study the frequency of stimulation required to achieve half-maximal tension in samples from stable human tissue was similar (9Hz) to reported studies (Eaton & Bates 1982; Kinder & Mundy 1987; Nurse *et al* 1991), implying that the control used in this study compares well to controls used in other centres.

There was no significant difference between the frequency of electrical stimulation to achieve half maximal tension of samples from stable and unstable bladders in the present study i.e. samples from both groups of patients were equally excitable. These results are in contrast to one study comparing human tissue from stable and idiopathic unstable bladders which demonstrates a shift in the frequency /response curve to the left i.e. biopsies from unstable bladders were more sensitive to lower frequencies of electrical stimulation and therefore more excitable compared to controls (Kinder & Mundy 1987).

The results from this present study are however agree with the finding in the samples from transected pig bladder, (designed to be an animal model of non-obstructed detrusor instability (Sethia *et al* 1990)), the model most closely representing the unobstructed detrusor instability of the experimental group. In this study the tissue from transected pig bladders was compared to tissue from control and obstructed bladders. The samples from obstructed pig bladders did demonstrate a significant difference, namely a decreased sensitivity to electrical stimulation, when compared to control samples whereas the unobstructed unstable demonstrated no such difference when compared to control samples. Other work comparing normal detrusor with obstructed detrusor shows a shift of the frequency /response curve to the right, indicating that tissue from obstructed bladder is less sensitive to electrical stimulation than tissue from normal bladders (Harrison *et al* 1987; Eaton & Bates 1982, Sibley 1984)

Biopsies from hyper-reflexic detrusor show either an increased sensitivity to electrical stimulation compared to normal detrusor (Kinder & Mundy 1987) or a decreased sensitivity in another study (Saito 1993) when compared to normal controls.

Thus there seems to be no consensus in the literature as to whether changes in the force/frequency relationship are associated with bladder dysfunction in vivo, the results varying within species and disease models. Our results provide no evidence for a change in the excitability of isolated detrusor smooth muscle taken from unstable or stable bladders in vitro .

4.1.i). Spontaneous Activity

There was no spontaneous activity observed in the muscle strips in this study but there were frequent spontaneous transient increases in intracellular $[Ca^{2+}]$ in isolated cells from both stable and unstable bladders with equal frequency. The significance of this is unknown but as the cells were isolated, in the majority of cases, using the same samples from which muscle strips (which showed no evidence of spontaneous activity) had been dissected, these transients are unlikely to have been sufficient to initiate a measurable contraction in the multicellular preparation. However they may be responsible for the altered tone in detrusor muscle if they are asynchronous in different cells. It is possible that the presence of this spontaneous activity in isolated cells may indicate dying cells, however many spontaneous transients occurred throughout experimentation and were not just a feature of loss of viability. Spontaneous Ca^{2+} transients may be of significance in the consideration of cell to cell communication, particularly if communication is enhanced in disease where such transients may assume a greater influence. This could be studied by investigating the cable properties of an isolated smooth muscle strip and correlating findings with the presence or absence of spontaneous Ca^{2+} transients in cells isolated from the same preparation. The spontaneous Ca^{2+} transients may be similar to the repetitive Ca^{2+} oscillations seen after stimulation of the cell by agonists indicating a derangement of intracellular Ca^{2+} metabolism (Fry & Wu 1998).

The presence of spontaneous activity in isolated detrusor smooth muscle strips is dependent on the length and resting tension of the preparation (Finkbeiner 1980). In this present study the strips were not stretched and were subjected to only 1g of tension, which may have accounted for the absence of spontaneous activity. In one study of spontaneous activity 5g of tension was applied to each strip, resulting in the observation of spontaneous contractile activity in 57% of the human preparations (Kinder & Mundy 1985). This spontaneous activity was not nerve-mediated, seemed to originate from the muscle itself and relaxed on the application of vasoactive intestinal peptide. Other work has compared spontaneous activity from a sample taken from an idiopathic unstable bladder which demonstrated an

increased number of spontaneous contractions to a control gleaned from a cadaveric bladder. However in this article not all strips dissected from adjacent areas of that bladder exhibited spontaneous activity (Brading 1997), thus all these results must be reviewed with caution and their significance is unclear.

Animal studies have also described the presence of spontaneous contractions in animals with bladder disease; the transected pig model of non-obstructed detrusor instability (Sethia *et al* 1990) and in obstructed rats (Malmgren *et al* 1990) and cats (Kato *et al* 1990).

There seems at present little consensus whether the observation of spontaneous mechanical activity is of importance as it may be artefactual due to overstretching of isolated muscle strips, and may not be consistent within strips dissected from the same bladder. However the presence of spontaneous Ca^{2+} transients may be of particular interest in certain circumstances where cell to cell communication may be enhanced.

4.2. Absolute tension and hypertrophy.

The isolated detrusor smooth muscle strips were dissected from biopsies after removing the mucosal layer. This process was performed with the help of a dissecting microscope but it was impossible to discern if the strip was cut with the muscle bundles in alignment, especially in the small endoscopic biopsies. The lengths were approximately equal at 3-4 mm and the weight of the strip was measured at the end of experimentation and in a fixed length preparation the weight of the strip was proportional to the cross sectional area. This method of normalisation of tension measurements yields identical results compared to those when cross-sectional area is measured directly and was preferred because in the small bundles cross-sectional area was not uniform and difficult to measure accurately (Fry, personal communication)

Hypertrophy of detrusor smooth muscle cells has been demonstrated in samples from obstructed bladders and such hypertrophy has been associated with changes in intracellular electrical coupling of smooth muscle from other sources and alteration of the contractile

164

function (Bortoff & Sillin 1986). The samples in our study were taken from patients who had unobstructed voiding and it was not known if there was any cellular hypertrophy. It remains a future experimental objective to measure the isolated cells to obtain an accurate measure of cell size and compare the mean size of the two experimental groups.

Collagen deposition in detrusor smooth muscle has been described in patients following abdomino-perineal resection and resulting denervation of the bladder (Neal *et al* 1982). Connective tissue infiltration of smooth muscle bundles has been shown in one study to be absent in normal human bladder and present in 12 out of 14 samples from patients with obstructed bladders (Gilpin *et al* 1985). In this study patients demonstrating evidence of obstruction at urodynamics were excluded, as were those with obvious neurological disease or previous major pelvic surgery. All patients in this study were under the age of 64 so the effects of ageing, demonstrated as an increase of fibrosis within muscle bundles, (Holm *et al* 1995) would be minimal. Bladders that had been previously damaged e.g. had undergone a hypoxic insult, may well have developed fibrosis so it was important to assess the absolute tension of each group to investigate this.

The absolute mean tensions observed in the isolated muscle strips, in terms of Newton's force per gram of wet tissue weight were similar in both groups: there was no statistical difference between the force of contraction generated at a fixed stimulation frequency or following the same pharmacological stimulation between the strips from stable or unstable bladders. Therefore it can be assumed that there was unlikely to be significant additional deposition of non-contractile connective tissue in the samples from unstable bladders used in this study.

The muscle strips showed no difference in the absolute force of contraction per unit weight between the two groups. This is in agreement with findings in another study involving idiopathic unstable and hyper-reflexic bladder biopsies (Kinder & Mundy 1987) where both groups of samples contracted with the same maximal tension per unit weight as the

controls. Connective tissue infiltration has not been shown in idiopathic instability but only infiltrating muscle bundles at increasing age or in the presence of outlet obstruction (Holm *et al* 1995) neither of these factors were present in the unstable group in this study the similarity of absolute tension of the two groups is not unexpected.

The muscle strips were of a sufficiently small diameter (0.5mm) to allow adequate diffusion of substrates throughout the sample, the response of the strip to the agonist carbachol was swift, within 3 seconds, following exposure and relaxed promptly on its removal. The average size of the preparations was similar to those strips dissected by other authors (Thomas & Fry 1996, Speakman *et al* 1987, using human and pig biopsies respectively) and smaller than other authors using human bladder (Saito *et al* 1993; Sibley 1984 and Eaton & Bates 1982) where strips varied from 1-2 mm in diameter. The muscle strips despite being smaller than many described in the literature maintained their contractile properties for several hours (usually between 3 and 5 hours) during experimentation and rarely showed signs of fatigue.

4.3 Denervation and:-

4.4 Supersensitivity (the response to carbachol).

These are considered together as it has been proposed that denervation supersensitivity is an underlying theme that may be responsible for all or most unstable detrusor contractions (Turner & Brading 1994). Denervation supersensitivity describes a feature of diseased muscle where the response to nerve stimulation is poor as there is a reduction in the nerve density which leads to a corresponding hypersensitivity to neurotransmitter. In clinical terms this would account for compensatory function in certain conditions e.g. hypertrophied bladder tissue has fewer nerve endings per unit area than normal bladder tissue and the individual cells are larger. Therefore if the tissue was more sensitive to

neurotransmitter this would compensate these factors and maintain function. In laboratory terms denervation is gauged by the ratio of electrical contraction to that produced by the maximum concentration of neurotransmitter.

It has been established that in this study there was no difference between samples isolated from stable and unstable bladders with respect to their excitability, the force /frequency relationships being similar in both groups. This is similar to the nerve transected pig model of idiopathic instability which showed no reduction in the nerve density when investigated microscopically (Sethia *et al* 1990). It would have been of interest to assess the nerve density of the samples in this study microscopically but as there was no electrophysiological evidence of a change in the excitability of the preparations a change in nerve density was not expected.

The sensitivity of the muscle preparations to muscarinic neurotransmitter was investigated by performing dose-response curves to carbachol i.e. the relationship between the concentration of carbachol, and the force of contraction of the muscle strip. The concentration of carbachol required to elicit the half maximal response, the EC_{50} was calculated for each preparation and the results of the two groups compared. There was no significant difference between the strips from stable and unstable bladders in this respect (mean stable EC_{50} = 1.3μM, range 0.78-2.0μM, the mean unstable EC_{50} = 1.3μM range 0.91-1.71μM). These values compare favourably with other studies using human tissue from stable bladders (Nilvebrant *et al* 1985; Saito *et al* 1993).

These results support previous work on human stable and idiopathic unstable detrusor muscle (Kinder & Mundy 1987) which also found no evidence of increased sensitivity to acetyl-choline in samples from hyper-reflexic bladder. Although they stated that there was a tendency for diseased muscle to have a greater response at lower concentrations of agonist than normal detrusor, this was not significant. In that study the ratio of electrical response / acetyl-choline response increased (as the samples from unstable or hyper-reflexic bladders

were more sensitive to electrical stimulation). Eaton & Bates (1982) also found no increased sensitivity to acetyl-choline in the obstructed unstable bladder specimens when compared to the stable controls.

However some authors working with human tissue have described an increased sensitivity to acetyl choline in muscle strips taken from obstructed bladders when compared to those from stable bladders. (Harrison *et al* 1987; Smith & Chapple 1994).

Most of the work demonstrating a supersensitivity of obstructed unstable bladder muscle to acetyl-choline has been performed using animal models in particular the experimental model of detrusor instability in the obstructed pig (Sibley 1985). This model, produced by placing a ligature around the proximal urethra in the young male pig, leads to progressive obstruction of the lower urinary tract and the development of unstable detrusor contractions during modified urodynamic testing in up to 64% of the animals. Physiological studies on detrusor smooth muscle strips from this model showed an increased sensitivity to acetyl-choline, a decreased sensitivity to nerve mediated stimulation and no difference in the response to adrenergic stimulation when compared to controls (Sibley 1987). Morphological studies showed an inverse correlation between the neuronal density of the bladder smooth muscle and the duration of the obstruction and concluded that this model demonstrated the features of post-junctional supersensitivity secondary to partial denervation (Speakman *et al* 1987). As mentioned earlier in this section, another pig model was described by the same group as an animal model of non-obstructive bladder instability. This model was produced by performing circumferential supratrigonal bladder transection on mini pigs. All pigs developed detrusor instability and muscle biopsies demonstrated a non specific supersensitivity to agonist similar to that observed in the obstructed pig model. However there was no concomitant loss of cholinergic nerves (Sethia *et al* 1990).

Changes to detrusor innervation in the presence of obstruction have also been demonstrated in humans, a decrease in innervation was present in men with prostatic hypertrophy, the nerve axons were damaged but there was no damage to the cell body. Following prostatectomy subsequent biopsies showed a regeneration of these nerves in 8 out of 10

patients (Cumming & Chisholm 1992). A reduction in the density of cholinergic receptors has been demonstrated in hyper-reflexic human biopsies compared to controls by the same workers that found no evidence to support a supersensitivity to cholinergic stimulation in hyper-reflexic bladder biopsies (Restorick & Mundy 1989).

The concept of supersensitivity is supported by work using a rabbit model of detrusor instability following mild outlet obstruction (Kato *et al* 1988) which demonstrated an increase of muscarinic receptors in detrusor muscle following obstruction compared to pre-operative controls. However there was no change in the response of the whole bladder preparation to bethanechol, although it was less sensitive to field stimulation. Outflow obstruction in the rat has not been associated with changes in the response of the detrusor biopsies to carbachol or electrical stimulation in two studies (Malmgren *et al* 1990; Andersson *et al* 1988).

The evidence from the obstructed pig model of instability clearly supports the hypothesis that detrusor instability, secondary to urinary outflow obstruction, is associated with a denervation supersensitivity to acetyl-choline. Support for this hypothesis in other models is not overwhelming, as the rabbit model suggests and there is no support from evidence derived using the rat model. The transected animal model designed to represent idiopathic instability shows no denervation but does demonstrate a non specific supersensitivity to agonist. The similarities of this model to human idiopathic instability are not yet established.

Bladder that compensates for ischaemia from partial obstruction becomes denervated by virtue of the development of hypertrophy (Levin 1999) and this can compound the problem causing a cycle of compensation, further hypertrophy, denervation and corresponding decrease in perfusion all leading to progressively deteriorating bladder function.

The results in the present study do not support the hypothesis of supersensitivity to acetyl-choline in human detrusor associated with idiopathic instability. There is no general

consensus of opinion that the unstable bladder in the human demonstrates supersensitivity to acetylcholine, this supersensitivity has not been found in a study using bladder biopsies from patients with idiopathic instability and its role as a factor in the pathphysiology of detrusor instability following outlet obstruction remains controversial.

4.5. Atropine Resistance.

In the small number of samples tested in the present study, 3 from stable and 1 from an unstable bladder, there was no atropine resistance demonstrated. Atropine, a muscarinic antagonist, has been shown to block nerve-mediated contraction in human bladder with no part of the contraction atropine resistant even in abnormal bladder (Kinder & Mundy 1987; Palfrey et al 1984; Chen et al 1994; Speakman et al 1985; Sibley 1984). However atropine resistance in varying degrees has been described in both humans and animals by several authors. Human work has demonstrated atropine resistance in disease of the bladder e.g. interstitial cystitis (Palea et al 1993; Ruggieri et al 1990), obstructed detrusor instability (Nerdgårdh & Kuhn 1983; Sjogren et al 1982) detrusor hyper-reflexia (Saito et al 1993) and more recently in an extensive study where atropine resistant contractions were noted in both obstructed and idiopathically unstable bladder, but not in normal bladder (Bayliss et al 1998). It has been hypothesised that in pathological bladders atropine resistance increases, and hyper-reactivity to ATP may occur: e.g. the severity of bladder instability in benign prostatic hypertrophy depends on the hyperactivity of non-cholinergic, non-adrenergic neurotransmitters (Sjogren et al 1982).

Atropine resistance in the rat urinary bladder has been demonstrated when the muscle strip was stimulated by short-train field stimulation (Carpenter 1977), antimuscarinics in another study inhibited, although not completely, the contraction of rat detrusor muscle in vivo (Hammarstrøm & Sjostrand 1984).

The presence of atropine resistance in both diseased human (in some studies) and animal bladder suggests a role for non-cholinergic neurotransmitters and this is now a topic of

increasing interest. In this work the number of preparations studied, on bladder samples from stable bladders is too small to be able to draw any conclusions.

The receptor mediated IP$_3$ excitation of cells requires more aerobic energy than membrane depolarisation (Levin *et al* 1999) and so alternative neurotransmitters acting by opening Ca^{2+} channels and depolarising the cell membrane may be of importance, particularly if the cell is trying to compensate for hypoxia. This could be investigated by assessing the ATP mediated contractile function in diseased bladders in the presence of hypoxia. Membrane depolarisation by a strong KCl solution and the effects of hypoxia are discussed in section 6.3 p177.

4.6. Summary of the characteristics of the preparations.

The characteristics of the muscle strips used in these experiments have been shown to be similar to preparations in other studies on human and some animal tissue. The strips were dissected or prepared in accordance with accepted published methods, responded to field stimulation which was proved to generate a nerve mediated response which was cholinergic and blocked by atropine. They did not demonstrate any spontaneous activity, but were not overstretched and contracted with a consistent absolute tension for many hours. Therefore these preparations were suitable for the designed experiments.

The two groups of samples were thus very similar in essential characteristics. As the clinical descriptions of the patients from which the samples originated were clearly defined and free as possible from confusing variables, such as old age, obstruction and neurological imperfection, this provided a good model on which to test the hypotheses of this study.

The samples were also unusual in being from unobstructed humans, a group from which samples are rarely obtained, thus providing valuable information with which other more plentiful supplies of samples can be compared. Thus the characteristics of these

experimental groups are important as there has been much less work published on unobstructed bladders, particularly samples from human bladders, than obstructed models of bladder disease. The study of unobstructed human bladder is of particular relevance to gynaecologists and urologists specifically concerned with female detrusor instability which is often not associated with bladder outlet obstruction.

5. The response to agonist.

5.1. The sensitivity of the isolated smooth muscle strip and the isolated smooth muscle cell with respect to muscarinic stimulation.

The relationship between the concentration of carbachol and the magnitude of the resultant transient rise of intracellular Ca^{2+} was measured using cells isolated from stable and unstable bladders in the present study to assess if the findings of the multicellular preparation were also true for the isolated single myocyte. The EC_{50} or concentration of carbachol required to achieve the half maximal Ca^{2+} transient was determined for each cell and the mean values for each group compared. The EC_{50} was similar (1.5 $\pm 1.30\mu M$ in the stable group and 1.3 $\pm 1.10\ \mu M$ for the unstable group) to the EC_{50} of the muscle strips. This implied that the transient rise of intracellular Ca^{2+} observed corresponds to the contraction measured at the same concentration of agonist in the isolated muscle strip. The calculated estimates of intracellular $[Ca^{2+}]$ from the calibration experiments correspond to a change of $[Ca^{2+}]$ from submicromolar to micromolar levels necessary to activate the contractile machinery as described previously (Wu *et al* 1995). These results give credibility to the value of the isolated single cell as an experimental model, demonstrating that, at least for carbachol stimulation, the measurement of Ca^{2+} transients can give information relating the Ca^{2+} metabolism of the cell to contraction.

There was no significant difference between cells isolated from stable or unstable bladders in the sensitivity of the cells to carbachol stimulation, in accord with the results using muscle strips.

5.2. The response to caffeine

The application of 10mM caffeine on isolated detrusor smooth muscle strips in the present study produced a contraction in only 4 out of 9 preparations studied. As all preparations were responding normally to field stimulation and carbachol stimulation it was assumed that the remaining preparations were not abnormal. As the affect of caffeine is intracellular, the molecule not only has to diffuse to the cell membrane as carbachol in previous experiments but then must diffuse through the cell membrane to the site of action.

Caffeine at the same concentration used for muscle strips produced reliable Ca^{2+} transients in all isolated detrusor myocytes examined. These transients were of the same magnitude as those evoked by carbachol, corresponding to the same intracellular Ca^{2+} concentration which would be sufficient to produce a contraction in the isolated strip. However in half of the preparations there was no contraction measured. Caffeine is an intracellular phosphodiesterase inhibitor and thus acts not only on the caffeine sensitive intracellular Ca^{2+} stores but also increases the production of cAMP which causes cell relaxation (Meisheri & Ruegg 1983; Watanabe *et al* 1992). Therefore caffeine affects the cell in both an excitatory fashion by evoking a transient rise in intracellular $[Ca^{2+}]$ and also by increasing the production of a relaxant cAMP, therefore the transient rise in Ca^{2+} may not necessarily lead to a contraction of the myocyte.

5.4. The response to KCl.

All preparations responded with a contraction in the isolated strips and a Ca^{2+} transient in the isolated myocytes to a concentration of 100mM KCl. This intervention was designed to investigate the effect of 2mM NaCN on membrane depolarisation. This method of

173

investigation uses a pharmacological agent (KCl) and therefore was useful as it could be used in the existing experimental set-up, thus gathering more information from the same preparations and provides equivalent information to using electrophysiological methods to depolarise the cell.

6. The effects of cellular hypoxia

6.1. The effect of cellular hypoxia on the force of contraction of isolated human detrusor smooth muscle strips

These experiments were pivotal to determine whether the poor contractile ability observed in bladder ischaemia was in some way due to the resulting cellular hypoxia from reduced aerobic activity. This might also apply to cases where no bladder ischaemia has been determined but to conditions when altered anaerobic metabolism for other reasons, might be a cause of poor contractile function.

Tyrode's solution containing 2mM NaCN caused a reduction in the force of the phasic contraction elicited by $1\mu M$ carbachol in all isolated smooth muscle strips. The control contraction measured in Tyrode's solution was taken as the average of the pre-and post intervention controls. Thus any fatigue of the preparation was taken into consideration. The nature of the contraction in 2mM NaCN was similar to the controls, in both solutions the preparation contracted rapidly and reversibly to carbachol in the superfusate. The muscle strips from stable bladders were significantly more affected by NaCN generating only 43 ± 16% of the control force, than those from unstable bladders which generated 56 ± 15% of the control contracture in the same conditions.

The differential response to NaCN in muscle from the two groups might have been a function of the initial tension generated by the muscle samples. However the absolute tension generated by carbachol was similar in the two groups and the proportional reduction of force was shown to be independent of the initial force of contraction of each

muscle strip. The investigation of possible reasons for these observations formed the basis of the subsequent experiments and will be discussed in later sections.

6.2. Comparison with other metabolic models

Anoxia has been shown to affect the contractile force of isolated rabbit detrusor strips (Levin *et al* 1981). Isolated detrusor smooth muscle strips were exposed to a superfusate equilibrated with 100% nitrogen, containing no glucose or other metabolic substrate. After 60 minute exposure to anoxic conditions the strip was returned to control oxygenated conditions and the response of the strip recorded on brief exposure to bethanechol, a muscarinic agonist. The force of contraction initially, following return of the strip to control conditions, was less than 5% of the pre-intervention control but the preparations gradually increased the force of contraction and the tension generated after 60 minutes in control conditions was equal to the pre-intervention control.

This animal study examined the effects of complete anoxia on isolated strips and confirmed the finding from the present study that hypoxia reduces the force of contraction. However the conditions created in the animal study were not representative of in vivo conditions even in a pathological state. Further work from the same group examined the effects of in vivo ischaemia on the rabbit urinary bladder by ligating the bladder vasculature for 1 hour (van Arsdalen *et al* 1983). Rabbits were either sacrificed immediately, after one week of recovery or as control. A decrease in the contractile response to bethanechol (to 58% of control) of isolated smooth muscle strips taken from the bladders immediately after the ischaemic insult was measured when compared to control strips . This reduction in force did not improve in the samples taken from animals allowed a 1 week recovery from the ischaemia. The reduction in force to 58% of control is somewhat less than that in the acute measurements in this study with samples from control bladders. There may be compensatory mechanisms limiting the damage of the ischaemia in vivo, e.g. cellular respiration would continue until the oxygen tension of the static blood remaining in the ligated vessel was very low, which may limit the effect of the ligation.

In functional studies of the same rabbit model in vitro anoxia and in vivo ischaemia were compared using the whole bladder (Levin et al 1983). The whole bladder model enabled studies of bladder contractile function to be measured together with a measurement of the ability of the bladder to empty. Anoxia was produced by equilibrating the whole bladder with nitrogen and ischaemia produced as before. The contractile response of the bladder to bethanechol did not decrease until the oxygen tension fell below 20% of normal; below this the response decreased rapidly. The bladder was able to recover fully from 60 minutes of in vitro anoxia whereas 60 minutes of in vivo ischaemia resulted in a permanent reduction in the contractile response to bethanechol. These findings are similar to the reversible reduction in the force of contraction, in the presence of cellular hypoxia in vitro, observed in the present study.

The urinary bladder uses glucose for intracellular energy production in the form of ATP. Muscarinic stimulation leads to an increase in aerobic respiration of glucose, as well as increased CO_2 and lactic acid production (Levin et al 1988). This study also demonstrated that the initiation of contraction requires more energy than sustaining it. The bladder has also been shown to utilise aerobic respiration and produce less glycogen in common with other smooth muscles, than skeletal muscle (Haugaard et al 1987). The contractile response to bethanechol has also been shown to be dependent on the pre-existing level of ATP in the cell prior to ischaemia or anoxia (Haugaard et al 1990).

The effect of chronic ischaemia on the glucose metabolism of the rabbit bladder has been investigated at the time of maximal functional impairment, two weeks after one vesical artery had been ligated (Lin et al 1989). The tissue was first superfused in glucose-free medium in order to deplete the pre-existing cellular ATP, ischaemic tissue was demonstrated to contain less glycogen than tissue from control bladders. This study demonstrated that ischaemic bladder tissue was able to respire anaerobically and produced less CO_2 than control bladders or tissue from the contralateral side of the vessel ligation.

Ischaemia has been shown to reduce detrusor contractile function and it is suggested this results from interrupted glycolysis. The precise mechanism whereby contractile function is reduced under these circumstances is unclear, as metabolic inhibition not only reduces the supply of high energy phosphates but also produces waste products; e.g. intracellular acidosis and extracellular hyperkalaemia, which themselves may influence contractile function and other processes in the cell preventing recovery of the cell after the insult.

The inhibition of oxidative phosphorylation by NaCN is a more specific way of investigating the effects of hypoxia although it also leads to an increase in the products of anaerobic respiration. A 2mM solution of NaCN has been shown to depress oxidative phosphorylation in ferret heart muscle (Allen *et al* 1985). In the present study a "dose/response" relationship between the concentration of NaCN and the attenuation of the carbachol evoked intracellular Ca^{2+} transient was established. The maximum attenuation was achieved at 2mM NaCN and so was used in these experiments.

This observed reduction in the force of the carbachol stimulated phasic contraction in the presence of 2mM NaCN may have been as a result of changes to membrane function, an intracellular active process or a secondary effect such as intracellular acidosis a change in the pH causing an alteration of contractile function (Liston *et al* 1991). The subsequent experiments were designed to find the particular mechanism affected.

6.3. The effect of 2mM NaCN on membrane events

In the present study 2mM NaCN attenuated the carbachol contracture of isolated detrusor smooth muscle strips. The mechanism of this attenuation was investigated by assessing the

177

effect of 2mM NaCN on the membrane stimulated contraction elicited by high extracellular KCl.

100mM KCl Tyrode's solution was used as the stimulus for the isolated smooth muscle strip studies. This is similar to 124mM KCl solution used to evoke contraction in a previous study (Saito *et al* 1993) which elicited a maximal response. 2mM NaCN had no effect on the KCl contracture, in preparations from either experimental group. This finding may indicate that oxidative phosphorylation may not be as necessary for membrane depolarisation to occur and to permit the influx of Ca^{2+} ions. Other metabolic studies, measuring ATP, have demonstrated that less energy in the form of cytosolic ATP utilisation, is required to activate the contractile apparatus by membrane depolarisation, compared to the use of a second messenger system, stimulated by muscarinic agonist (Kamm & Stull 1989, Levin 1988), even though the force of the initial contractures stimulated by both KCl and bethanecol were the same. These studies were performed on rabbit and the results from these and from the present work correlate well.

However as the force of the KCl contraction was not affected by 2mM NaCN this might suggest that membrane depolarisation did not require any energy in the form of ATP; which was not found from the rabbit studies, so it may be that the KCl contracture can utilise anaerobic metabolism to a larger extent than the muscarinic stimulated contracture and is less affected by the reduction of oxidative respiration. This might be studied by biochemical assays of cytosolic enzymes involved with anaerobic respiration (See section 6.8 p183).

6.4. The effect of 2mM NaCN on the caffeine contracture.

These results demonstrated that the effect of cellular hypoxia appears to be on intracellular Ca^{2+} storage mechanisms, as there is an attenuation of force for both the carbachol and caffeine evoked contractions but no effect of cellular hypoxia on membrane events, represented by the KCl contracture.

Further study might include repeating these experiments in the presence of a Ca^{2+} channel blocker e.g. diltiazem to exclude the possible actions of carbachol and caffeine directly on membrane Ca^{2+} movements.

Cellular hypoxia has been shown to depress human detrusor contractility in other work (Thomas & Fry 1996). In this study cellular hypoxia was induced by a low PO_2 in the superfusate, 5mM Na azide or 10mM 2-deoxy-glucose. Contractility was reduced in the steady-state in all conditions of cellular hypoxia and substrate depletion, it was shown that these interventions acted directly on the muscle cells themselves, rather than the excitatory nerves. The reduction of tension was preceded by a transient increase in force, the presence and timing of which was variable. Simultaneous measurements of extracellular pH indicated that this initial transient increase in contractility was associated with a transient extracellular alkalosis.

6.5. The effect of cellular hypoxia on the carbachol, caffeine and KCl evoked Ca^{2+} transient

The mechanism by which cellular hypoxia exerted an effect is thought to be intracellular from the results of the isolated muscle strip experiments. This hypothesis was tested using isolated single human detrusor smooth muscle cells loaded with a Ca^{2+} fluorescent indicator Fura-2. Both the carbachol- and caffeine-evoked Ca^{2+} transients were reduced in the presence of 2mM NaCN compared to control conditions, the KCl transient was unchanged. These results were obtained by measuring the magnitude of the transient and also by calculating the estimated $[Ca^{2+}]$ by calibration of the fluorescence signal.

These results are consistent with the findings of the isolated smooth muscle strips and so add further evidence to support the hypothesis that cellular hypoxia reduces the force of contraction of human detrusor smooth muscle by affecting mechanisms involving the release or some other aspect of the metabolism of Ca^{2+} from intracellular stores. Figure D1

illustrates the correlation between the results obtained from muscle strips and those obtained from isolated single cells.
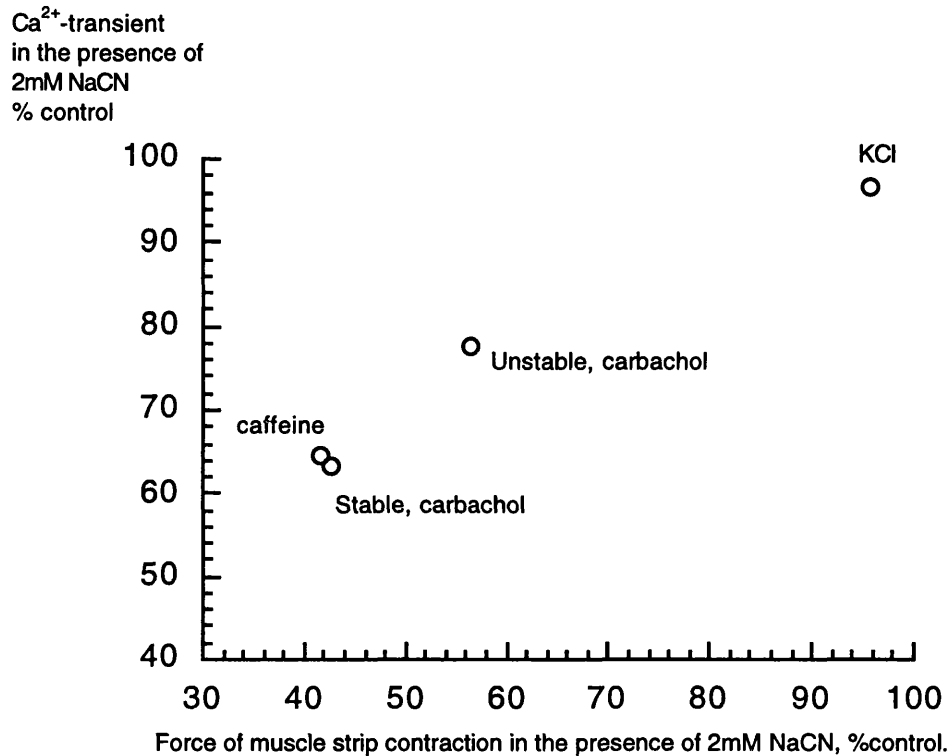
Figure D1. A plot of the mean force of contraction vs the mean magnitude of the Ca^{2+} transient in the presence of 2mM NaCN. Values are expressed as a percentage of the control force or transient. The individual points are labelled separately according to the stimulus evoking the response. The results of the carbachol stimulation are separated into the mean obtained from samples from stable bladders (stable, carbachol) and samples from unstable bladders (unstable carbachol).

Figure D1 illustrates that the Ca^{2+}-transient data compares well with the data from muscle strip preparations. This is important as conclusions can be drawn from the results from both experimental techniques, the effects of 2mM NaCN are similar in both the isolated single cell and multicellular preparations. It would be expected that further experimentation

on an isolated single cell preparation would yield similar results in the multicellular preparation.

The effect of 2mM NaCN on the carbachol stimulation of preparations from unstable bladders was less than those from stable bladders exhibited in both preparations. This is important as the difference between the experimental groups translates well from isolated single cell to isolated muscle strip, therefore it is reasonable to suggest that the difference also translates to the whole organ. This means that if the whole bladder acts in the same way as expected (similar to the isolated in vitro preparation) the observed difference between the two experimental groups may have clinical importance.

6.6. The effect of cellular hypoxia on intracellular pH

Cellular hypoxia, by decreasing oxidative phophorylation leads to an increase in anaerobic respiration and the corresponding accumulation of lactic acid, shown in rabbit bladder ischaemia (Haugaard *et al* 1987).

This accumulation and corresponding expected decrease in pH could lead to a decrease in contractile function. In vivo studies of rat uterine smooth muscle have shown a fall pH following occlusion of the uterine artery, together with a depression of contractile force (Harrison *et al* 1994). It was suggested in this study that the fall of pH from 7.32 to 7.00 was responsible for the depression of contractile force.

In the human bladder cellular hypoxia, generated by a low PO_2 and Na azide gave rise to an extracellular acidosis, preceded by a transient alkalosis, but this was not observed with 2-deoxyglucose. All interventions produced a contractile decay in the isolated muscle strips (Thomas & Fry 1996). An increase in extracellular $[H^+]$ has been shown to attenuate the magnitude of caffeine-induced Ca^{2+} transients and affect the resting $[Ca^{2+}]$ but not those evoked by carbachol (Fry *et al* 1994).

It was therefore appropriate to measure the effect of 2mM NaCN on intracellular pH in the isolated myocytes.

2mM NaCN produced a transient cellular alkalosis, which corresponded with the findings in the Thomas study (Thomas & Fry 1996) where an alkalosis was associated with a transient increase in contractile force. Cellular hypoxia generated by the addition of 2mM NaCN in ferret heart muscle also caused a transient alkalosis (Allen *et al* 1985). NaCN is a mild base and so this at least partly explains this observation in both ferret myocardium and the present study.

After the transient alkalosis pH_i returned to control values and there was no significant acidosis observed in the present study. In the contracting ferret heart, after the transient alkalosis a persistent acidosis developed. The strips in this experiment underwent continued stimulation and so the build up of anaerobic respiration would have been more marked (Allen *et al* 1985). The myocytes in the present study were not stimulated as the aim was to assess the effect of 2mM NaCN on the resting cell and not the effect of $[H^+]$ on the contractility of the myocyte.

The results of the present study show that 2mM NaCN does not produce a steady state rise in the concentration of H^+ in the resting cell. Therefore the attenuation in force of the carbachol evoked contraction and the attenuation of the carbachol and caffeine evoked Ca^{2+} transients are not explained by a change of resting intracellular pH. Another mechanism must be responsible.

6.7. The effect of NaCN on the resting concentration of Ca^{2+} in the cell

2mM NaCN produced a significant rise in the resting $[Ca^{2+}]$ in 29 out of 32 cells. This rise in the fluorescence output from the cells was converted to estimated absolute $[Ca^{2+}]$ from the calibration curves obtained in the absence and presence of 2mM NaCN. The value of converting the fluorescence signals to $[Ca^{2+}]$ was that the small effect of NaCN on the Fura-2 fluorescence was accounted for, which would have been hidden if the raw fluorescence signals alone were compared. A similar rise has been shown to occur in rabbit detrusor myocytes in the presence of ischaemia (Levin, personal communication).

The observation of an increased Ca^{2+} fluorescence in the presence of 2mM NaCN could have been artefactual and a result of NaCN affecting the dissociation of the flourescent indicator. The calibration experiments where the effect of NaCN on the intensity of the fluorescent signal was tested, show that NaCN changes the calibration curve shifting it slightly to the right. From this experiment a K_d was calculated in the presence of 2mM NaCN to compensate for this and although the difference in the value of K_d was very small the concentrations estimated using the appropriate K_d for each condition.

Thus one explanation of unprovoked and multiple contractions and Ca^{2+}-transients could be a raised $[Ca^{2+}]$ in hypoxia. This has been shown in other cells to reduce the ability of the cell to regulate the sarcoplasmic $[Ca^{2+}]$ and thus reduce control of the normal cycle of contraction and relaxation. For example when the heart is exposed to high concentrations of cardiac glycosides, the enhanced influx of Ca^{2+} due to Na^+ pump blockade and the secondary influx of Ca^{2+} via Na^+- Ca^{2+} exchange leads to an inability of the sarcoplasmic reticulum to store the increased Ca^{2+}. This results in irregular electrical activity, arrythmias in the heart and low amplitude phasic contractions (Fry *et al* 1999).

Therefore NaCN may be affecting the release of Ca^{2+} or its uptake from intracellular stores. This warrants further study as the increase of sarcoplasmic Ca^{2+} caused by NaCN is not known to originate from a depletion of sarcoplasmic Ca^{2+} stores or an inability of the stores to cope with an added influx of extracellular Ca^{2+} ions as described in the heart. This could be investigated by performing the experiments in the presence of a Ca^{2+} channel blocker such as diltiazem or in a Ca^{2+} free solution.

6.8 The differences between samples from stable and unstable bladders.

The samples were grouped into those from stable or unstable bladders according to current international standards on clinical history and examination and urodynamic findings. The samples from unstable bladders were significantly less affected by cellular hypoxia than

those from stable bladders with respect to the carbachol contracture and carbachol evoked Ca^{2+}-transient. The effect of cellular hypoxia on the caffeine evoked Ca^{2+}-transient was similar in cells from bladders from both groups.

These findings can not be explained by differences in the electro-mechanical characteristics of the samples, both groups exhibited the same contractility and excitability and contracted with similar force per unit wet weight.

One proposal might be that the samples from unstable bladders already have developed compensatory mechanisms to help overcome a lack of aerobic energy. A similar compensatory cycle has been proposed to accompany bladder outlet obstruction (Levin *et al* 1989). This cycle commenced with partial outlet obstruction leading to an increase in bladder mass. This hypertrophy if not accompanied by an increase of unit bladder blood flow would lead to ischaemia, a reduction of contractile function and a stimulus to further hypertrophic growth. This compensation of loss of unit contractile activity through muscle growth however cannot be maintained indefinitely and eventually the organ will decompensate. The importance of understanding the progress of this compensation/ decompensation process is to determine when the positive benefits of hypertrophy are outweighed by its negative effects. Initially anaerobic metabolism would increase intracellular H^+ production which would cause an increase in detrusor contractility (Liston *et al* 1991) and would provide a compensatory process. Applying this to the unobstructed model the activation of free radicals and their intracellular accumulation in ischaemia could cause specific damage to the sarcoplasmic reticulum and mitochondria which would lead to an increase in anaerobic metabolism to provide the same compensatory process. If the cycle continued the damage caused by the accumulation of free radicals would not be compensated by an initial increase in contractility as above.

This cycle should be applied with caution to the clinical situation affecting idiopathic instability as a previous ischaemic insult could cause damage to the mitochondria exacerbating the defect in aerobic metabolism. The cell would therefore need to be more reliant on the less effective anaerobic metabolism. As membrane events require less oxidative energy than receptor mediated contraction the cell may be more sensitive to cell

coupling and may have different connections to other cells giving greater communication, this is an area that requires more research as outlined by Fry *et al* (1999).

To investigate the hypothesis that samples from idiopathic bladders use more energy derived from anaerobic metabolism than those from stable bladders, biochemical analyses could be used. Decreased oxidative metabolism could be as a result of decreased mitochondrial activity which can be demonstrated by reductions of the specific activities of citrate synthase and malate dehydrogenase as well as a decreased ATP synthesis which has been found in the partially obstructed rabbit model (Haugaard *et al* 1992). These enzymes could be measured in the human using samples from unstable and stable groups and a reduction if present in the enzyme activity of the unstable group would support the hypothesis that cells from unstable bladder have reduced aerobic metabolism. Further experiments could investigate if these enzymes were more abundant in the unstable group implying a compensatory mechanism for providing ATP in the absence of adequate functioning mitochondria.

In the clinical picture the hypothesis that as samples from idiopathically unstable bladder may have developed a compensatory mechanism by a reduction in aerobic energy the patients might then suffer the consequences of such compensated bladder function. Detrusor smooth muscle from the rabbit has been shown to contract with less force in anaerobic conditions, moreover bladders that contract using anaerobic energy are much less effective at emptying than would be expected from the reduction of force generated. Whole rabbit bladders left larger than expected residuals in aerobic conditions (Malkowicz *et al* 1986). So one might expect a human compensated bladder to contract less effectively than a normal bladder possibly becoming more damaged by continuing dysfunction as described. Once a bladder has begun to compensate and damage has occurred it is possible that the correction of the aetiological agent in the case of obstruction in men may not result in the cessation of bladder dysfunction. This may explain why some men do not gain relief from

their irritative symptoms following prostatectomy, and could be applied to idiopathic instability if bladder ischaemia has been present as part of a physiological process (pregnancy) or has passed unnoticed. Studies could be performed retrospectively on acute retention to investigate this, the severity or length of time a patient has been in acute retention may be reflected in the activity of the anaerobic metabolism the patient subsequently has.

A prospective study could be designed to consider any change of metabolism in women undergoing colposuspension. Samples taken at operation could be compared with samples taken endoscopically (possibly using a flexible cystoscope) at an interval post operatively and comparisons made with reference to the incidence of de novo instability in this group of women.

7. Summary

Cellular hypoxia has been shown to cause a reduction in the contractile force of isolated human detrusor smooth muscle following muscarinic stimulation.

A difference between biopsies from stable and unstable bladders was recorded; the cellular mechanism responsible for any change in function was investigated; the products of anaerobic metabolism in the cell was measured and the pH of the cell in the presence of tissue hypoxia was determined.

These aims were investigated using biopsy samples from bladders from patients whose clinical descriptions were clearly defined and were very similar in essential characteristics. The experiments were conducted using accepted published methods.

Possible artefacts that may have affected the interpretation of the results were investigated. The intracellular pH was measured in the presence of 2mM NaCN and was shown not to be significantly different to control solution.

The increased production of NADH was measured utilising a technique not yet applied to detrusor smooth muscle and thus demonstrated the mitochondrial action of 2mM NaCN.

The results show that cellular hypoxia reduced the force of contraction of isolated detrusor smooth muscle in response to carbachol. The attenuation of force was less marked in isolated detrusor smooth muscle from unstable bladders. Cellular hypoxia had no effect on membrane events, as measured by the use of KCl. Cellular hypoxia increased the resting $[Ca^{2+}]_i$ and attenuated both the carbachol- and caffeine- induced Ca^{2+}-transients demonstrating that cellular hypoxia affects the intracellular Ca^{2+} metabolism and intracellular Ca^{2+} stores.

8. Conclusion

This study demonstrates that hypoxia reduces the contractile function of isolated human detrusor, the site of this action is intracellular. As biopsy samples from unstable bladders were less affected by hypoxia this may indicate that a hypoxic insult has previously occurred or a process altering the cellular metabolism has occurred and the detrusor is better able to function in transient cellular hypoxia. This has important implications for clinical practice both in the prevention of bladder ischaemia and in the pharmacological treatment of abnormal detrusor function, which is currently focused on transmembrane Ca^{2+} flux.

References

Abrams, P.H., Farrar, D.H., Turner-Warwick, R.T., Whiteside, C.G. & Feneley, R.C.L. (1979). The results of prostatectomy: a symptomatic and urodynamic analysis of 152 patients. *J. Urol.*, **121**, 640-642.

Abrams, P.H., Blaivis, J.G., Stanton, S.L. & Andersen, J.T. (1988). The standardisation of terminology of lower urinary tract function. *Scand. J. Urol. Supplementum*, **144.**

Abrams, P.H. (1997). Lower urinary tract symptoms in women, who to investigate and how. *Br. J. Urol.* **80**, (suppl 1), 43-48.

Aidley, D.J. (1989). The physiology of excitable cells. *Cambridge University Press. Cambridge U.K.*

Allen, D.G., Morris, P.G., Orchard, C.H. & Pirolo, J.S. (1985). A nuclear magnetic resonance study of metabolism in the ferret heart during hypoxia and inhibition of glycolysis. *J. Physiol.*, **361**,185-204.

Amis, E.S. & Blaivis, J.G. (1991). Neurogenic bladder simplified. *Radiol. Cin. North Am.*, **29**, (3), 571-580.

Andersson, P.O., Andersson, K.E., Farenkrug, J., Mattiasson, A., Sjorgren, C. & Uvelius, B. (1988). Contents and effects of substance P and vasoactive intestinal peptide in the bladder of rats with and without infravesical obstruction. *J. Urol* .**140**, 168-172.

Andersson, K.E., Holmquist, F., Fovaeus, M., Hedlund, H. & Sundler, R. (1991). Muscarinic receptor stimulation of phosphoinositide hydrolysis in the human isolated urinary bladder. *J. Urol.* **146**, 1156-1159.

Appell, R.A. (1997). Clinical efficacy and safety of tolterodine in the treatment of the overactive bladder: a pooled analysis. *Urology* **50**, (6A), 90-96.

Artibani, W. (1997). Diagnosis and significance of idiopathic overactive bladder. *Urology* **50**, (6A), 25-32.

Askoy, M.O., Murphy,R.A. & Karim, K.E. (1982). Role of Ca^{2+} and myosin light chain phosphorylation in regulation of smooth muscle. *Am. J. Physiol.* **242**, c109-c116.

Azadzoi, K.M., Pontari, M., Vlachiotis, J. & Siroky, M.B. (1996). Canine bladder blood flow and oxygenation: changes caused by filling, contraction and outlet obstruction. *J.Urol.* **155**, 1459-1465.

Baskin, L.S., Howard, P.S., Duckett, J.W., Snyder, H.M. & Macarak, E.J. (1993).Bladder smooth cells in culture. 1. Identification and characterisation. *J.Urol.* **149**, 190-197.

Bates, C.P. (1971). A clinical study of the dynamics of voiding and of the sphincter mechanism. *Ann. R. Coll. Surg. England.* **49**, 18-34.

Bates, C.P., Bradley, W.E., Glen, E.S., Melchior, H., Rowan, D., Sterling, A.M., Sundin, T., Thomas, D., Torrens, M., Turner-Warwick, R., Zinner, N.R. & Hald,T. (1981).Fourth report on the standardisation of terminology of lower urinary tract function. *Br. J. Urol.* **53**, 333-335.

Batista, J.E., Wagner, J.R., Azadzoi, K.M., Krane, R.J. & Siroky, M.B. (1996). Direct measurement of blood flow in the human bladder. *J.Urol.* **155**, 630-633.

Bayliss, M., Newgreen, D. & Fry, C.H. (1998). Changes to the functional properties of human detrusor smooth muscle and its innervation with disease. *J. Physiol.* **507.P.** 73P.

Bellringer, J.F., Ward, J. & Fry, C.H. (1994). Intramural pH changes in the anaesthetised rabbit bladder during filling. *J. Physiol.* **480. P.** 82-83P.

Benham, C.D., Bolton, T.B. & Lang, R.J. (1985). Acetylcholine activates and inward current in single mammalian smooth muscle cells. *Nature.* **316**, 345-346.

Blackford, H.N., Murray, K., Stephenson, T.P. & Mundy, A.R. (1984). Results of transvesical infiltration of the pelvic plexus with phenol in 116 patients. *Br. J. Urol.* **56**, 647-649.

Bond, M.D. & Van Wart, H.E. (1984). Characterisation of the individual collagenases from clostridium histolyticum. *Biochem.* **23**, 3085-3091.

Bortoff, A. & Sillin, L.F. (1986). Changes in intracellular electrical coupling of smooth muscle accompanying atrophy and hypertrophy. *Am. J. Physiol.* **250**, (*Cell Physiol* 19), C292-C298.

Brading, A.F. & Widdecombe, J.H. (1977). The use of lanthanum to estimate the numbers of extracellular cation exchanging sites in the guinea-pig taenia coli and its effects on transmembrane monovalent ion movements. *J. Physiol.* **266**, 255-273.

Brading, A.F. (1987). The physiology of bladder smooth muscle. In Torrens, M. & Morrisson, J.F.B. (eds). *The physiology of the lower urinary tract.* Springer-Verlag, London, 161-191.

Brading, A.F.& Turner, W.H. (1994). The unstable bladder: towards a common mechanism. *Br. J. Urol.* **73**, 3-8.

Brading, A.F. (1997). A myogenic basis for the overactive bladder. *Urology* **50**, (6A) 57-67.

Bristow, S.E., Pridie, A.K., Bates, D. & Neal, D.E. (1998). Neuromodulation of phasic detrsuor activity by temporary S3 nerve root stimulation. *Br. J. Urol.* **80** (suppl 2), 91-95.

Burnstock, G. (1972). Purinergic nerves. *Pharmacol. Rev..***24**, 509-581.

Burnstock, G. (1990). Innervation of the bladder and bowel. In: *Nerobiology of incontinence (Ciba Foundation Symposium).* Wiley & Sons, Chichester. **151**, 2-26.

Cardozo, L., Stanton, S.L. & Williams, J.E. (1979).Detrusor instability following surgery for genuine stress incontinence *Br. J. Urol.* **51**, 204-207.

Cardozo, L.(1991). Urinary incontinence in women : have we anything new to offer? *B.M.J.* **303**, 1453-1457.

Cardozo, L. & Cutner, A. (1997). Lower urinary tract symptoms in pregnancy. *Br. J. Urol.* **80**, (supp 1) 14-23.

Carpenter, F.G. (1977). Atropine resistance and muscarinic receptors in the rat urinary bladder. *Br. J. Pharmacol,* **59**, 43-49.

Chen, T.F., Doyle, P.T. & Ferguson, D.R. (1994). Inhibition in the human urinary bladdder by gamma-amino-butyric acid. *Br, J, Urol.* **73**, 250-255.

Craig, R. & Megerman, J. (1977). Assembly of smooth muscle myosin into side-polar filaments. *J. Cell. Biol.* **75**, 990-996.

Cumming, J.A. & Chisholm, G.D. (1992). Changes in detrusor innervation with relief of outflow obstruction. *Br. J. Urol.* **69**, 7-11.

Cutner, A., Carey, A. & Cardozo, L. (1992). Lower urinary tract symptoms in early pregnancy. *J. Observation Gynaecol.* **12**, 75-78.

Daniel, E.E. (1985). Subcellular membrane fractions in analysis of control of smooth muscle function. *Experimentia*, **41**, 905-913.

Delancey, J. (1988). Anatomy and mechanisms of structures around the vesical neckposition that might affect its closure. *Neurourol. Urodynam.* **7**, 161-162.

Delancey, J. (1989). Pubovesical ligament: a separate structure from the urethral supports. *Neurourol. Urodynam.* **8**, 53-61.

Delancey, J. (1990). Anatomy and physiology of urinary incontinence. *Clinical Obstet. Gyn.* **33**, (2), 298-307.

De Groat, W.C. & Booth , A.M. (1980). Inhibition and facilitation in parasympathetic ganglia of the urinary bladder. *Federation Proceedings.* **39**, 2990-2996.

De Groat, W.C. (1997). A Neurologic basis for the overactive bladder. *Urology* **50** (6A), 36-52.

Duchen, M.R. & Biscoe, T.J. (1992). Mitochondrial function in type 1 cells isolated from rabbit arterial chemoreceptors.*J. Physiol.* **450**, 13-31.

Dunn, M. (1974). A study of the bladder blood flow during distension in rabbits. *Br. J. Urol.* **46**, 67-72.

Eaton , A.C. & Bates, C.P.(1982). An in vitro physiological study of normal and unstable detrusor muscle. *Br. J. Urol.* **54**, 653-657.

Elbadawi, A., Yalla, S. & Resnick, N. (1993). Structural basis of geriatric voiding dysfunction II. Ageing detrusor : normal vs impaired contractility. *J. Urol.* **150**, 1657-1667.

Enhorning (1961). Simultaneous recording of the intravesical and intrurethral pressure. A study on urethral closure in normal and stress incontinent women. *Acta Chir. Scand. (suppl.)* **276**, 1-68.

Farrar, D.J., Osborne, J.L. & Stephenson T.P. (1976). A urodynamic view of bladder outflow obstruction in the female. Factors influencing the results of treatment. *Br. J. Urol.* **47**, 815-822.

Farrar, D.J. & Turner-Warwick, R. (1979). Outflow obstruction in the female. *Urol. Clin. North Am.* **6**, 217-225.

Fowler, C.J., Christmas, T.J. & Chapple, C.R. (1988). Abnormal electromyographic activity of the urethral sphincter, voiding dysfunction, and polycystic ovaries: A new syndrome? *B.M.J.* **297**, 1436-1438.

Fowler, C.J. (1994). Intravesical capsaicin for the treatment of detrusor hyper-reflexia. *J. Neurol. Neurosurg. Psych.* **57**, 169-173.

Fowler, C.J. (1998) Investigational techniques. *Eur. Urol.* **34**, (supp 1), 10-12.

Fry, C.H., Powell, T., Twist, V.W. & Ward, J.P.T. (1984). Net calcium exchange in adult rat ventricular myocytes: and assessment of mitochondrial calcium accumulating capacity. *Proc, R. Soc. Lond.* **B223**, 239-254.

Fry, C.H., Powell, T., Twist, V.W. & Ward, J.P.T. (1984). The effects of sodium, hydrogen and magnesium ions on mitochondrial calcium sequestration in adult rat ventricular myocytes. *Proc, R. Soc. Lond.* **B223**, 239-254.

Fry, C.H. & Palfrey, E.L.H. (1986). An in vitro investigation of normal and abnormal human bladder smooth muscle. *J. Physiol.* **372**, 37P.

Fry, C.H., Gallegos, C.R.R. & Montgomery B.S.I. (1994). The actions of extracellular H^+ on the electrophysiological properties of isolated human detrusor smooth muscle cells. *J. Physiol.* **480**, (1), 71-80.

Fry, C.H., Relle, C., Birns, J. & Cooklin, M. (1996). Longitudinal impedence of isolated guinea-pig detrusor smooth muscle. *J. Physiol.* **501**,P. 113-114.

Fry, C.H. & Wu, C. (1998). The cellular basis of bladder instability. *Br. J. Urol.* **81**, 1-8.

Fry, C.H., Wu, C. & Mundy, A.R. (1999). Bladder instability and detrusor smooth muscle function. *Exp. Physiol.* **84**.

Fujii, K., Foster, C.D., Brading, A.F. & Parekh, A.B. (1990). Potassium channel blockers and the effect of chromakalin on the smooth muscle of the guinea-pig bladder. *Br. J. Pharmacol.* **99**, 779-785.

Gabella, G. & Uvelius, B. (1990). Urinary bladder of the rat: fine structure of normal and hypertrophic musculature. *Cell and Tissue Research.* **262**, 67-79.

Gallegos, C.R.R. & Fry, C.H. (1994). Alterations to the electrophysiology of isolated human detrusor smooth muscle cells in bladder disease. *J. Urol.* **151** 754-758.

Ganitkevich, V.Y., Shuba, M.F. & Smirnov, S.V. (1987). Calcium dependent inactivation of potential-dependent calcium inward current in an isolated guinea-pig smooth muscle cell. *J. Physiol.* **392**, 431-449.

Ganitkevich, V.Y. & Isenberg, G. (1991). Depolarisation-mediated intracellular Ca^{2+} transients in isolated smooth muscle cells of guinea-pig urinary bladder. *J. Physiol.* **435**, 187-205.

Ganitkevich, V.Y. & Isenberg, G. (1992). Caffeine-induced release and reuptake of Ca^{2+} by Ca^{2+} stores in myocytes from guinea-pig urinary bladder. *J. Physiol.* **458**, 99-117.

Ganitkevich, V.Y. & Isenberg, G. (1992). Contribution of Ca^{2+}-induced Ca^{2+} release to the $[Ca^{2+}]_i$ transients in myocytes from guinea-pig urinary bladder. *J. Physiol.* **458**, 119-137.

German, K., Bedwani, J., Davies, J., Brading, A. & Stephenson, T.P. (1994). An assessment of the contribution of vesico-elastic factors in the aetiology of poor compliance in the human neuropathic bladder. *Br. J. Urol.* **74**, 744-748.

Gilpin, S.A., Gosling, J.A. & Barnard, R.J. (1985). Morphological and morphometric studies of the human obstructed and trabeculated urinary bladder. *Br. J. Urol.* **57**, 525-529.

Gosling, J. (1979). The structure of the bladder and urethra in relation to function .*Urol. Clin. of North Am.* **6**, (1), 31-38.

Gosling, J., Dixon, J.S. & Critchley, H. (1981). A comparative study of the human external and periurethral levator ani muscles. *Br. J. Urol.* **53**, 35.

Gosling, J., Gilpin, S.A., Dixon, J.S. & Gilpin, C.J. (1986). Decrease in the autonomic innervation of human detrusor muscle in outflow obstruction. *J. Urol.* **136**, 501-504.

Griffiths, D. (1998). Clinical aspects of detrusor instability and the value of urodynamics. A review of the evidence. *Eur. Urol.* **34**, (suppl), 13-15.

Grynkiewicz, G., Poenie, M., & Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved flourescent properties. *J. Biol. Chem.* **260**, 3440-3450.

Hammarstrom, M. & Sjostrand, N.O. (1984). Comments on atropine resistance of the neurogenic contractile response of the rat detrusor muscle. *Acta Physiol. Scand.* **122**, 475-481.

Harrison, S.C.W., Hunnan, G.R., Farman, P., Ferguson, D.R. & Doyle, P.T. (1987). Bladder instability and denervation in patients with bladder outflow obstruction. *Br. J. Urol.* **60**, 519-552.

Harrison, S.C.W., Sibley, G.N., Doyle, P.T. & Ferguson, D.R. (1988). The use of endoscopic detrusor muscle biopsies for in vitro muscle strips studies. *Br. J. Urol.* **61**, 210-212.

Harrison, S.C.W., Ferguson, D.R. & Doyle, P.T. (1990). Effect of bladder outflow obstruction on the innervation of the rabbit urinary bladder. *Br. J. Urol.* **66**, 372-379.

Harrison, N., Larcombe-McDouall, J.B., Earley, L. & Wray, S. (1994). An in vivo study of the effects of ischaemia on uterine contraction, intracellular pH and metabolites in the rat. *J. Physiol.* **476**, 349-354.

Harris, D.R.(1995). Smooth muscle cell culture: a new approach to the study of human detrusor physiology and pathphysiology. *Br. J. Urol.* **75**, (suppl. 1.) 18-26.

Hashtaani, H. & Suzuki, H. (1995). Electrical and mechanical responses produced by nerve stimulation in detrusor smooth muscle of the guinea-pig. *Eur. J. Pharm.* **284**, 177-183.

Haugaard, N., Wein, A.J. & Levin, R.M. (1987). In vitro studies of glucose metabolism of the rabbit urinary bladder. *J. Urol.* **137**, 782-784.

Haugaard, N., Hypolite, J., Wein, A.J. & Levin, R.M. (1990). Correlation between contractile force and metabolism in the rabbit urinary bladder. *Neurourol . Urodynam.* **9**, 358-359.

Haugaard, N., Potter, L.,Wein, A.J. & Levin, R.M. (1992). Effect of partial obstruction of the rabbit urinary bladder on malate dehydrogenase and citrate synthase activity. *J. Urol.* **147**, 1391.

Heslington, K. & Hilton, P. (1995). A comparison of ambulatory monitoring and conventional cystometry in asymptomatic female volunteers. *Nuerourol. Urodynam.* **14**, 533-534.

Himpens, B. & Somlyo, A.P. (1988). Free Ca^{2+} and force transients during depolarisation and pharmacomechanical coupling in guinea-pig smooth muscle. *J. Physiol.* **395**, 507-530.

Hodgkinson, C.P., Ayers, M.A. & Drukker, B.H. (1963). Dyssynergic detrusor dysfunction in the apparently normal female. *Am. J. Obstet. Gynaecol.* **87**, 717-728.

Hodgkinson, C.P. & Drukker, B.H. (1977). Infravesical nerve resection for detrusor dyssynergia. *Acta. Obstet. Gynaecol. Scand.* **56**, 401-408.

Holm, N.R., Horn, T. & Hald, T. (1995). Detrusor in ageing and obstruction. *Scand. J. Urol. Nephrol.* **29**, (1), 45-49.

Hoyle, C.H.V., Chapple, C. & Burnstock, G. (1989). Isolated human bladder: evidence for and adenine dinucleotide acting on the P2x- purinoceptors and for purinergic transmission. *Eur. J. Pharm.* **174**, 115-118.

Iacovou, J.W., Hill, S.J., Birmingham, A.T. & Bates, C.P. (1989). Calcium dependence and inositol phosphate accumulation in human detrusor muscle. *Neurourol. Urodynam.* **8**, 418-419.

Iacovou, J.W., Hill, S.J., Birmingham, A.T. (1990). Agonist induced contraction and accumulation of inositol phosphates in the guinea-pig detrusor : Evidence that muscarinic and purinergic receptors raise intracellular Ca^{2+} by different mechanisms. *J. Urol.* **144**, 775-779.

Ingelman-Sundberg, A. (1959). Partial denervation of the bladder. A new operation for the treatment of urge incontinence in women. *Acta. Obstet. Gynaecol. Scand.* **38**, 487-502.

Ingelman-Sundberg, A. (1975). Urge incontinence in women. *Acta. Obstet. Gynaecol. Scand.* **54**, 153-156.

Ingelman-Sundberg, A. (1978). Partial bladder denervation for detrusor dyssynergia. *Clin. Obstet. Gyn.* **21**, (3), 797-809.

Inoue, R. & Brading, A.F. (1990). The properties of the A.T.P. induced depolarisation and current in single cells isolated from guinea-pig urinary bladder. *Br. J. Pharmacol.* **100**, 619-625.

Ishida, Y. & Paul, R.J. (1990). Effects of hypoxia on high energy phosphagen content, energy metabolism and isometric force in guinea-pig taenia coli. *J. Physiol.* **424**, 41-56.

Jackson, S. (1997). The patient with an overactive bladder, symptoms and quality of life issues. *Urology* **50**, (6A) 18-24.

Jorgensen, L., Lose, G., Mortensen, S.O., Molsted, Pedersen, L. & Kristensen, K.J. (1988). The Burch colposuspension for urinary incontinence in patients with stable and unstable detrusor function. *Neurourol. Urodynam.* **7**, 435-441.

Kamm, K.E. & Stull, J.T. (1989). Regulation of smooth muscle contraction elements by second messengers. *Ann. Rev. Physiol.* **51**, 299-313.

Kato, K., Wein, A.J., Radzinski, C., Longhurst, P.A., M^cguire, E.J., Miller, L.F., Elbadawi, A. & Levin, R.M. (1990). Short term functional effects of bladder outlet obstruction in the cat. *J. Urol.* **143**, 1020-1025.

Kidata, S., Ikey, Y., Hasui, Y., Nishi, S., Yamaguchi, T. & Osada, Y. (1992). Bladder dysfunction in elderly men with subclinical brain magnetic resonance imaging lesions. *J.Urol.* **147**, 1507-1509.

Kinder, R.B. & Mundy, A.R. (1985). Inhibition of spontaneous contractile activity in isolated human detrusor muscle strips by vasoactive intestinal polypeptide. *Br. J. Urol.* **57**, 20-23.

Kinder, R.B. & Mundy, A.R. (1985). Atropine blockade of nerve mediated stimulation of the human detrusor. *Br. J. Urol.* **57**, 418-421.

Kinder, R.B. & Mundy, A.R. (1987). Pathophysiology of idiopathic detrusor instability and detrusor hyper-reflexia. *Br. J. Urol.* **60**, 509-515.

Klarskov, P., Gestenberg, T. & Hald, T. (1984). Vasoactive intestinal polypeptide influence on lower urinary tract smooth muscle function from human and pig. *J. Urol.* **131**, 1000-1004.

Klarskov, P. (1987a). Non-cholinergic, non-adrenergic nerve mediated relaxation of the pig and human detrusor muscle *in vitro*. *Br. J. Urol.* **59**, 414-419.

Klarskov, P. (1987b). Non-cholinergic, non adrenergic inhibitory nerve responses of bladder outlet smooth muscle *in vitro*. *J.Urol.* **60**, 337-342.

Klevmark, B. & Kulseng- Hanssen, S. (1991).Continence mechanism in the female. *Scand. J. Urol. Nephrol. (suppl.)* **138**, 35-40.

Klockner, U. & Isenberg, G. (1985). Ca^{2+} currents of caesium loaded smooth muscle cells (urinary bladder of the guinea-pig). *Pfug. Archiv.* **405**, 340-348.

Kuru, M. (1965).The nervous control of micturition. *Am. J. Physiol.* **45**, (3), 426-494.

Lang, R.J. (1989). Identification of the major membrane currents in freshly dispersed single smooth muscle cells of guinea-pig ureter. *J. Physiol.* **412**, 375-395.

Lepor, H., Gup, D., Shapiro, E. & Bauman, M. (1989). Muscarinic cholinergic receptors in the normal and neurogenic human bladder. *J. Urol.* **142**, 869-874.

Levin, R.M., High, J. & Wein, A.J. (1981). Metabolic and contractile effects of anoxia on the rabbit urinary bladder. *J.Urol.* **128**, 194-196.

Levin, R.M., Brendler, K., Van Ardsalen, K.N. & Wein, A.J. (1983). Functional response of the rabbit urinary bladder to anoxia and ischaemia. *Neurourol. Urodynam.* **2**, 233-243.

Levin, R.M., Ruggieri, M.R., Gill, H.S., Haugaard, N. & Wein, A.J. (1988). Effect of bethanechol on glycolysis and high energy phosphate metabolism of the rabbit urinary bladder. *J.Urol.* **139** 646-649.

Levin, R.M., Hypolite, J., Ruggieri, M.R., Longhurst, P. & Wein, A.J. (1989). Effects of muscarinic stimulation on intracellular calcium in the rabbit bladder. Comparison with metabolic response. *Pharmacology* **39**, 69-77.

Levin, R.M., Saito, M., Wein, A.J., Packard, D., Cohen, A. & Haugaard, N. (1993). Effect of partial outlet obstruction on choline acetyl transferase activity in the rat and the rabbit. *Neurourol. Urodynam.* **12**, 255-261.

Levin, R.M., Monson, F.C., Haugaard, N., Buttyan, R., Hudson, A., Roelofs, N., Sartore, S. & Wein, A.J. (1995). Genetic and cellular characteristics of bladder outlet obstruction. *Urol. Clin North. Am.* **22**, (2), 263-283.

Levin, R.M., Haugaard, N., Hypolite, J., Wein, A.J. & Buttyan, R. (1999). Metabolic factors influencing lower urinary tract function. *Exp. Physiol.* **84**.

Lin, A.T-L., Wein, A.J., Gill, H.S. & Levin, R.M. (1988). Functional effect of chronic ischaemia on the rabbit urinary bladder. *Neurourol. Urodynam.* **7**, 1-12.

Lin, A.T-L., Monson, F.C., Kato, K., Haugaard, N., Wein, A.J. & Levin, R.M. (1989). Effect of chronic ischaemia on glucose metabolism of rabbit urinary bladder. *J.Urol.* **142**, 1127-1133.

Liston, T.G. Palfrey, E.L.H., Raimbach, S.J. & Fry, C.H. (1991). The effects of pH changes on human and ferret detrusor muscle function. *J. Physiol..* **342**, 1-21.

McCullough, W.L., Golland, I.M. & Clark, A.D. (1997). Acute urinary retention after T.A.H. *Br. J. Urol.* **80**, (suppl 1) 91-92.

Maggi, C.A. Scanticioli, P., Patacchini, R., Cellerini, M., Turini, D. Barbanti, G., Beneforti, P., Rovero, P. & Meli, A. (1988). Contractile response of the human isolated urinary bladder to neurokinins; involvement of the NK-2 receptors. *Eur. J. Pharm.* **145**, 335-340.

Mallet, V., Hosker, G., Smith A.R.B. & Worrell, D. (1994). Pelvic floor damage and childbirth, a neurophysiological follow-up study. *Neurourol. Urodynam.* **13**, 357-358.

Malkowitz, S.B., Wein, A.J., Elbadawi, A., Van Ardsalen, K., Ruggieri, M. & Levin, R.M. (1986). Acute biochemical and functional alterations in the partially obstructed rabbit urinary bladder. *J. Urol.* **136**, 1324.

Malmgren, A., Uvelius, B., Andersson, K.E. & Andersson, P.O. (1990). On the reversibility of functional bladder changes induced by infravesical outflow obstruction in the cat. *J. Urol.* **143**, 1026-1031.

Malmgren, A., Andersson, K.E., Andersson, P.O., Fovaeus, M. & Sjogren, C. (1990). Effects of chromakalin (BRL 34915) and pinacidil on normal and hypertrophied rat detrusor muscle in vitro. *J. Urol.* **143**, 828-834.

Malone-Lee, J.(1997). Drug treatment for urge incontinence in the elderly. *Urology* **50**, (6A) 86.

Marston, S.B. & Smith, C.W.J. (1985). The thin filaments of smooth muscles. *J. Muscle Res. Cell Motil.* **6**, 669-708.

Massey, J.A. & Abrams, P.H. (1988). Obstructed voiding in the female. *Br. J. Urol.* **61**, 36-39.

Masters, J.G. & Ramsden, P.D. (1997). Bladder outlet obstruction in women: aetiology, management and outcome. *Br. J. Urol.* **80**, (suppl 1) p91.

Mattiasson, A., Andersson, K., Elbadawi, A., Morgan, E. & Sjogren, C. (1987). Interaction between adrenergic and cholinergic nerve terminals in the urinary bladder of the rabbit, cat and man. *J. Urol.* **137**, 1017-1019.

Mayo, M.E., Lloyd-Davies, R.W. Shuttleworth, K.E.D. & Tighe, J.R. (1973). The damaged human detrusor; functional and electron microscopic changes in disease. *Br. J. Urol.* **45**, 116-125.

Meisheri, K.D. & Ruegg, J.C. (1983). Dependence of cyclic A.M.P. induced relaxation on Ca^{2+} and calmodulin in skinned smooth muscle of the guinea-pig taenia-coli. *Pfug. Archiv.* **399**, 315-320.

Minezaki, K.K., Suleiman, M.S. & Chapman, R.A. (1994). Changes in mitochondrial function induced in isolated guinea-pig ventricular myocytes by calcium overload. *J. Physiol.* **476**, (3), 459-471.

Montgomery, B.S.I. & Fry, C.H. (1992). The action potential and net membrane currents in isolated human detrusor smooth muscle cells. *J. Urol.* **147**, 176-184.

Montgomery, B.S.I., Thomas, P.J. & Fry, C.H. (1992). The actions of extracellular magnesium on isolated human detrusor muscle function. *Br. J. Urol.* **70**, 262-268.

Morita, T., Ando. M., Kilhara, K. & Oshima , H. (1993). Species differences in cAMP production induced by beta adrenoceptor subtypes in urinary bladder smooth muscle. *Neurourol. Urodynam.* **12**, 185-190.

Mostwin, J.L. (1985). Receptor operated intracellular Ca^{2+} stores in the smooth muscle of the guinea-pig bladder. *J. Urol.* **133**, 900-905.

Mostwin, J.L. (1991). Current concepts of female pelvic anatomy and physiology. *Urol. Clin. North Am.* **18**, (2), 175-195.

Mundy, A.R. & Stephenson, T.P. (1985). "Clam" ileocystoplasty for the treatment of refractory urge incontinence. *Br. J. Urol.* **57**, 641-646.

Mundy, A.R. (1988). Detrusor instability. *Br. J. Urol.* **62**, 393-397.

Neal, D.E., Bogue, P.R. & Williams, R.E.(1982). Histological appearances of the nerves of the bladder in patients with denervation of the bladder after excision of the rectum. *Br. J. Urol.* **54**, 658-666.

Nergardh, A. & Kinn, A.C. (1983). Neurotransmission in activation of the contractile response in the human urinary bladder. *Scan. J. Urol. Nephrol.* **17**, 153-157.

Nilvebrant, L., Andersson, K. E. & Mattiasson, A. (1985). Characterisation of the muscarinic cholinoceptors in the human detrusor. *J. Urol.* **134**, 418-423.

Norton, P.A. (1990). Prevalence and social impact of urinary incontinence in women. *Clinical Obstet. Gynaecol.* **33**, (2), 295-297.

Nurse, D.E., Restorick, J.M. & Mundy, A.R. (1991). The effect of chromakalim on the normal and hyperreflexic human detrusor muscle. *Br. J. Urol.* **68**, 27-31.

Pacaud, P. & Bolton, T.B. (1991). Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. *J. Physiol.* **441**, 477-499.

Palea, S., Artibani, W., Ostado, E., Trist, D.G. & Pietra, C. (1993). Evidence of purinergic neurotransmission in human urinary bladder affected by interstitial cystitis. *J.Urol.* **150**, 2007-2012.

Palfrey, E.L.H., Fry, C.H. & Shuttleworth, K.E.D. (1984). A new in vitro microperfusion technique for the investigation of human detrusor muscle. *Br. J. Urol.* **56**, 635-640.

Pope, A.J., Shaw, P.J.R., Copcoat, M.J. & Worth, P.H.L.(1990). Changes in bladder function following a surgical alteration in outflow resistance. *Neurourol. Urodynam.* **9**, 503-508.

Raz, S., Caine, M & Zeigler, M. (1972). The vascular component in the production of intraurethral pressure. *J.Urol.* **108**, 93-96.

Restorick, J.M. & Mundy, A.R. (1989). The density of cholinergic and alpha and beta adrenergic receptors in the normal and hyper-reflexic human detrusor. *Br. J. Urol.* **63**, 32-35.

Rink, T., Tsien, R.Y. & Pozzan, T. (1982). Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell Biol.* **95** 189-196.

Robertson, A., Griffiths, C. & Neal, D. (1996). Conventional urodynamics and ambulatory monitoring in the definition and management of bladder outflow obstruction. *J. Urol.* **155**, p506.

Ruggieri, M.R., Whitmore, K.E. & Levin, R.M. (1990). Bladder purinergic receptors. *J. Urol.* **144**, 176-179.

Saito, M., Kondo, A., Kato, T., Hasegawa, S. & Miyake, K. (1993). Response of the human neurogenic bladdder to KCl, Carbachol and $CaCL_2$. *J. Urol.* **149**, 408-411.

Saito, M., Kondo, A., Kato, T. & Miyake, K. (1993). Response of the human urinary bladder to angiotensins: a comparison between neurogenic and control bladders. *J.Urol.* **149**, 408-411.

Saito, M., Wein, A.J. & Levin, R.M. (1993). Effect of partial outlet obstruction on contractility : a comparison between severe and mild obstruction. *Neurourol. Urodynam.* **12**, 573-583.

Schneider, P., Hopp, H.H. & Isenberg G. (1991). Ca^{2+} influx through ATP-gated channels increments $[Ca^{2+}]_i$ and inactivates Ca^{2+}_i in myocytes from guinea-pig urinary bladder. *J. Physiol.* **440**, 479-496.

Seki, N., Karim, O.M.A. & Mostwin, J.L. (1992). The effect of experimental urethral obstruction and its reversal on changes in passive electrical properties of detrusor muscle. *J. Urol.* **148**, 1957-1961.

Sethia, K.K., Brading, A.F. & Smith J.C. (1990). An animal model of non-obstructive detrsuor instability. *J. Urol.* **143**, 1243-1246.

Sibley, G.N.A. (1984). A comparison of spontaneous and nerve mediated activity in bladder muscle from man, pig, and rabbit. *J. Physiol.* **354**, 431-443.

Sibley, G.N.A. (1985). An experimental model of detrusor instability in the obstructed pig. *Br. J. Urol.* **57**, 292-298.

Sibley, G.N.A. (1987). The physiological response of the detrusor muscle to experimental bladder outflow obstruction in the pig. *Br. J. Urol.* **60**, 332-336.

Sjogren, C., Andersson, K.E., Husted, S., Mattiasson, A. & Moller-Madson, B. (1982). Atropine resistance of transmurally stimulated isolated human bladder muscle. *J. Urol.* **128**, 1368-1371.

Sjogren, C., Andersson, K.E. & Mattiasson, A. (1985).Effects of vasoactive intestinal polypeptide on isolated urethral and urinary bladder smooth muscle from rabbit and man. *J. Urol.* **133**, 136-140.

Smith, D.J. & Chapple, C.R. (1994). In vitro response of human bladder smooth muscle in unstable obstructed male bladders: a study of pathological causes. *Neurourol. Urodynam.* **13**, 414-415.

Sokal, R.R. & Rohlf, F.S. (1969). Biometry. *W.H. Freeman & Co.* San Francisco, USA

Speakman M.J., Brading, A.F., Dixon, J.S. Gilpin, C.J. & Gosling, J.A. (1987). Bladder outflow obstruction- a cause of denervation supersensitivity. *J. Urol..* **138**, 1461-1466.

Speakman, M.J., Walmsley, D. & Brading, A.F. (1988). An in vitro pharmacological study of the human trigone, a site of non-adrenergic, non cholinergic neurotransmission. *Br. J. Urol.* **61**, 304-309.

Speakman M.J., Brading, A.F., Dixon, J.S. Gilpin, C.J. & Gosling, J.A. (1991). Cystometric, physiological and morphological studies after relief of bladder outflow obstruction in the pig. *Br. J. Urol.* **68**, 243-247.

Thomas, A.P. & Delaville, F. (1991). The use of fluorescent indicators for the measurement of cytosolic free calcium in cell populations and single cells. In McCormack & Cobbold eds: *Cellular Calcium, a Practical Approach.* In I.R.L. Press (at Oxford University Press) New York.

Thomas, P.J. & Fry, C.H. (1996). The effects of cellular hypoxia and extracellular ion accumulation in isolated human detrusor smooth muscle. *J. Urol.* **155**, 726-731.

Thomas, T.M., Plymat, K.R., Blanni, J. & Meade, T.W. (1980). Prevalence of urinary incontinence. *B.M.J.*.**281**, 1243-1245.

Todd, J.K. & Mack, A.J. (1969). A study of human bladder detrusor muscle. *Br. J. Urol.* **41**, 448-454.

Turner-Warwick, R. (1979). Observations on the function and dysfunction of the sphincter and detrusor mechanisms. *Urol. Clin. North Am.* **6**: 13 .

Uvelius, B. & Gabella, G. (1980). Relationship between cell length and force production in urinary bladder smooth muscle. *Acta Physiol. Scand.* **110**, 357-365.

Uvelius, B. (1986). Detrusor smooth muscle in rats with alloxan-induced diabetes. *J. Urol.* **136**, 949-952.

Van Arsdalen, K.N., Wein, A.J. & Levin, R.M. (1983). The contractile and metabolic effects of acute ischaemia on the rabbit urinary bladder. *J. Urol.* **130**, 180-182.

Van Waalwijk van Doorn, E.S.C., Remmes, A. & Jankenegt, R.A. (1992). Conventional and extramural ambulatory urodynamic testing of the lower urinary tract in female volunteers. *J. Urol.* **47**, 1319.

Van Karrebraeck J. (1988). The role of electrical stimulation in voiding dysfunction. *Eur. Urol.* **34**, (suppl 1) 27-30.

Venn, S.N. & Mundy, A.R. (1988). Long term results of augmentation cystoplasty. *Eur. Urol.* **34**,(suppl 1) 40-42.

Vereecken, R.L. & Das, J. (1985). Urethral instability; related to stress and/or urge incontinence? *J. Urol.* **134**, 698-701.

Watanabe, C., Yamamoto, H., Hirano, K., Kobayashi, S. & Kanaide, H. (1992). Mechanisms of caffeine induced contraction and relaxation of rat aortic smooth muscle. *J. Physiol.* **456**, 193-213.

Wheeler, M., Martin, T.V. & Weiss, R.M. (1995). Effect of carbachol and norepinephrine on phosphatidyl inositol hydrolysis and cAMP levels in guinea-pig urinary tract. *J. Urol.* **153**, 2044-2049.

Wu, C., Kentish, J.C. & Fry, C.H. (1995). Effect of pH on myofilament Ca^{2+} sensitivity in alpha-toxin permeabilized guinea-pig detrusor smooth muscle. *J. Urol.* **154**, 1921-1924.

Yang, A., Mostwin, J.L., Rosenschein, N.B. & Zerhouni, E.A.(1991). Pelvic floor descent in women, dynamic evaluation with fast M.R. imaging and cinematic display. *Radiology* **179**, 25-33.

Yaun, X-Y, Goldman, W.F. & Todd, M.L.(1993). Hypoxia reduces potassium currents in cultured rat pulmonary but not mesenteric arterial myocytes. *Am. J. Physiol.* **264**, L116-L123.

Zderic, S.A., Sillen, U., Liu, G-H., McSnyder, H., Duckett, J.W., Wein, A.J. & Levin, R.M. (1993). Developmental aspects of bladder contractile function; evidence for an intracellular calcium pool. *J. Urol.* **150**, 623-625.

Zderic, S.A., Sillen, U., Liu, G-H., McSnyder, H., Duckett, J.W., Gong, C.& Levin, R.M.(1994). Developmental aspects of excitation-contraction coupling of rabbit bladder smooth muscle. *J. Urol.* **152**, 679-681.

Zinner, N. R., Sterling, A.M. & Ritter, R.C. (1980). The role of inner softness in urinary incontinence. *Urology* **16**, 115-117.

Zinner, N.R. (1998). Clinical aspects of detrusor instability and the value of urodynamics. *Eur. Urol.* **34**, (suppl 1) 16-19.