# CHAPTER 6

## Committee 2

# Cell Biology

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# Cell Biology

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## INTRODUCTION

This aim of this report is to highlight features of the cell biology of the urinary tract that will lead to a better understanding of the pathophysiology of urinary and faecal incontinence. Research strategy aims to understand as completely as possible the normal cell biology of the system and to characterise changes that occur which are associated with lower urinary tract and gastrointestinal tract dysfunction. It is important to realise that it is difficult to differentiate between changes to tissue and cell function that cause pathophysiological changes, or are secondary to the development of dysfunction. However, even if such a distinction cannot be drawn the description of such changes is useful as they identify potentially useful targeted models for the development of drugs or other diagnostic or corrective approaches.

Since the previous consultations knowledge of the cell biology of the lower urinary and gastrointestinal tracts has increased significantly. Understanding of the cell biology of smooth muscle function has increased significantly in some areas (eg detrusor), whilst in others (eg urethra, trigone) it remains less clear. Urothelial function has come under greater investigation, especially with respect to its role in afferent responses, although its transport properties are also being appreciated as playing an important role in the homeostatic function of the lower urinary tract. Since the first consultation the human genome has also been published in full, and the identification of abnormal genetic markers in lower urinary and gastrointestinal tract dysfunction promises to generate new approaches to manage these conditions. The increased understanding of the cell biology of these tissues has also facilitated tissue-engineering approaches to develop functional implants. Finally this committee has also described the cell physiology of the lower gastrointestinal tract. Many conditions associated with lower urinary tract dysfunction have parallels in the lower gastrointestinal tract, and the two systems have similar and integrated physiological controls. Therefore a more integrated approach can only increase our understanding of both systems.

This report has attempted to reflect the tenor of recent advances, as well as emphasise the most important previous work, to achieve the above aims. All the referenced material is from peer-reviewed publications, the majority of which has appeared subsequent to the first consultation.

## I. CELL PHYSIOLOGY OF LOWER URINARY TRACT SMOOTH MUSCLE

### 1. Contractile activation of detrusor smooth muscle

A first volume of this consultation [1] outlined the cellular basis of contractile activation in detrusor smooth muscle : the role of M3 receptor activation and the generation of intracellular inositol trisphosphate ($IP_3$). $IP_3$ releases $Ca^{2+}$ from intracellular stores by binding to $IP_3$-receptors on the membrane of the store itself [2], and the $Ca^{2+}$ released, when combined with calmodulin, activates the contractile proteins. Relaxation occurs when the intracellular [$Ca^{2+}$] falls, either by sequestration into the SR, or by its removal from the cell via $Na^+/Ca^{2+}$ exchange [3]. Maintenance of adequate filling of $Ca^{2+}$ stores involves $Ca^{2+}$ influx through membrane $Ca^{2+}$ channels. [2]. Moreover there is increasing evidence that detrusor cells are functionally coupled through gap junctions [4], which gives the opportunity for information to spread between contiguous cells to form a functional syncitium. Such syncitial transfer would be better

within, compared to between, muscle bundles [5] due to the significant separation between bundles. Thus information transfer would not be co-ordinated on a whole organ basis as in the heart for example, but would allow local coordination of responses. A number of changes to this basic scheme have been reported that are associated with bladder dysfunction. Although it is not known if they cause such dysfunction or are secondary they do represent targets that are associated with abnormal function and thus may offer more targeted approaches to regulate aberrant bladder activity.

## 2. $Ca^{2+}$ CHANNELS AND DETRUSOR MUSCLE

$Ca^{2+}$ channels play an important role in initiating contraction in many muscles. However, in detrusor a somewhat different function is ascribed as cholinergic activation occurs independently of changes to the resting membrane potential. Two classes of $Ca^{2+}$ channel are present in detrusor, an L-type and a T-type channel [6] and the possible $Ca^{2+}$ entry through these channels is large. The function of the L-type channel has been most thoroughly investigated and in detrusor it regulates the filling of intracellular $Ca^{2+}$ stores during resting states [2]. This is achieved by regulation of the membrane potential by $Ca^{2+}$-activated $K^+$ channels, such that when the intracellular $[Ca^{2+}]$ is low (for example, immediately after a contraction when some $Ca^{2+}$ efflux occurs), the conductance of these channels is reduced, the membrane depolarises and L-type $Ca^{2+}$ channels open to restore $Ca^{2+}$ balance in the cell. The scheme is illustrated in **Figure 1**. These channels have also been reported to show a prolonged-open state induced by large depolarisations [7]. In principle therefore the presence of depolarising mechanisms in detrusor (i.e via purinergic transmission in the overactive human bladder) would induce further $Ca^{2+}$ influx through these $Ca^{2+}$ channels, and act as a positive feedback mechanism to enhance detrusor contractility. The cellular processes that regulate the long-open state are unclear, but it is not due to channel phosphorylation [8, 9].

The demonstration that T-type $Ca^{2+}$ channels also exist in detrusor is of interest because they open at more negative membrane potentials, and in principle will allow some $Ca^{2+}$ influx even at resting potentials. Their actual function remains to be evaluated however. On the one hand, selective blockade of these channels with low (micromolar) $Ni^{2+}$ concentrations reduces spontaneous contractions in detrusor [10], however they seem to exert no significant effect on similar spontaneous alterations to membrane poten-

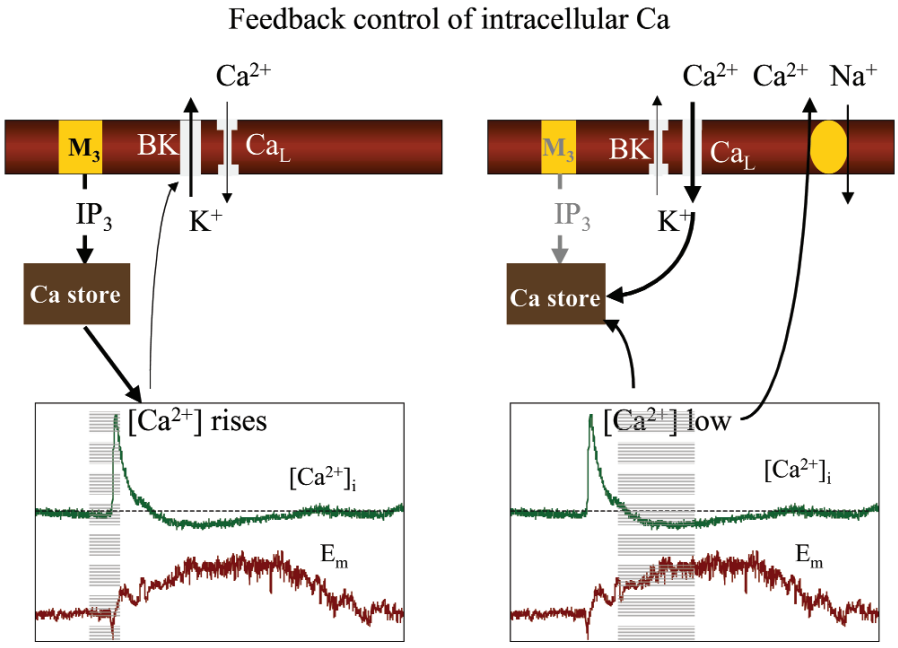


***Figure 1. Feedback control of intracellular $Ca^{2+}$ in detrusor smooth muscle.***
***Left-hand scheme: The intracellular $[Ca^{2+}]$ is high after release from intracellular stores, following muscarinic receptor activation. The raised intracellular $[Ca^{2+}]$ opens $Ca^{2+}$-activated $K^+$ channels (BK channels) and hyperpolarizes the cells. This in turn reduces the opening probability of membrane $Ca^{2+}$ channels. The experimental traces from which the scheme is derived is shown at the bottom of the panel. Right-hand scheme: The intracellular $[Ca^{2+}]$ falls, following sequestration by intracellular Ca-stores and removal from the cell via $Na^+$-$Ca^{2+}$ exchange and thus BK channels close, causing membrane depolarisation. Membrane $Ca^{2+}$ channels open to permit $Ca^{2+}$ influx and thus restore the intracellular $Ca^{2+}$ balance – see [2].***

tial - where L-type $Ca^{2+}$ channel activity is more important [11]. It is important to resolve their exact roles in regulating intracellular $Ca^{2+}$ activity, and hence contractile function, not only because they clearly modulate cellular $Ca^{2+}$ levels, but also because the proportion of T-type, compared to L-type, channels, increases in detrusor from overactive bladders [12].

## 3. Spontaneous contractions and detrusor muscle

Spontaneous contractile activity can be measured in vitro in nearly all detrusor preparations [13], and in vivo from animal and human bladders [14, 15]. In addition, activity is increased in vitro using tissue from patients with over-active bladders or animals with outflow tract obstruction [16-18]. These contractions could in principle manifest themselves as in vivo bladder phenomena either by contributing to detrusor contractions associated with bladder over-activity or by increasing resting detrusor muscle tone and thus decreasing bladder compliance during filling. It remains to be shown unequivocally that in vitro spontaneous activity is related to whole bladder phenomena. However, conditions that increase spontaneous activity in isolated preparations enhance bladder wall stiffness [19], and low level pressure fluctuations in the bladder have been recorded which have been speculated to be manifestations of underlying muscular activity [20].

Much is known about the manipulation of spontaneous contractions, from which it is concluded that they are not of neural origin, but are initiated in the muscle mass itself [10, 21, 22]. However, little is known of their origin. Agents that modulate spontaneous activity also affect bladder function. For example, $Ca^{2+}$ channel antagonists, such as nifedipine, inhibited detrusor instability in a rat model of outflow obstruction [23] and ATP-dependent $K^+$ channel openers such as pinacidil and cromakalim suppressed bladder over-activity [24]. In addition, intravesical instillation of the cholinergic antagonist atropine is effective in over 40% of neuropathic instability, but only in less than 20% in idiopathic instability [25]. It is possible that neurogenic over-activity has a greater reliance on afferent neural input, in which acetylchoine released from the urothelium (see section 3.1) may facilitate afferent excitation. However, the observations suggest a distinct non-neural, myogenic process underlying idiopathic detrusor instability.

Spontaneous contractions should be more evident when $[Ca^{2+}]_i$ is raised, by analogy to other tissues such as myocardium [26]. Human detrusor exhibits a higher resting $[Ca^{2+}]_i$, more frequent spontaneous $Ca^{2+}$ spikes and a higher sensitivity to muscarinic agonists than other mammals, and may pre-dispose human detrusor to greater spontaneous activity. Detrusor exhibits spontaneous membrane potential variations, which generally oscillate around a range that could activate T-type $Ca^{2+}$ channels, with less frequent larger spikes that could activate L-type $Ca^{2+}$ channels [11]. In addition, spontaneous action potentials have been recorded in isolated preparations that rely on $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels, but whose frequency is modulated by $K^+$ channel activity. The occurrence of electrical activity was closely correlated with spontaneous contractile activity, suggesting a direct causal relationship between the two variables [27]. More recently, spontaneous electrical activity has been shown to be linked to contractile activity through spontaneous $Ca^{2+}$ transients [28] (**Figure 2**). Furthermore, spontaneous simultaneous variations of potential and $[Ca^{2+}]_i$, are enhanced in cells isolated from unstable bladders [29] and suggests a cellular basis for irregular detrusor contractions

Two current hypotheses for spontaneous contractions are that they originate from $Ca^{2+}$ sparks within individual myocytes, or from pacemaker cells that lie near the smooth muscle bundles. Potential pacema-

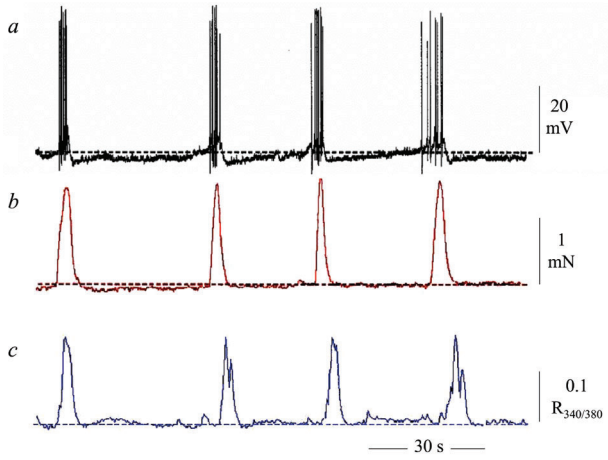


***Figure 2. Spontaneous electromechanical activity in detrusor smooth muscle Simultaneous recordings of (a) action potentials, (b) isometric tension and (c) intracellular $[Ca^{2+}]$ in a guinea-pig detrusor strip. The intracellular $[Ca^{2+}]$ scale is a relative change of a fluorescence ratio, R, when illuminated alternately at 340 and 380 nm. An increase of the ratio R340/380 represents an increase of the intracellular $[Ca^{2+}]$. Modified from [28].***

ker cells (interstitial cells) will be discussed in more detail below.

$Ca^{2+}$ sparks are subcellular events that represent $Ca^{2+}$ release from one, or a very few, sites within the cell and have been recorded in many cells. These could spatially and temporally summate to generate a cellular $Ca^{2+}$ wave, and if sufficiently frequent and widespread, could be manifest as a localised spontaneous contraction. Indeed $Ca^{2+}$ waves have been reported to spread throughout multicellular detrusor preparations [30]. $Ca^{2+}$-oscillations may arise by activating oscillator units of ryanodine and $IP_3$ receptors, as implicated in vascular or ureteric smooth muscle [31,32]. Preliminary data (C Wu & CH Fry, unpublished data, **Figure 3**) show that $Ca^{2+}$ sparks may indeed be recorded in isolated human detrusor cells. Sparks are associated with transient outward currents that can be blocked by ryanodine and suggests that $Ca^{2+}$ from intracellular stores are indeed responsible for these subcellular $Ca^{2+}$ transients.

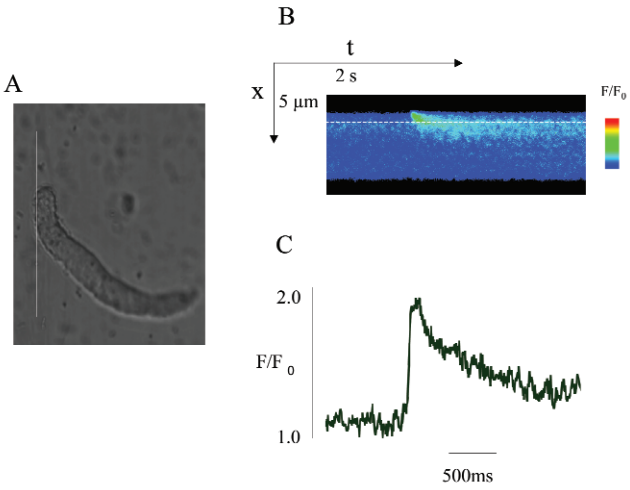


***Figure 3. A $Ca^{2+}$ spark in an isolated detrusor smooth muscle cell. A spontaneous $Ca^{2+}$ spark recorded from an isolated guinea-pig detrusor myocyte.***
***A: The image was obtained from repeated line scans (50 Hz) through the white line shown superimposed on the image of the isolated detrusor myocyte.***
***B: Successive line scans positioned side-by-side to show the temporal change of the intracellular [$Ca^{2+}$] along the axis of the line scan. The scale shown on the right-hand side indicated an increasing [$Ca^{2+}$].***
***C: A temporal scan through the point indicated by the white dotted line in part B. Scan produced by a BioRad 2100 confocal system with Ca-Green as the fluorescent ligand, excitation at 488 nm with an argon laser, emission collected at 515-530 nm.***

## 4. Intercellular coupling and detrusor muscle

There has been considerable debate as to whether detrusor myocytes are functionally coupled via the intracellular space, and if so what function this would serve. Although, cholinergic activation is not associated with an action potential, changes to membrane potential occur throughout the contracting and relaxed phases [27], and would be enhanced when purinergic activity was evident.

Furthermore, ions and small molecules could also diffuse between cells. Electrophysiological measurements indicate that detrusor behaves as a functional electrical syncitium [4, 33], although to a lesser extent than in some other smooth muscles [34], and this has been corroborated by the demonstration of connexin45 protein in detrusor [35]. Connexins are the component proteins of gap junctions and the type found in detrusor forms pores of relatively low conductance, indicating that electrical coupling is relatively poor, compared to tissue such as myocardium and myometrium.

The unresolved question remains the consequence of electrical coupling in detrusor tissue, especially as connexin density and intercellular electrical coupling are reduced in tissue from unstable bladders [35]. Qualitative estimates of changes to two important determinants of the electrophysiological behaviour of tissue, namely conduction and excitability, have been made in human detrusor from stable and overactive bladders [36]. A decrease of connexin distribution was found to increase electrical excitability, but decrease the conduction velocity of electrical signals from any sites. Thus, these changes would enable electrical activity to generate local events that would persist more readily. The quantitative extent of the spread of electrical activity is a future direction that will determine if local electrical activity can precipitate localised contractile responses.

## 5. Urethral skeletal muscle

The skeletal muscle component of the urethra wall (rhabdosphincter) forms an incomplete ring of skeletal muscle around the urethra [37]. In human samples more than 60% of the muscle fibres are slow, fatigue resistant type I ; most of the rest are fast type IIa fibres, which are also relatively fatigue resistant [38]. In women, the number of muscle fibres reduces with age [39], and in women with stress incontinence the volume of the skeletal muscle bulk was decreased [40]. Such a loss of muscle fibres has been linked to

an increase of their apoptosis [41]. Abnormal electromyographic (emg) activity from these muscles has been recorded, most notably in a group of women with impaired voiding and associated abnormal progesterone status [42]. It was proposed that the abnormal emg activity arose from aberrant electrical transmission between adjacent skeletal muscle cells (ephaptic transmission), although this remains to be demonstrated.

Pudendal efferents that innervate the skeletal muscle end in cholinergic end-plates. However, the cell bodies in Onuf's nucleus have an interesting profile, with large numbers of noradrenergic and serotonergic terminals whose activation augments sphincter contraction. This has resulted in the development of serotonin (5-HT) and noradrenaline uptake inhibitors, such as duloxetine to enhance the sphincteric function of the rhabdosphincter [43]. In addition, neuronal nitric oxide synthase (nNOS) has been detected in the muscle fibre membranes, especially near the neuromuscular junction, and in nerve fibres and intramural ganglia [44, 45]. However, nNOS inhibition did not exert any functional effects on urethral function, so that the significance of these observations remains unclear.

Interest in culturing skeletal muscle cells has arisen from the possibility of autologous transplantation, as a means to minimise stress incontinence. Human cells that respond to cholinergic stimulation can be maintained in culture [46]. Furthermore, muscle-derived progenitor cells have been successfully implanted into an animal model, which has greatly reduced intrinsic urethral skeletal musculature following denervation [47].

## 6. URETHRAL SMOOTH MUSCLE

The urethra is characterised by an internal layer of longitudinal smooth muscle, and an outer, thinner layer of circular smooth muscle. Many of the mechanical and electrical characteristics of this tissue have been outlined in the first volume of this consultation [1]. It has been proposed that, at least in the male, the longitudinal muscle shortens during micturition, and the circular muscle contracts during continence, in the female the smooth muscle layers are thought to play a more minor role [48]. However, there are significant regional differences within the urethra, as well as male-female variability, to make a homogeneous description of function difficult.

Control of the contractile function of urethral smooth muscle is complex, with evidence of adrenergic and cholinergic receptors and modulation via volatile gases, such as nitric oxide (NO) and carbon monoxide (CO). The presence of particular receptors and intracellular pathways does not itself demonstrate that urethral muscle function is controlled by neural pathways that would release receptor agonists and modulators. However, sympathetic control is regarded as pre-dominant in maintaining contraction, mediated by $\alpha_1$ receptors, probably the subtype $\alpha_{1A}$ [49]. $\alpha_2$ receptors have been located in the urethra and have been proposed to act as pre-junctional modulators [50], or regulate blood flow [51]. Relaxation by β-receptors has also been investigated, and in dog and rat the effects were weaker than in bladder and mediated by $\beta_2$ receptors, rather than $\beta_3$ receptors as in detrusor muscle [52]. A similar pattern is suggested in pig urethra [53], which is often preferred as an animal model to mimic human urethral properties. Such selectivity may be useful in the development of bladder-relaxing agents that affect less outflow resistance. Cholinergic contractile mechanisms are also important. One study has suggested that this may be especially so in the proximal part of the urethra and bladder neck [54].

There is also evidence for relaxation mediated via the volatile gases NO and CO, which ultimately exert a role through cellular cyclic nucleotides. NO, produced by nitric oxide synthase in cholinergic nerves is the more important, and is the key process that induces urethral relaxation during voiding [55]. There is evidence, in a rabbit model, that in diabetes mellitus NO-mediated relaxation is impaired [56], possibly due to reduced generation of cyclic nucleotides, even though NOS activity seems to be increased. Administration of arginine, the substrate for NO production, restored the ability of the urethra to relax [57]. However whether this recovery of function was mediated by a restoration of tissue cyclic nucleotide levels was not measured. Nevertheless, the study indicated that the nitrergic system could be modulated in disease, to restore function towards normal urethral.

The cellular physiology of urethral smooth muscle has received less attention than detrusor smooth muscle. Electrical activity characterised as spikes, slow waves and spike complexes superimposed on slow depolarising waves has been recorded from isolated preparations [58]. The spikes were similar to those recorded from isolated urethral myocytes and the slow waves from interstitial cells [59]. The spike complexes may represent recordings from muscle cells driven by pacemaker cells, as recorded in intes-

tinal preparations [60]. Two types of ion channels underlie the depolarising phases of activity, $Ca^{2+}$ channels and $Ca^{2+}$-activated $Cl^-$ channels. The $Ca^{2+}$ channels themselves are of two types, an L-type and a T-type channel, the latter activated at more negative membrane potentials [61-63]. Repolarisation and maintenance of the resting potential is mediated by a number of $K^+$ channels that include : $Ca^{2+}$ activated $K^+$ channels, a voltage-sensitive channel and an ATP-regulated channel, $K_{ATP}$ [64, 65]. The relative significance of these different ion channels to determine the overall electrophysiological characteristics of urethral smooth muscle remains to be established.

Several ion channel modulators have been investigated to identify agents that may, in particular, enhance urethral relaxation. $K_{ATP}$ channel modulators have been most intensively studied [65, 66]. However, it is of interest that the T-type $Ca^{2+}$ channels that play a role in membrane depolarisation, may be different in the urethra and bladder, as judged by their relative sensitivity to $Ni^{2+}$ [12, 62]. If so this offers a further avenue to manipulate selectively urethral function over bladder activity.

## 7. Interstitial cells/myofibroblasts and lower urinary tract function

There has been recent interest in non-muscle cells located in the walls of the urinary tract, located either in the sub-urothelial space or the detrusor layer, and called variously interstitial cells or myofibroblasts. It is unlikely that these cells are a homogeneous group as many of their functions and features at different sites vary. In some tissues (e.g. gut [67] and brain [68]) they have been found in two states - an activated and a stellate-transformed myofibroblast - and this spectrum may be so at different sites in the lower urinary tract walls. **Figure 4** shows micrographs of isolated cells from the urethral smooth muscle layer and from the sub-urothelial space in the bladder wall. The former cells are large and have long projections whereas the sub-urothelial myofibroblasts are smaller without extensive branches. Such cells do have well-defined morphological and immuno-histochemical characteristics [69-71]. Most generally myofibroblasts are defined from the identification of cytoskeletal proteins, i.e vimentin, desmin and α-smooth muscle actin, and whether they express one or a number of these has been used to sub-classify them [71]. In addition some populations of cells [72] react positively to the proto-oncogene c-kit, but this is not a ubiquitous finding [59, 73].

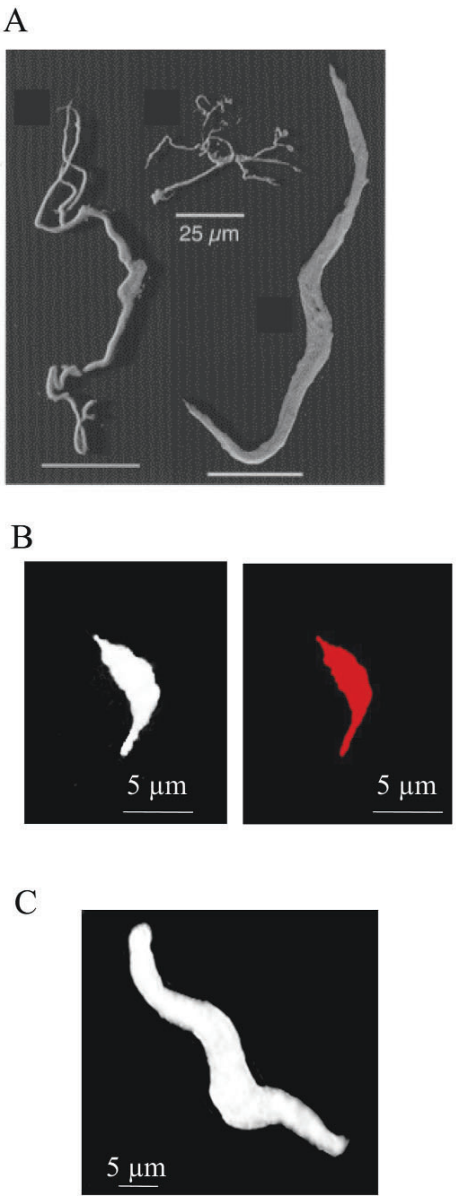


***Figure 4. Isolated interstitial cell/myofibroblasts***
***A: Interstitial cells (ICC - left, middle) and a smooth muscle cell (SMC - right) obtained from rabbit urethra. Images obtained from confocal image stacks incubated with antibodies to anti-vimentin (ICC) or anti-myosin (SMC), modified from [59]. B: Sub-urothelial myofibroblasts obtained from guinea-pig bladder. Dark field image (left) and fluorescent image with a vimentin-antibody stain (see [73] for methods). C: Guinea-pig detrusor smooth muscle myocyte, dark field image for comparison with B.***

Such cells, characterised to various degrees, have been found throughout the lower urinary tract, not only in the bladder [72-75] and urethra [59], but also in prostate tissue [76, 77] and the ureter [78]. With respect to muscle layer cells in the lower urinary tract, those in the bladder and urethral wall have

received recent attention, as it has been postulated by some that they may act as pacemaker cells to drive the smooth muscle layer. This is extrapolated from studies of interstitial cells of Cajal in the G-I tract where they are coupled electrophysiologically to smooth muscle cells, and whose pacemaking function has been carefully characterised [60, 79]. In the lower urinary tract comparable experiments have not been done, so that any pacemaking activity remains to be unequivocally demonstrated.

However, in the urethra, cells have been isolated that show spontaneous electrical activity, that could be modulated by noradrenaline, and mediated by current flow through $Ca^{2+}$-activated $Cl^-$ channels [59]. In view of similarities in interventions that modulate spontaneous electrical activity in multicellular urethral preparations [80] and in these interstitial cells it has been proposed that the latter could perform a pacemaking function.

In the detrusor smooth muscle layer the situation is less clear. Extrapolation of function to the detrusor from tissues such as the G-I tract and urethra may not be justified. The functional activities of the G-I tract and bladder differ, with the former exhibiting continuous peristaltic activity and thus requiring continued co-ordination of activity, whilst the bladder remains more quiescent until voiding is initiated. Several studies have described interstitial-like cells in the smooth muscle layer of animal bladders [72,81, 82]. **Figure 5** shows c-kit positive cells located in the detrusor layer, and furthermore are more abundant in samples from neuropathic bladders. A recent careful study using human tissue from patients with normal or neuropathic bladders however could not distinguish such cells from fibroblasts [83]. Connexin43 labelling - a gap junction protein that is a feature of myofibroblasts and interstitial cells - is not abundant in the detrusor muscle layer of the bladder wall, but is in other places such as the sub-urothelial space [35] : in detrusor the main connexin labelling is from connexin45.

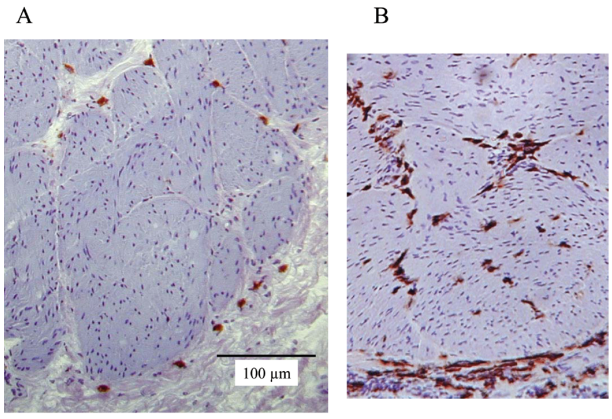


***Figure 5 . c-kit positive staining cells in the detrusor layer A: Human detrusor bundle from a stable bladder, showing c-kit positive cells (brown) around the exterior of a muscle bundle. B: c-kit positive cells in a detrusor bundle from a bladder showing overactivity after spinal cord damage.***

Functionally extrapolation from G-I tract to bladder is also not always possible. In isolated detrusor bundles $Ca^{2+}$ waves propagate throughout the bundle, and their generation and propagation are dependent on voltage-dependent $Ca^{2+}$ channels [82] and disrupted by gap junction blockers such as 18β-glycyrrhetinic acid. This study proposed that interstitial-like cells, present both within and around muscle bundles assist in the propagation of such signals, rather than initiate them. At a cellular level, c-kit positive detrusor cells exhibit spontaneous or agonist (carbachol)-mediated changes of intracellular $Ca^{2+}$ [72]. Spontaneous electrical activity in detrusor and GI-tract shows different sensitivities to modulators of $Ca^{2+}$ channel activity and cellular metabolism [27, 84] and thus has fundamental differences. Thus interstitial-like cells are found throughout the muscle layers of the urinary tract, but their functions as yet remain to be properly evaluated

Sub-urothelial myofibroblasts will be considered in the Neural Control report, where their apposition to the afferent system implies a different role.

## II. TRANSMITTERS AND DETRUSOR FUNCTION

The lower urinary tract receives a rich innervation that utilises several neurotransmitters. Ganglia are also present in the bladder wall, most of which are in parasympathetic pathways that supply smooth muscle, although there could also be an involvement in local reflexes [85, 86].

### 1. Muscarinic systems

#### *a) Post-junctional receptors*

Autonomic nerve fibres form a dense plexus among the detrusor smooth muscle cells, most of which contain acetyl cholinesterase. While they occur in profusion throughout the muscle coat, some muscle bundles are more richly innervated than others, a situation exacerbated in some pathologies [87]. The majority of fibres are excitatory cholinergic [88], and

contraction of the normal stable human bladder is mediated almost exclusively through muscarinic receptor stimulation as it is completely abolished by atropine [13]. There are five distinct genes (m1-m5) for muscarinic receptors, which correspond to five receptor subtypes (M1-M5) [89]. Muscarinic receptors are coupled to G-proteins (**Table 1**), which lead either to phosphoinositide hydrolysis and mobilization of intracellular Ca2+ (M1, M3 or M5), or inhibition of adenylate cyclase activity (M2, M4).

Normal human detrusor and salivary glands possess the muscarinic receptor subtype m3 gene and express M3 protein, although m3 expression in less uniform in detrusor compared to salivary gland. In addition detrusor possesses m2 and abundant M2 expression, but not m1/M1 [90-92]; by contrast salivary glands exhibit m1/M1 but not m2/M2 (**Figure 6**). However, subtype-specific anti-muscarinic drugs may not necessarily be effective, as although M2 receptors predominate in receptor binding studies of the bladder, M3 receptors are thought to mediate contraction. **Figure 7** shows also that the expression of m2 and m3 receptors is variable ; especially in detrusor muscle compared to salivary gland, and will contribute to the variability of response of antimuscarinic receptor antagonists between patients. Moreover, muscarinic receptors desensitise, mediated by receptor phosphorylation by guanosine phosphate binding (G) protein coupled receptor kinase (GRK) [93-95]. GRK2 expression is significantly lower in detrusor from patients with obstructed bladders due to benign prostatic hyperplasia (**Figure 8**) [95] : this may contribute to the over-activity observed in these patients.

In addition to acetylcholine released from peripheral nerve endings, non-neuronal release from the bladder urothelium or suburothelial space has been measured. This release increased with the age of the subject, and is also augmented when the bladder is stretched [96] ; its significance is unclear at present.

***b) Pre-synaptic mechanisms***

There has been less attention focussed on pre-synaptic regulation of neurotransmitter release, However, in several animal species pre-synaptic M1 receptors exert a facilitatory action on acetylcholine release acting, through a phospholipase C - PKC mechanism; whilst M2/M4 receptors may also attenuate release [97, 98]. Moreover, this mechanism may be enhanced in pathological conditions such as spinal cord injury. Muscarinic receptor activity is increased in this condition, and furthermore there is evidence of a sub-type shift from M1 to M3 receptors [99, 100].

## 2. ADRENERGIC SYSTEMS

***a) Adrenergic β-receptors***

During the urine storage phase, using animal models, sympathetic nerve activity increases [101], and both detrusor relaxation via β-receptors and urethral smooth muscle contraction via α1-receptor have been reported. There are three β-receptor subtypes (β1, β2, β3, **Table 2**) and detrusor relaxation is partly mediated via β2-receptors [102, 103].

Originally the β3-receptor was thought only to regulate fat metabolism. More recently, an important role for the β3-receptor in mediating detrusor relaxation has also been suggested. β3 mRNA has been identified in human and animal detrusor [104-107] and indeed represents the great majority of the β-adrenoreceptor mRNA [106]. Several β3-adrenoreceptor agonists [108-112] and antagonists [108, 109, 113] have been synthesised and have permitted demonstration that the β3-adrenoreceptor plays the predominant function role in relaxing detrusor [106, 113]. Experiments with animal models have shown that β3-adrenoreceptor agonists suppress bladder overactivity without affecting residual volume [114], and without significant effects on cardiovascular function [114, 115]. In addition bladder function is affected more than urethral function, showing a selective effect within the lower urinary tract.

***b) Adrenergic α-receptors***

These also have been subclassified into 3 subtypes [116] ($\alpha 1_A$, $\alpha 1_B$, $\alpha 1_D$, **Table 2**), although the nomenclature has been recently reorganised. Urethral and prostatic smooth muscle contraction is mediated mainly by the $\alpha 1_A$ subtype ; in contrast to peripheral artery, which is via the $\alpha 1_B$ receptor [117, 118]. Improvement of lower urinary tract symptoms in patients with BPH by α1-blockade is thought to be due to relaxation of these smooth muscles. However, two novel actions of α1-blockade have been reported. Doxazosin and terazosin induce apoptosis of prostate stroma and epithelium, mediated via the $\alpha 1_D$-receptor [119, 120]. Doxazosin also affects prostate stromal differentiation by altering the myosin heavy chain isoform from SM1 to SM2, and reducing the sensitivity of the cell to noradrenaline [121, 122].

α1-mediated contraction in detrusor is of minor importance. However, in detrusor over-activity due to outlet obstruction or neurogenic causes, a background shift from a predominant β-mediated relaxation, to α-mediated contraction has been suggested

*Table 1. Muscarinic receptor subtypes.*

| Subtype | M1 | M2 | M3 | M4 | M5 |
|---|---|---|---|---|---|
| G-protein | Gq/11 | Gi/o | Gq/11 | Gi/o | Gq/11 |
| Gene /chromosome | CHRM1 /11q12-13 | CHRM2 /7q35-36 | CHRM3 /1q43-44 | CHRM4 /11p12-11.2 | CHRM5 /15q26 |
| Location | brain, glands, sympathetic ganglia | heart, hindbrain, smooth muscle | smooth muscle, glands,brain | basal forebrain, striatum | substantia nigra |

*Table 2 .Classification of adrenergic beta (β)-receptors and alpha1 (α1)-receptors.*

| | | | |
|---|---|---|---|
| β receptor subtypes | β1 | β2 | β3 |
| Coupled G-protein | Gs | Gs | Gs, Gi/o |
| Gene/chromosome | ADRB1/10q24-26 | ADRB2/5q31-32 | ADRB3/8p11-12 |
| Number of amino acids | Human 477 | Human 413 | Human 408 |
| α1 receptor Subtypes | $\alpha 1_A$ | $\alpha 1_B$ | $\alpha 1_D$ |
| Other names | $\alpha 1_{a/c}$ | $\alpha 1_b$ | $\alpha 1_{A/D}$, $\alpha 1_{a/d}$ |
| Coupled G-protein | Gq/11 | Gq/11 | Gq/11 |
| Gene/chromosome | ADR1A/8 | ADR1B/5q33 | ADR1D/20p13 |

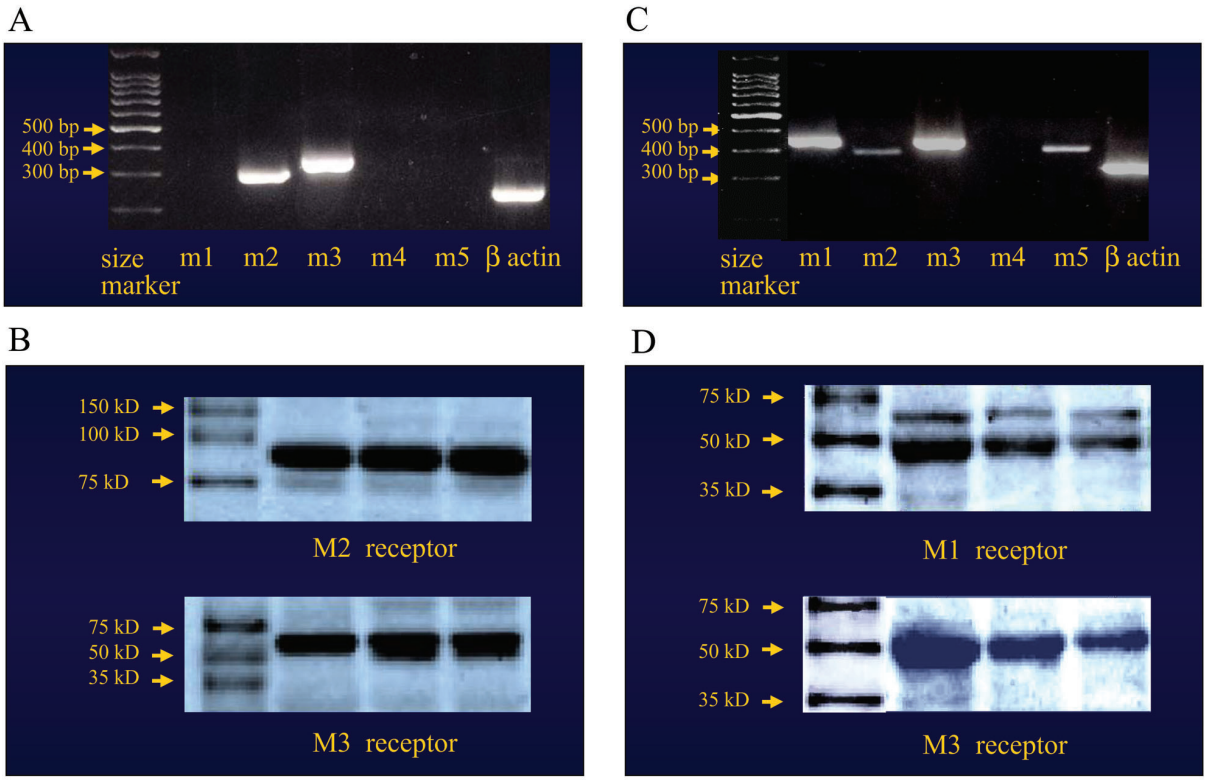


*Figure 6. Electrophoresis of RT-PCR products and Western blots of muscarinic receptor products in human detrusor smooth muscle and salivary glands. A: RT-PCR products of m1-m5 from human detrusor smooth muscle, β-actin is also shown as a control. B: Western blots from four samples of M2 and M3 products. C: RT-PCR products of m1-m5 from human salivary gland, β-actin is also shown as a control. D: Western blots from three samples of M1 and M3 products. In all gels standard weight markers (kbase units parts A & C; kDalton units parts B & D) are shown to the left of the sample traces.*

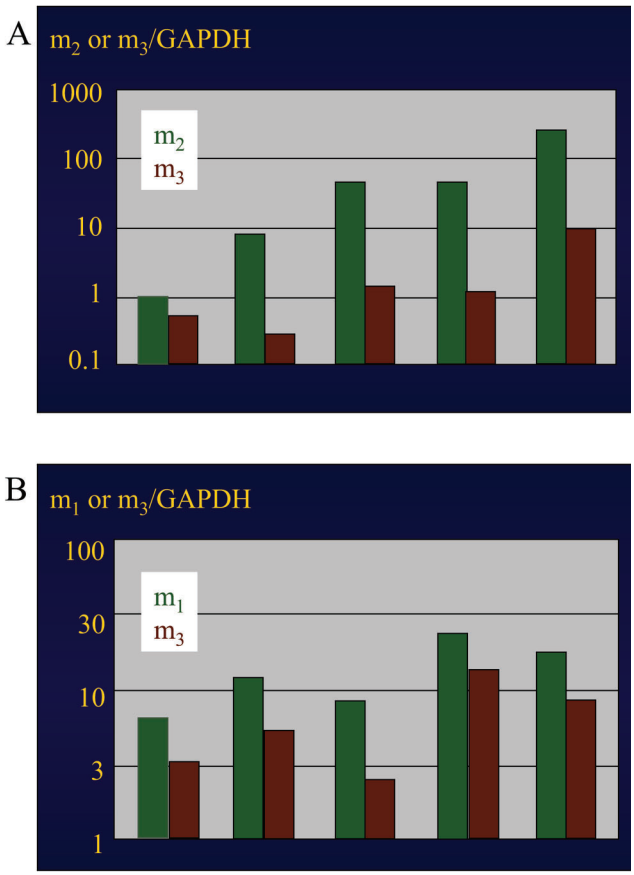


*Figure 7. Quantitative RT-PCR of muscarinic receptor products from human detrusor smooth muscle and salivary glands A: m2 and m3 products from human detrusor smooth muscle using five samples. B: m1 and m3 products from human salivary glands using five samples. In each plot the ordinate has a logarithmic axis. The amounts of muscarnic receptor products are expressed as a ratio compared to a 'housekeeping' gene GAPDH, of reasonably constant expression*

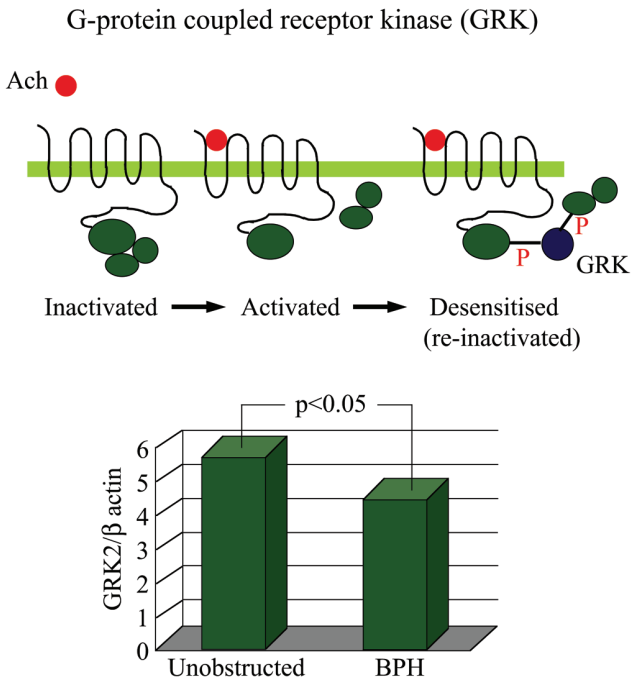


*Figure 8 . Muscarinic receptor desesitisation by G-protein coupled receptor kinase (GRK). A: Left and middle panels show show binding of acetylcholine (Ach) to the receptor and dissociation of G-protein, resulting in activation of intracellular signal transduction. Right panel shows inactivation of dissociated G-protein by phosphorylation through GRK. B: Expression of G-protein coupled receptor kinase (GRK)2 protein in human detrusor smooth muscle by Western blotting in normal (unobstructed, left bar) and obstructed (right bar) bladders ($p<0.05$). (GRK)2 levels are expressed as a proportion of β-actin, that is produced at constant levels in different experimental groups.*

[123, 124]. In normal human detrusor mRNA analyses for $\alpha 1_d$, $\alpha 1_a$, and $\alpha 1_b$ are 66%, 34%, and 0%, respectively [123]. In rat detrusor the distribution is 25%, 70%, and 5%, but this changes in favour of the $\alpha 1_d$ subtype in the obstructed bladder (75%, 23%, and 2% respectively) [124]. Although the function of the $\alpha 1_D$ receptor is unknown, these data may be correlated to the fact that $\alpha 1_D$ receptor inhibition improves lower urinary tract symptoms in patients with BPH, especially those with over-active bladder. The effect of $\alpha 1_D$ receptor on detrusor bladder function may be similar to that on prostatic tissue. There is little evidence for inhibitory nervous pathways to the bladder, however, sympathetic fibres terminate on vesical parasympathetic ganglia and inhibit neurotransmission and symapathetic activity seems to increase compliance [101, 125].

## 3. Purinergic systems

ATP is not a significant excitatory transmitter in normal human bladder, but is probably co-released with acetylcholine. However, in several human pathologies associated with bladder over-activity ATP is an additional functional transmitter [126, 127]. Several P2X purinoceptor subtypes have been demonstrated in the bladder by immunohistochemistry. In the rat, $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_6$ receptors have been identified in the smooth muscle, but only the $P2X_1$ is present on the cell surface membrane [128]. This is in addition to the identification of $P2X_3$ on sensory nerves [128, but see 129], $P2X_3$, $P2X_4$ and $P2X_5$ on urothelial cells [128, 129], and $P2X_6$ receptors on the sub-urothelial basement membrane [130]. Similarly mouse detrusor expresses only the $P2X_1$ receptor on the cell surface membrane [130], with $P2X_2$, and

$P2X_4$ at other sites. Additionally, $P2X_2$, $P2X_4$ and $P2X_7$ receptors were localised on an unidentified cell between the muscle bundles.

Functionally, human detrusor can be contracted via $P2X_1$ receptors [131-133], although there is evidence of inhibitory P2Y receptors in other primate detrusor [134]. It has been suggested in animal detrusor that the two transmitters have separate functions, and that ATP supports the initial rapid phase of contraction, and acetylcholine the more prolonged phase [135,136] - although this is difficult to demonstrate in isolated human preparations. The rapid purinergic contraction reflects rapid activation through ionotropic $P2X_1$ receptors, whilst cholinergic activation involves activation via metabotropic muscarinic receptors. A $P2X_1$ selective knockout mouse showed no apparent difference in bladder morphology or function, and generated contractile responses to carbachol akin to control muscle strips [130] : it was suggested that the dual purinergic and cholinergic systems act as failsafe for bladder contraction although there is no direct evidence for this.

It was stated above that in human detrusor a purinergic component of contraction is specifically associated with several bladder pathologies, such as bladder obstruction and idiopathic detrusor over-activity. Moreover, the purinergic component is especially evident at low stimulation frequencies [137]. However, the appearance of purinergic activity in the unstable bladder was not associated with major differences in the distribution of $P2X_1$ immunoreactivity on the cell membrane [137]. This agrees with functional experiments whereby ATP-mediated intracellular $Ca^{2+}$-transients were identical in cells isolated from normal and over-active human bladders [133].

Thus other reasons for the appearance of purinergic contractions in the over-active bladder must be sought, and include : i) more ATP is released from motor nerves ; ii) ATP is broken down less effectively in the nerve-muscle junction and more is available to activate detrusor. The first possibility has not been tested, yet evidence exists for the second. Firstly, ectonucleotidase activity is reduced in detrusor samples from over-active bladders [138]; secondly, pre-treatment with the non-specific ATPase apyrase reduces the strength of nerve-mediated contractions in detrusor samples from over-active bladders, but not from stable bladders [139] ; thirdly inhibition of the intrinsic ecto-ATPase with the ATP analogue ARL 67156 (6-N,N-diethyl-D-[β,γ]-$Br_2$-methylene-ATP) enhances the response to motor nerve stimulation or exogenously added ATP in guinea-pig and human detrusor [139, 140]. One report has shown a significant positive correlation of purinergic, and negative correlation of cholinergic, neurotransmission with age [141] ; whether this is related to altered ectonucleotidase activity has not been determined.

***a) Detrusor myography***

Activated $P2X_1$ receptors act as non-specific cation channels and hence depolarise the cell, which subsequently opens L-type $Ca^{2+}$ channels initiating $Ca^{2+}$ influx to further raise the intracellular [$Ca^{2+}$] [133]. Although the precise functional role of the purinergic transmission pathway remains to be established, the appearance of additional purinergic activation in the over-active human bladder has a number of consequences :

- a functional ionotropic transmitter in the over-active bladder offers a method to detect altered neurotransmitter activity.
- it may enhance contractile activity in the over-active bladder
- an excitatory transmitter active only in the over-active bladder offers an attractive drug target.

Previous attempts to record bladder electromyograms (emg) [142] have been criticised because the signals may be contaminated by movement artefacts [143] and in stable human bladders no electrical activity would be anticipated on nerve-mediated activation, as this is predominantly cholinergic and per se should be electrically silent. Recently, extracellular electrodes have been developed that will record unequivocally voltages attributable to purinergic activation of detrusor smooth muscle [144, 145]. **Figure 9** shows that the signals are unaffected by atropine, reversibly abolished by α,β-methylene ATP (which desensitises muscle purinergic receptors [146]), and are dependent on extracellular $Ca^{2+}$ - all features expected of an ionotropic purinoceptor-mediated response. Their usefulness as a diagnostic tool to record emg signals in over-active bladders remains to be developed.

***b) P1-receptor feedback control of the nerve-muscle junction***

The nerve-muscle junction is further complicated by the fact that ectonucleotidase activity generates ultimately the P1 receptor agonist, adenosine. Adenosine exerts a negative inotropic effect on nerve-mediated contractions from detrusor, as in other smooth muscles [147-149]. Thus, adenosine provides a nega-

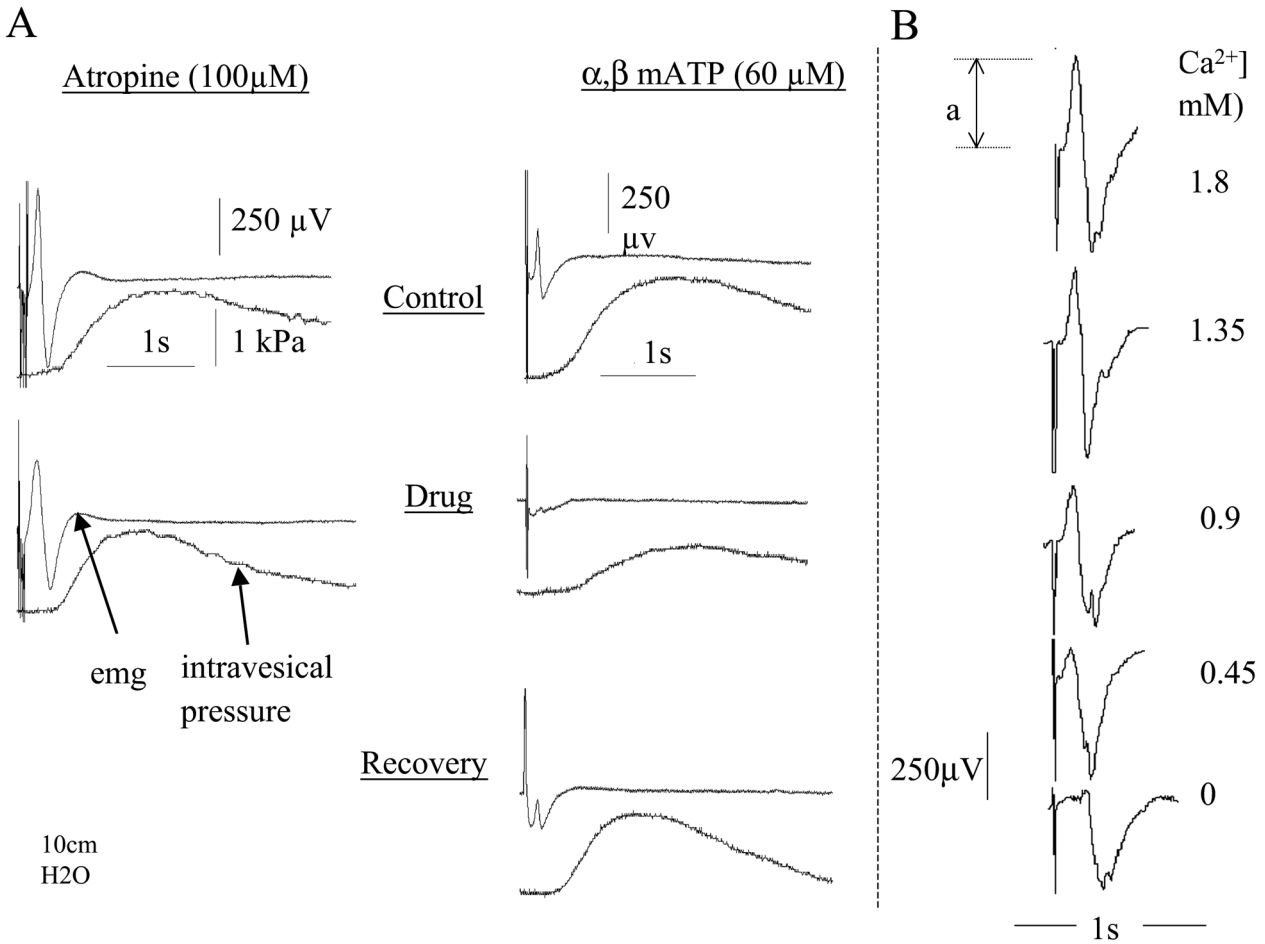


***Figure 9. Electromyograms recorded from isolated guinea-pig bladders Simultaneous measurements of electromyographic signals (emg) and intravesical pressure. A: pairs of recordings in control conditions (top), in the presence of 100 µM atropine or 60 µM α,β mATP (α,β methylene ATP, middle), and after removal of the intervention in the case of α,β mATP (bottom). The emg signal was unaffected by atropine, but was reversibly attenuated by α,β mATP. B: the effect of reducing the extracellular [Ca] on the emg response. The Ca-dependent signal is here denoted by the amplitude of the signal 'a'. Adapted from [24]***

tive-feedback control over the muscle contraction. Several P1 receptor sub-types exist [150]. Adenosine has an action on the motor nerve via the A1 subtype, as the specific agonist N6-cyclopentyladenosine mimics its action However, adenosine also exerts a direct action on the detrusor myocyte by a A2 receptor action [149]. A scheme of excitation-contraction coupling in detrusor to illustrate in particular the features concerned with purinergic signalling is shown in **Figure 10.**

## 4. Other systems

### *a) Nitric oxide*

Nerves staining positively for nitric oxide synthase have been localised to the bladder wall and it is presumed that nitric oxide may be released [75]. Controlled release of NO has been shown to relax detrusor muscle [151], although this is not a ubiquitous finding [152]. In pre-contracted detrusor preparations, electrical stimulation generates phasic relaxations which themselves are attenuated by ODQ (α blocker of adenylate cyclase) or inhibitors of NO production such L-NAME [153]. In abnormal conditions, such as with obstruction and spinal cord injury NO production appears to be raised [154, 155]. However, knockout mice that lack neuronal NOS have normal lower urinary tract function [156], and those with no inducible NOS are not particularly abnormal.

### *b) Bioactive peptides*

Less is known about the action of peptides such as vasointestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and neuropeptide Y. Neurones that stain positively to these molecules have been detected in the bladder wall, although there is discussion if they supply the detrusor layer or are located more

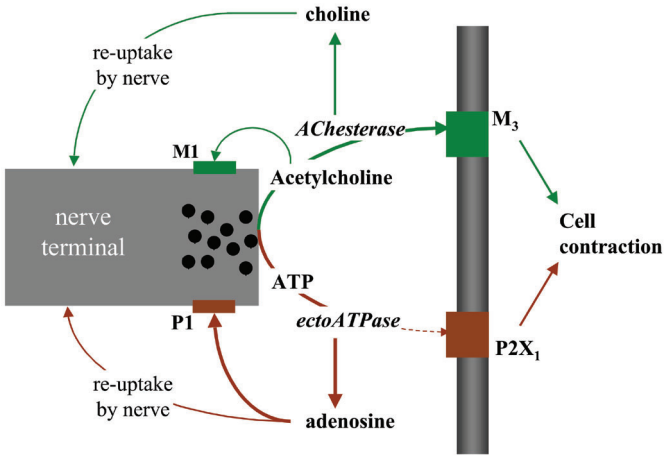


***Figure 10. Schematic diagram of the events occurring at the nerve-muscle interface in detrusor smooth muscle. The top half shows the release of acetylcholine, and its subsequent removal by acetylcholinesterase. The bottom half shows the release of ATP and its subsequent removal by ectonucleotidase (ectoATPase). ATP is ultimately degraded to adenosine that can act on pre-junctional sites via a P1 receptor, probably the A1 subtype. ATP acts on the muscle cells via P2X1 receptor. The dotted line after ectoATPase has acted on ATP represents the small amount (is any) of ATP that reaches the muscle cell membrane, especially in the stable bladder.***

in the sub-urothelial space [85, 157-159]. Moreover their distribution is altered in pathological conditions, such as spinal cord injury [160]. VIP attenuates carbachol-induced contractions in human tissue, probably by elevating cAMP and cGMP levels, although its effect on detrusor from other species is variable [161] : CGRP exerts no significant action [159]. Additionally, it has been proposed that VIP has an antioxidant effect and can protect nerves in the detrusor layer from damage induced by low $PO_2$ or low substrate concentrations [162]. With respect to neuropeptide Y ; in human detrusor, receptors were not localised, and moreover the peptide was unable to exert a pre- or post-synaptic effect on contractile function - in contrast to actions on animal tissue [163, 164].

### *c) Serotonin (5-HT) receptors*

The main action of serotonin (5-HT) is within the central nervous system, where the 5-$HT_{1A}$ receptor appears to be involved in the regulation of micturition [165]. However, there is evidence also for a peripheral action, with most evidence suggesting a pre-synaptic site on parasympathetic nerves to increase the action of acetylcholine. In pig and human detrusor preparations, [166] this effect is mediated via of 5-$HT_4$ receptors, whilst in rabbit detrusor 5-$HT_3$ receptors fulfil this role [167]. There is also evidence that this pre-synaptic action is attenuated in human tissue from neurogenic bladders, although agonist affinity was unchanged [168].

### *d) Tachykinins and neurokinin (NK) receptors*

Bladder distension is reported to exert some contractile responses on the detrusor mediated by axonal reflexes: these sensory-motor functions of the afferent collaterals appear to involve the release of tachykinins, such as substance P and neurokinin A, from the afferent endings in muscle. Tachykinins cause a relaxation of detrusor tone and can be blocked by specific NK-2 antagonists [170].

## III. BIOMECHANICAL PROPERTIES OF MUSCLE

The contractile characteristics of muscles depend not just on the ability of individual myocytes to generate force but also on the orientation of muscle cells and fibres within the tissue mass, as well as the mechanical properties of the extracellular matrix. A consideration of the biomechanical properties of muscular tissues is therefore important to have a full understanding of how muscle cell activity is translated to changes to wall tension of a muscular organ.

### 1. Passive mechanical properties of the intact bladder

### *a) Compliance - normal bladder*

It is important to differentiate between compliance of the whole bladder, and compliance of the bladder wall material. The relationship between compliance of whole bladder and bladder wall material is not simple. Compliance of a whole bladder can actually be increased in bladders with decreased compliance of the bladder wall material, if the bladder becomes larger [171]. Most of what is found in the literature is, unfortunately, on whole bladder compliance only. Bladder compliance (C) describes the ratio of the change in bladder volume ($\Delta V$) for an increment of detrusor pressure ($\Delta Pdet$), during bladder filling, generally expressed in units of ml.cm $H_2O^{-1}$ [172]. The normal bladder is a very compliant organ such that a large increase in volume is accompanied by only small changes in pressure. If the volume rise is rapid, there will be a large initial increase of pressure, which will then decline in a time-dependent man-

ner to a new steady-state. This visco-elastic phenomenon is also known as stress-relaxation [173]. It is important when calculating compliance that the pressure value used is that at steady-state [174]. Moreover the measurement of visco-elastic properties depends on preceding perturbations and this may account for some of the variability observed in the literature [175]. In vitro contractile studies of detrusor smooth muscle support this observation that bladder tone is dependent on the visco-elastic properties of the bladder wall and not on neural input [176]. In animal models, as will be discussed below, during in utero bladder development compliance increases and in contrast it declines with age in the adult bladder [177, 178].

***b) Compliance - obstructed bladder***

There are several reproducible animal models of bladder outflow obstruction (BOO) [179-181]. Filling cystometry of relaxed obstructed rat bladders [182] showed an increased compliance as defined above. The compliance of the bladder wall material was, however, decreased. Filling cystometry of whole obstructed foetal bovine bladders also demonstrates increased bladder compliance and partial stress relaxation [183]. In vitro contractility studies on detrusor strips obtained from the same obstructed bladders showed that they generated less force than the control group on nerve-mediated stimulation. This was attributed to a reduction in tissue elasticity and not altered myogenc function per se [184]. Studies with whole rabbit bladders also showed that obstruction diminished the capacity for stress relaxation [185]. The relationship between compliance and collagen content or type is considered below. Recent work has been directed to examining the cellular basis of compliance changes during obstruction. One interesting approach has been to apply cyclical stretch and relaxation to detrusor myocytes [186].

## 2. Properties of passive muscle

Bladder, urethra and pelvic floor tissue have, as with all biological tissues, an exponential stress-strain relation. The passive mechanical properties depend on the visco-elastic properties of the muscle and the surrounding stroma [174, 176, 187, 188]. Tissue collagen and elastin are generally thought to be intimately related to tissue compliance. Many collagen isoforms have been described, and with respect to passive mechanical properties types I and III are the most important [189]. Both isoforms are arranged in banded fibrils but have different mechanical properties. An increased type III:I ratio is found in non-compliant adult bladder tissue (see below).

## 3. Passive mechanical properties of urinary bladder - collagen subtypes

The relaxed normal bladder has a high compliance during filling at physiological filling rates. This may be coupled to the ability of the collagen III fibrils to rearrange in conformation and orientation during filling [190]. The synthesis of collagen by cells in the bladder wall seems to be dependent on their stretch, but independent of nervous input [177]. During development there is a correlation between the collagen III:I ratio of the bladder and its compliance. In the foetal bovine bladder the increase in compliance during the second and third trimester parallels a decreased ratio of collagen III:I. On the other hand, the adult bovine bladder has a decreased compliance and an increased III:I ratio [177].

In non-compliant human bladder there is increased expression of type III collagen mRNA. In addition there is greater deposition of type III collagen [191] mainly between detrusor muscle bundles [192], a fact which explains the increased type III:I ratio [193]. In similar bladders decreased elastin and elastin gene expression also occurs [194]. Obstructed rat bladder has both increased total collagen content [195] and raised expression of both types I and III collagen [196], which can explain the decreased compliance of the bladder wall material [182]. Other pathological states have also reported changes of compliance. An increase of compliance was measured in the severely obstructed foetal sheep (**Figure 11**), and correlated with a greater deposition of extracellular collagen, although the subtype was not determined [184, 197].

## 4. Passive mechanical properties of urethra and pelvic floor tissue

The relation between urinary continence and compliance of the pelvic floor tissue is complex. Decreased para-urethral or pubocervical fascial collagen concentration occurs in stress incontinent women [198, 199]. By contrast, paraurethral tissue from premenopausal stress incontinent women had a 30% higher collagen concentration with a similar increase of the diameter of collagen fibrils [200]. In premenopausal stress-incontinent women the collagen III:I ratio was increased and collagen cross-linking was decreased [201]. With post-menopausal stress-incontinent women data are less consistent. Stress-inconti-

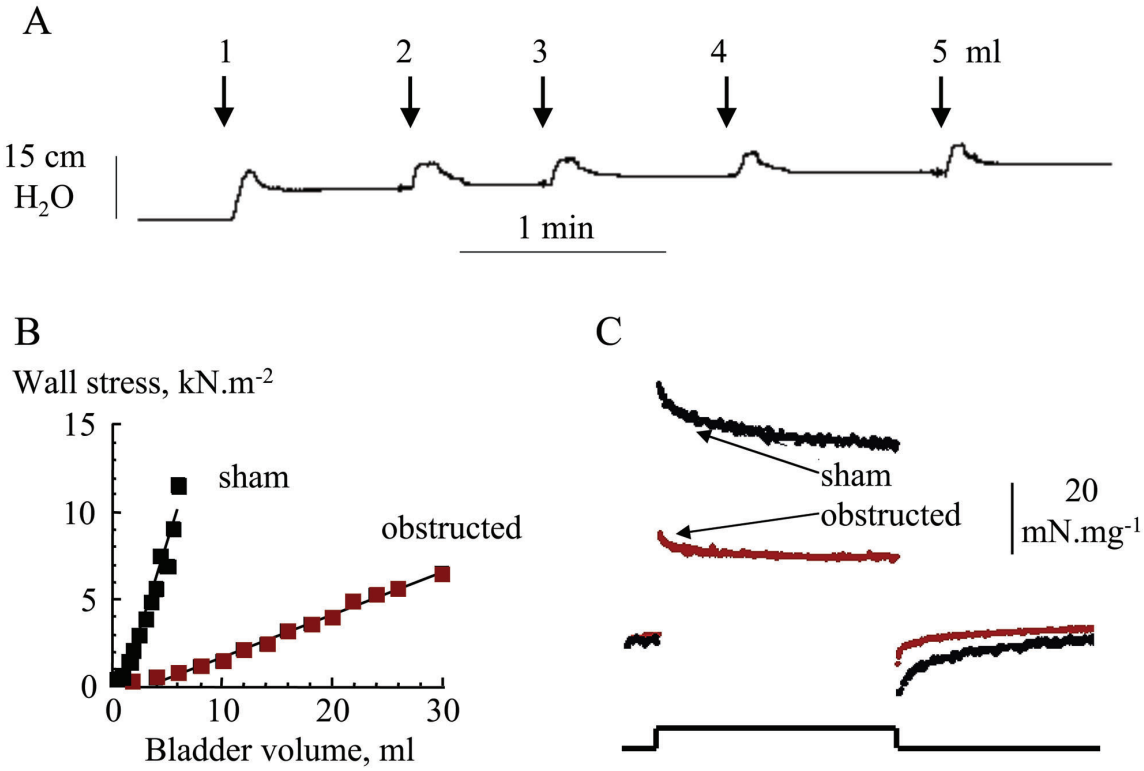


***Figure 11. Pressure-volume and stress-strain relationships in normal and obstructed fetal sheep bladders.***
***A: Intravesical pressure in an obstructed fetal sheep bladder during step increments of volume. B: calculated stress-strain relationships from sham-operated and obstructed fetal sheep bladders from pressure-volume relationships as shown in part A, using Laplace's Law. C: Stress-strain data for isolated strips from sham-operated and obstructed fetal sheep bladders. Strips (resting length 5 mm) were stretched by 1 mm and isometric tension changes recorded (see [184] for details).***

nent women showed either no difference [202], or significantly reduced collagen type I and III content [203], in paraurethral collagen concentration compared to control groups. In rabbit urethra, compliance increases during pregnancy [204], but it is not known whether this is linked to alterations in collagen.

## 5. Mechanical properties of contracting muscle

The role of the activated contractile machinery is to produce force and/or shortening, and the contractile machinery of the urogenital organs is similar to that of other smooth muscles [1]. There are two types of filaments interacting during contraction. Thin filaments containing mainly the globular protein actin (which exists in three isoforms, α, β, and γ) are anchored to dense bodies, which contain alpha-actinin. Dense bands in turn are associated with the dense bodies and are attached to the inner surface of the cell membrane. The major component of the thick filaments is myosin, and in smooth muscle consists of two isoforms, SM1 and SM2. The cells also contain intermediate filaments (of intermediate diameter between thin and thick filaments). In mature smooth muscle the intermediate filaments mainly contain desmin, but in developing smooth muscle vimentin is dominant. The intermediate filaments do not participate in contraction but constitute a cytoskeleton [205].

Each myosin molecule contains two intertwined polypeptide chains. The free N-terminal regions are arranged as globular myosin heads [206]. These heads or cross-bridges can, upon activation, attach to sites on the thin filament when a translatory movement is performed to generate force. The attachment is then broken and the myosin head is tilted back to its original position by energy released from hydrolysis of one molecule of ATP. Repeated cross-bridge cycles produce a sustained contraction and the force output depends on the number of active cross-bridges. When the load is lower than the isometric condition, thick and thin filaments slide past each other and the muscle shortens. The lower the load, the higher the shortening velocity. Maximum shortening velocity against zero load is proportional to maximum crossbridge turnover rate, and myosin ATPase activity.

$Ca^{2+}$ activates the contractile machinery through two different mechanisms [206]. The myosin molecule contains a 20 kDa regulatory light chain situated in the head region, which is phosphorylated by a kinase in the presence of a $Ca^{2+}$-calmodulin complex, to activate the myosin head. In addition, the inhibitory action of caldesmon (a thin filament-associated protein) on actin-myosin interaction is reversed by $Ca^{2+}$.

There is a characteristic relationship between cell length and force production. Active force is dependent on the overlap between thin and thick filaments as in other muscles, but smooth muscle generates active force over a far wider range of muscle lengths than striated muscle. This reflects the need of smooth muscle to be able to generate force, despite large variations in the degree of passive stretch. One reason for this flexibility might be that the thick filaments can successively interact with a number of thin filaments during cell shortening.

The relation between shortening velocity, v, and the force against which a muscle is contracting F, is hyperbolic (**Figure 12**), and can be approximated by the Hill equation [207]:

$$v = \frac{b(F_0 - F)}{(F + a)}$$

where Fo is the isometric force and a and b are constants. In clinical urodynamics, this equation is transformed to related equations describing the hyperbolic relationship between detrusor pressure and flow rate (the so called urethral resistance relation).

Maximum shortening velocity ($v_{max}$) varies between smooth muscles : detrusor muscle is a fast smooth muscle with a $v_{max}$ of 0.2-0.3 muscle lengths per second [208]. In addition to the 20 kDa light chains, the myosin heads also contain 17 kDa light chains (LC17). These exist in one acidic (LC17a) and one basic (LC17b) isoform. Muscles with a low relative amount of LC17b have a high $v_{max}$ [209]. Another factor that influences vmax is the relative number of myosin heads that have a 7 amino acid insert [206, 208]. The higher the relative number of myosin heads with insert, the higher the $v_{max}$.

## 6. Mechanical properties of contracting detrusor muscle

**Figure 13** shows a length-tension relation for normal rabbit detrusor muscle [210] and is similar to that for normal human detrusor [211]. This shows that despite huge differences in bladder mass and capacity (with the consequent variability of wall tension according to Laplace's Law) the force generating ability of individual smooth muscle cells is similar. The active length-tension relation of the bladder wall can however be influenced by pathophysiological conditions. Infravesical obstruction [212], or hyperdiuresis secondary to diabetes mellitus induce a rightward shift of the bladder circumference-force curve that might contribute to retention of urine.

Muscle from abnormal bladders show a number of mechanical changes. Hypertrophic rat detrusor has a lower maximal active force per unit cross-sectional area [213], which could be due to a decreased myo-

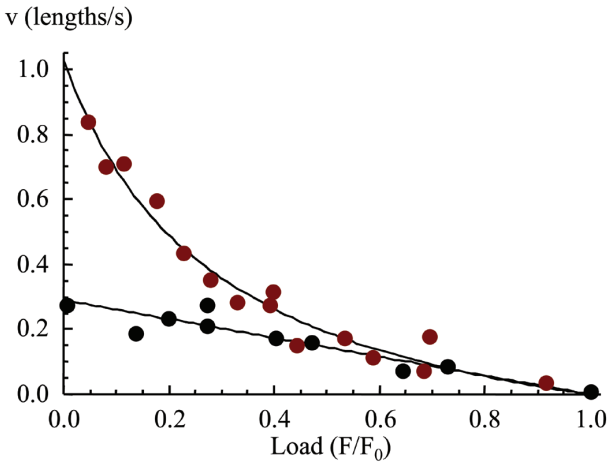


*Figure 12. Force-velocity relationships on urethral smooth muscle. Mean force-velocity relationships of circular (black) and longitudinal (brown) rabbit urethral smooth muscle strips. The shortening velocity, v, is plotted as muscle lengths per second as a function of a load, F, as a proportion of maximal load, F0. The lines are fits of equation 1 through the data points. Modified from [218].*

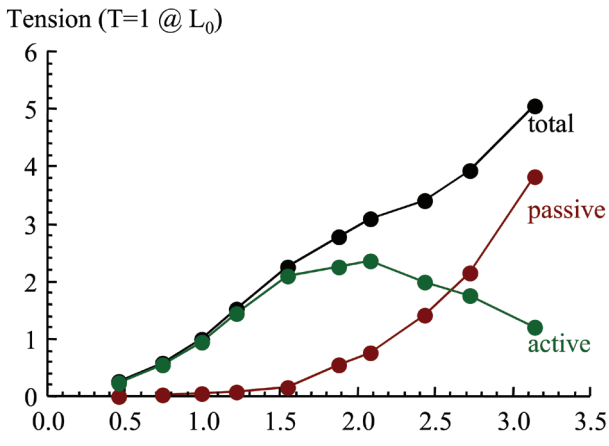


*Figure 13. Length-tension relationships of detrusor smooth muscle Mean isometric length-tension relationships of rabbit detrusor smooth muscle. Curves for the active and passive components, as well as the total force (active+passive force) are shown. The length of the strip is a proportion of that of a strip isolated when the bladder had a volume of 10 ml, L0. Tension is expressed as a proportion of active tension at L0. Modified from [210].*

sin concentration [209]. However, rat detrusor from obstructed bladders shows a relative increase in SM1, and muscle from obstructed human bladders has a similar SM1/SM2 ratios compared to that from normal bladders [214, 215]. In hypertrophic rat detrusor from obstructed bladders a lowered $v_{max}$ is measured compared to normal bladders as well as a decreased rate of force development and relaxation [208, 211]. These changes have been postulated to be due to a decreased relative amount of myosin heads with the 7-amino acid insert (above) and a relative increase in LC17b : both changes would decrease $v_{max}$. A role for the Rho kinase system has also been suggested to influence detrusor relaxation [216], but requires further study.

### 7. Mechanical properties of contracting urethral muscle

Less is known about urethral muscle mechanics. The urethra and periurethral tissue, including the prostate capsule, contain fast and slow striated fibres [37, 48, 217] intermingled with smooth muscle. Circular and longitudinal smooth muscles are present; the former would maintain urethral tone during bladder filling and the latter funnel the proximal urethra during bladder emptying. Circular smooth muscle in the rabbit urethra has a much lower vmax than its longitudinal counterpart (Figure 12) [218]. In smooth muscle from other sources, the molecular structure of the light chain is different in phasic and tonic types [219], although this is not known for urethral tissue.

## IV. THE UROTHELIUM

The urothelium permits the bladder to store urine without permitting significant fluxes of solutes or water that might affect underlying tissues, such as detrusor, or indeed alter the composition of other extracellular fluid compartments. In addition it has been more recently proposed that the urothelium has a sensory function by initiating a chemically-mediated process that senses bladder volume.

### 1. Structure of the urothelium

The urothelium is a transitional epithelium [220] and in humans has a baseline turnover rate of about 150 days. If damaged the turnover rate is dramatically increased to re-establish urothelial barrier function as rapidly as possible. In the collapsed state the urothelium looks to be composed of up to seven cell layers, whereas in the distended state it is thinned to an appearance of three cell layers. This is reflected in the presence of three distinct cell populations: a basal layer (cell diameter 5-10 $\mu$m) ; an intermediate layer (about 20 $\mu$m diameter) and a superficial layer of polygonal cells (often called umbrella cells) with a range of "diameters" from 50 to 150 $\mu$m, depending on the degree of stretch. The increase of cell diameter is accompanied by an increase of nuclear dimensions. Normally germinal cells in the basal layer divide, about 3-4 cells fuse to form the intermediate layer and about 6-7 of these intermediate cells fuse to form the surface umbrella cells [221]. The most striking cells are those in the superficial layer. In addition to their size and large multiple nuclei, they have two other features:

a) 70 to 90% of the apical surface area is occupied by polygonal-shaped protein plaques, about 0.5 $\mu$m in diameter and 12 nm thick. The remaining area is lipid membrane, about 8 nm thick, that separates and surrounds each plaque, and is called the hinge area. Each plaque contains about 1000 subunits with a centre-to-centre spacing of 16 nm. Each subunit has six-fold symmetry and is composed of an inner ring of six large, and an outer ring of six smaller particles [222]. The subunits are formed of five proteins collectively called uroplakins. Two have four transmembrane domains (UPIa and UPIb), with a molecular mass of 27 and 28 kDa respectively. The others are type 1 transmembrane proteins (UPII and UPIIIa & UPIIIb) of molecular mass 15 and 47 kDa respectively. UPIa associates with UPII and UPIb associates with UPIIIa or UPIIIb.

b) the cytoplasm of the surface cells has many fusiform or discoidal vesicles, formed by two apposing plaques joined by hinge membrane. The vesicles and the surface membrane plaques are joined together by a dense network of cytoplasmic filaments that attach to tight junctions at the apical/lateral membrane interface, and desmosomes in the basolateral membrane [223-225]. The function of these vesicles in modulating surface area during bladder filling is addressed below.

Surface umbrella cells are joined by tight junctions, which are composed of five to six bands of interconnecting strands. Solute movement across the urothelium can follow either a transcellular (through the cell) or paracellular (through the tight junction and the lateral intercellular space in the three cell layers) pathway. Impedance analysis suggests that surface

cells are not strongly coupled to the lower cell layers by gap junctions, and other studies [226, 227] showed that intermediate and basal cell layers do not offer a significant barrier to the flow of substances between urine and blood. Thus the major barrier that impedes the movement of substances from urine to plasma is the parallel combination of the tight junction and the surface umbrella cells. Both pathways have very low permeabilities to electrolytes and non-electrolytes.

## 2. Physiological Function - barrier functions

The bladder must have at least four properties to enable it to store urine, and maintain its composition similar to that elaborated by the kidney : it should be impermeable to urinary constituents ; possess an active transport system ; maintain a minimum surface area to volume ratio; and be inert to urinary constituents.

## 3. Permeability

The permeability of the urothelium has been assessed for several substances, and it is very impermeable to the major ions in urine ($Na^+$, $K^+$ and $Cl^-$) [228-230]. The transepithelial resistance is a measure of how well the urothelium impedes the movement of ions, and is as large as 87 $k\Omega cm^2$ when bathed in physiological saline, or about 17,000 times greater than the proximal tubule membrane. Since the urothelium is a layer of cells in parallel with tight junctions, the resistance of either of these two pathways must be greater than 87 $k\Omega cm^2$. Tight junction resistance has been estimated to be >150 $k\Omega cm^2$, and that of the cellular pathway ranges between 6 and 150 $k\Omega cm^2$, depending on whether $Na^+$ transport is high or low. In turn, cellular resistance is the sum of the apical and the basolateral membrane resistance, most of which is located at the apical membrane (4 to 150 $k\Omega cm^2$). Thus resistance measurements show that the urothelium has a low but finite permeability to ions. Will this finite permeability significantly change the composition of the urine during bladder storage? **Table 3** shows that there are minimal changes in urine volume, $Na^+$ and $Cl^-$ content, but a significant decrease of urea. For the conditions described in the legend, some 36% of the excreted urea will be recycled in 24 hours. Other measurements suggest that $K^+$ has a similar flux to $Na^+$ and $Cl^-$.

Parsons and colleagues have proposed that a glycosaminoglycan (GAG) layer, on the apical membrane, represents a major permeability barrier to small electrolytes and non-electrolytes [231]. However, there are several observations that would refute this : a) there is no significant electrical resistance between the bathing solution and the surface of the apical membrane ; b) nystatin (an antibiotic which increases selectively the permeability of surface cell membranes) rapidly and extensively decreases transepithelial resistance ; c) the $Na^+$ channel blocker amiloride rapidly diffuses through the GAG layer and blocks membrane $Na^+$ channels ; d) alteration of the luminal [$Na^+$] rapidly alters membrane transport; e) enzymatic cleavage of the GAG does not alter ion transepithelial permeability [232]. These observations do not preclude the possibility that the GAG layer is a permeability barrier for large hydrophilic or hydrophobic molecules or micro-organisms.

A proposed function of the asymmetric unit membrane and the uroplakins is to decrease the permeability of the apical membrane to solutes and water. This hypothesis was tested using a UPIII knockout mouse [233]. The apical membrane of KO mice lacked plaques and possessed microvilli instead of ridges, in cross section the cellular vesicles were described as discoidal not fusiform, and the surface cells

***Table 3. Permability of the urothelium to solutes.***
***Permeabilities were determined from isotopic flux measurements of in vitro bladder. Fluxes were calculated using the flux equation, a transepithelial gradient of 500 mM for urea (urine to blood), 900 mOsm-kg$^{-1}$ for water (urine to blood) and 100 mM for Na and Cl (this gradient could be in either direction), a bladder volume of 100 ml and a surface area of 104 cm$^2$. Change is the percent change in bladder composition of the substance over a one-hour period, a positive change means that the bladder gained the substance, negative means the bladder lost substance and a ± means either a gain or loss of substance depending on the direction of the gradient. Some data from [230]***

| Substance | Permeability | Flux | % Change/hr |
|---|---|---|---|
| Urea | $4.5x10^{-6}$ cm.s$^{-1}$ | 0.7 mmol.hr$^{-1}$ | - 1.5% |
| Water | $5.15x10^{-5}$ cm.s$^{-1}$ | 0.3 ml.hr$^{-1}$ | + 0.3% |
| Sodium | $1.1x10^{-8}$ cm.s$^{-1}$ | 0.4 $\mu$mol.hr$^{-1}$ | ± 0.0008% |
| Chloride | $1.8x10^{-8}$ cm.s$^{-1}$ | 0.7 $\mu$mol.hr$^{-1}$ | ± 0.013% |

were smaller. Although transepithelial resistance, rate of ion transport, and urea fluxes where not different between bladders from KO mice and control, water fluxes were two-fold greater in KO mice bladder compared to control. Of interest is that the water flux of mouse bladder is at least three times lower compared to other mammals (rabbit, cat, rat, and guinea pigs), whilst the urea flux is similar among these species. Since this difference in water permeability cannot be accounted for by UPIII (all of the bladders contain UPIII) then it must also be determined by the lipid composition of the membrane. Thus the structure or lipid composition of the membrane that determines water permeability does not seem to be the same as those that determine urea permeability.

## 4. Transport properties

Mammalian urothelium actively absorbs $Na^+$, and with similar NaCl concentrations on either side of the urothelium a spontaneous transepithelial potential of -20 to -120 mV is established (lumen negative), with a resistance ranging from 85 to 6 $k\Omega cm^2$ and a short-circuit current between 1 to 20 $\mu A.cm^{-2}$ [229, 230, 234]. $Na^+$ was solely responsible for the transepithelial potential [230], with other ions moving passively. Active $Na^+$ transport was rapidly blocked by the $Na^+$ channel blocker amiloride, applied luminally, and more slowly by serosal addition of ouabain to block the Na-pump. The rate of transepithelial $Na^+$ transport was directly dependent on the permeability of the apical membrane to $Na^+$; amiloride decreased and aldosterone increased $Na^+$ permeability. Although ouabain inhibits $Na^+$ exit across the basolateral membrane, it also decreases transepithelial transport by indirectly decreasing apical membrane $Na^+$ permeability [228], and was proposed as a mechanism by which epithelial cells could match the rate of $Na^+$ entry with exit.

A model for active $Na^+$ transport by the urothelium has been proposed using these and other similar observations. $Na^+$ crosses the apical membrane through $Na^+$ channels down a net electrochemical gradient of 130 mV (transmembrane potential -55 mV, extracellular:intracellular $Na^+$ gradient 120:7 mM) [235]. Immunolocalization studies have confirmed the presence of $Na^+$ channels in the luminal membrane of mammalian urothelium as well as in cytoplasmic vesicles [236] with an individual conductance of 9 pS [237, 238]. Intracellular $Na^+$ ions exit across the basolateral membrane via the Na/K ATPase (3 $Na^+$ in exchange for 2$K^+$) so that pump turnover raises cell $K^+$ activity to 70-90 mM [239, 240]. These $K^+$ ions exit this membrane via $K^+$ channels [241] and ensure that the basolateral membrane voltage is -55 mV with respect to the serosal solution. In addition the basolateral membrane is permeable to $Cl^-$ and has a small but finite permeability to $Na^+$.

Other ion channels have been described, although their function is less clear. In addition to the amiloride-sensitive $Na^+$ channel, the apical membrane contains three other channels : a) a non-selective cation channel, insensitive to amiloride [238] ; b) a separate non-selective channel that is unstable in the cell membrane (these two are degradation products of the amiloride-sensitive channel) ; c) a stretch-activated channel [242], that is permeable to $Na^+$ and $K^+$, blocked by high amiloride (>100 $\mu$M), $Ba^{2+}$, $Gd^{3+}$ and TEA and has been proposed to be a route for $K^+$ secretion. The basolateral membrane contains, in addition to the Na/K ATPase and $K^+$ channel, a large (64 pS) $Cl^-$ conductance [243] that is responsible for the passive distribution of $Cl^-$ under physiological conditions [240]. This channel is also permeable to $HCO_3^-$, and less so to $Na^+$, the latter can account for the $Na^+$ permeability of the basolateral membrane.

These cells also regulate their volume during osmotic stress [244]. A raised serosal solution osmolality causes rapid cell shrinkage, a decrease of basolateral membrane $K^+$ and $Cl^-$ permeability and activation of $Na^+/H^+$ and $Cl^-/HCO_3^-$ exchangers. The net effect, in concert with Na/K ATPase activity, increases cell [$K^+$] and [$Cl^-$], and hence increases water flux to restore cell volume. Water movement across the basolateral membrane is facilitated by aquaporin 2 and 3 that has been localized to the basolateral membrane of the surface cells, and membranes of the intermediate and lower cell layers [245]. The basolateral membrane of superficial cells and the membrane of the lower cell layers also contain a urea transporter (UT-B) [246, 247], whose possible function may be to reduce the accumulation of urea, of luminal origin, in the cell.

A recurring question is whether the above transport systems can alter urine composition. Based on a bladder area of 104 $cm^2$, a urine [Na] of 10 mM and volume of 100 ml, after 8 hours of storage the [Na] would decrease from 10 mM to 7 mM at a high $Na^+$ transport rate of 10 $\mu A/cm^2$. However, it has to remembered that for every $Na^+$ transported, either a $Cl^-$ is also absorbed or a $K^+$ secreted to maintain electroneutrality. Based on known $Cl^-$ and $K^+$ permeabilities it may be concluded that $K^+$ is the predominant

counterion, so that little net osmolality change would occur [238, 239]. A recent study showed that urine composition changes as it passes down the ureters [248]. Renal pelvis pH, osmolality, Na and K were all lower than that in the bladder. pH, osmolality and Na increased toward plasma levels, whilst K (which was above plasma levels) increased further, suggesting at least $K^+$ secretion in the ureters. Non-electrolytes such as urea were not measured, nor was the anion composition. This observation suggests that the ureter can alter urine composition and that the transport properties of the ureters might be different than bladder. **Figure 14** summarises the transport functions of the apical cell layer.

## 5. Storage properties

To minimize the flux of substances between urine and blood, the ratio of urine volume to surface area should be minimised. This may be accomplished by the addition of cytoplasmic vesicles into the apical membrane during bladder filling and their removal during contraction [249, 250]. This was tested by measuring surface area when the bladder was stretched. Initially stretch was accommodated by an unfolding of apical membrane, while further stretch required an insertion of cytoplasmic vesicles [228]. This was confirmed by morphometric analysis: the area of apical membrane to vesicles is about 1:3 [225, 251]. Vesicular movement was subsequently shown to require an intact microfilament system [251]. There is no change in the basolateral membrane area of the surface cells [252] ; unknown is whether the length of the tight junctions change. During micturition, the bladder collapses and vesicles are returned to the cell cytoplasm awaiting another round of filling and voiding. Recent evidence has questioned this simple model of vesicular recycling. Even though there is an increase in apical membrane surface during stretch, there is a rapid cycling of this membrane despite maintained stretch [252]. It was speculated that this rapid cycling might be to fine-tune the increase in surface area, and to remove old membrane.

Stretch also increases protein secretion into the urine, raises cell cAMP levels [252] and stimulates ATP release into the blood side [253]. The cellular basis of vesicle fusion has been recently reviewed [254]. In brief, stretch releases ATP [252] into the serosal compartment, which binds to purinergic (P2X) receptors on the basolateral membrane of the surface cells. This stimulates an increase of the intracellular $[Ca^{2+}]$, and in turn stimulates vesicle fusion. An increase of intracellular $[Ca^{2+}]$ (and a release of NO) is also induced by capsaicin and blocked by the vanilloid receptor (TRPV1) antagonist capsazepine. Some TRP ion channels are polyfunctional proteins, containing both an ion channel and an enzymatic domain. The thermo-TRPs, a subset of TRP ion channels, are activated by distinct physiological temperatures, and are involved in converting thermal information into afferent activity [255]. TRPM8 is a novel TRP ion channel family, and is activated by menthol and cool temperature ; its mRNA is expressed in the urothelium [256]. An interesting observation [257] is that TRPV1-knockout mice do not release ATP in response to stretch, and do not increase membrane surface area in response to stretch. The role of the vanilloid receptor in ATP release and exocytosis is not known.

ATP released from the urothelium, by conditions such as stretch, can also communicate ultimately with the CNS to provide information about the degree of stretch and perhaps the composition of the urine. This latter aspect is considered in more detail in the Neural Control committee report.

The molecular basis of vesicle fusion has also been studied. By analogy with synaptic vesicles, the apical membrane and the fusiform/discoidal vesicles contain SNAP 23 (synaptosomal associated protein of 23 kDa), and the SNARES ; synaptobrevin and syntaxin. Another key component for synaptic vesicle fusion are the Rab proteins and Rab 27b has been found in the whole bladder [258] and in umbrella cells [256]. Unlike synaptic vesicles, there was no evidence for clatherin suggesting that coated pits are not involved in endocytosis [259]. These authors proposed that exocytosed vesicles are not always retrieved into the cytoplasm, but might be released into the urine.

## 6. Inert properties

The barrier function of the bladder should not be compromised by normal urinary constituents (**Table 4** for composition). For most substances this is true, however several alter dramatically the permeability of the urothelium. **Table 5** outlines the effects of selected substances on urothelial barrier function. Variation of luminal [Ca] does not alter amiloride-sensitive $Na^+$ transport but has small, reversible effects on transepithelial resistance, via the amiloride-insensitive current [260]. Low pH reversibly decreased both amiloride-sensitive and amiloride-insensitive transport [260]. High luminal osmolality does not alter barrier function. At low pH (<4.5)

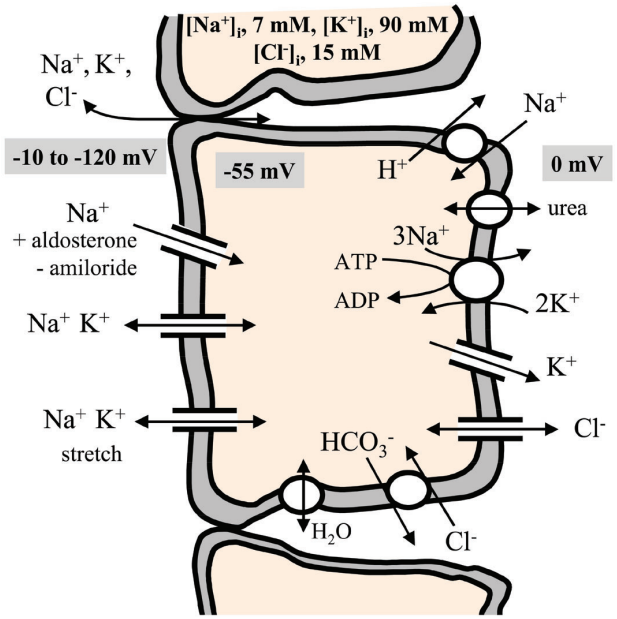


*Figure 14 . Model of trans-epithelial Na+ transport across the urothelium. Na+ crosses the apical membrane through Na+ channels (ENaC), blocked by amiloride; the density can be increased by aldosterone. Stretch-activated and degradation of ENaC, cation-selective channels are also present. Na+ entry is driven by the low intracellular [Na+] (7mM) and at times the negative-inside membrane potential. Intracellular Na+ are extruded across the baso-lateral membrane by the Na/K ATPase. K+ that enter via the Na/K ATPase exit across the basolateral membrane via K+ channels. The outward movement of K+ generates a voltage across the basolateral membrane which is 55 mV, cell interior negative. The basolateral membrane possesses also a Cl- channel and under normal conditions Cl - are passively distributed. During cell shrinkage the K+ and Cl- channels close and parallel Cl -/HCO-3 and Na+/H+ exchanger are activated resulting in an increase in cell KCl content, osmolality and consequent flow of water through aquaporins. Urea movement across the basolateral membrane is facilitated by the urea transporter UT-B.*

*Table 4. Composition of urine and plasma.*

| Substance | Urine Concentration | Plasma Concentration |
|---|---|---|
| Sodium | 50-250 mM | 135-145 mM |
| Potassium | 25-115 mM | 3.5-5.5 mM |
| Chloride | 75-365 mM | 95-107 mM |
| Glucose | 1 mM | 5 mM |
| Urea | 170-500 mM | 4 mM |
| Osmolality | 250-1250 mOsm.kg$^{-1}$ | 295 mOsm.kg$^{-1}$ |
| Calcium | <10 mM | 2 mM |
| Protein | 10-200 mg.l$^{-1}$ | |
| Volume | 780-1800 ml.day$^{-1}$ | |

*Table 5. Effect of various agents on bladder permeability.*

| Substance | Transport | Rt | Reversible | Ref |
|---|---|---|---|---|
| **Luminal side** | | | | |
| Low calcium | + | - | Yes | [259] |
| High calcium | - | + | Yes | [259] |
| Low pH | - | + | Yes | [259] |
| High pH | 0 | 0 | | [259] |
| 2 M urea (lumen) | 0 | 0 | | [259] |
| Fatty acids (low pH) | + | - | Yes | [261] |
| Trypsin | - | + | No | [259] |
| Urokinase | - | + | No | [263] |
| Kallikrein | - | +- | No | [259] |
| Eosinophil perox | + | - | * | [265] |
| Eosinophil MBP | + | - | * | [266] |
| Histones | + | - | * | [267] |
| Polymyxin B | + | - | * | [268] |
| Colistin | + | - | * | [269] |
| **Blood side** | | | | |
| 1 M urea (blood) | + | - | * | [280] |

*Rt : transepithelial resistance. + increase. - decrease.*
** degree of reversibility of transport and Rt is time dependent. At short time (minutes) it is fully reversible at longer times it does not completely reverse. Eosinophil MBP - eosinophil major basic protein ; Eosinophil perox - Eosinophil peroxidase*

volatile fatty acids such as butyrate, acetate, succinate or proprionate increase rapidly transport, and decrease transepithelial resistance [261].

That urinary constituents alter urothelium barrier properties was first suggested when apical membrane $Na^+$ channel density was found to be 8-fold lower than cytoplasmic vesicles, even though the apical membrane is composed of fused vesicles [262]. $Na^+$ channels are hydrolysed by trypsin (Table V), thus the difference in channel density might be due to urinary proteases, such as urokinase and kallikrein. Urokinase is a component of the fibrinolytic system and converts plasminogen into plasmin. The role of tissue kallikrein is unknown but is released into the tubule lumen from the principal cells of the distal tubule. Exposure of bladder epithelium to kallikrein or urokinase decreased the density of apical membrane $Na^+$ channels. Kallikrein in fact converts $Na^+$ channels into amiloride-insensitive non-selective cation channels and these, in turn, to the unstable cation channels [260, 263]. These proteases however do not alter tight junctions properties.

Protamine decreases dramatically trans-epithelial resistance. Disruption of the GAG layer was initially proposed as a reason [231], but subsequently it was found that this was due to an induced membrane conductance [264]. The induced route was permeable to $Na^+$, $K^+$, $Cl^-$, gluconate and 12 kdalton dextran, was modulated by $Ca^{2+}$ and $Mg^{2+}$, and its generation was dependent on the cell having a negative membrane potential. Other naturally occurring or synthetic cationic proteins produce similar effects, and include eosinophil peroxidase [265], eosinophil major basic protein [266], histones [267], polymyxin B [268], colistin [269], synthetic cationic peptides [264] and the amino terminal end of vimentin [270]. At long exposure times all of these cationic proteins cause an irreversible loss of urothelial barrier function, possibly due to increased ion influx and cell swelling.

## 7. Urothelial-detrusor interactions

The urothelium also releases a factor, or factors (UDIF), that decreases the force of smooth muscle contraction in response to muscarinic stimulation [271]. This factor(s) is released in response to either field stimulation or muscarinic agonists, and modulates the contractile action of these agonists [263]. The molecular identity of UDIF is unknown, however pharmaco-logical studies suggest that it is not NO, a prostaglandin, prostacyclin, adenosine nucleotide, catecholamine, GABA, or one that acts via apamin sensitive potassium channels. The possible role of endothelin remains to be investigated in this context, as endothelin receptors have been characterized on detrusor smooth muscle [272]. It is of interest that this inhibitory response it attenuated in a foetal model of bladder obstruction, compared to control animals [184]. This demonstrates that this dampening response can be modulated under circumstances that are associated with bladder overactivity.

## 8. Alteration to urothelial function

Barrier function can be compromised by other factors including, chemical exposure, inflammation, radiation, and bacterial infection. The causes are multifaceted; for example exposure of the bladder lumen to cyclophosphamide produces a hemorrhagic cystitis. Inflammation, as induced by sensitisation to ovalbumen, results in a rapid decrease in transepithelial resistance [273] and increase of urea flux [274]. This aberrant function is due to loss of tight junctions, focal loss of surface cells and alteration in apical membrane structure [273]. The underlying mechanism not unknown but might involve mast cell degranulation [275] and subsequent action of granule contents on the urothelium.

Irradiation-induced cystitis reduces barrier function by a loss of surface cells. Gene therapy with manganese superoxide dismutase (MnSOD) does not protect the bladder from the immediate effects of such cystitis, however it does permit a more rapid recovery of urothelial barrier function. This suggests that such therapy protects the intermediate and basal cells from irradiation, thus accelerating healing of the urothelium [276]. In addition to loss of barrier function, 24 hours post-irradiation urothelial cells produce more NO, compared to non-irradiated cells or irradiated cells that also received MnSOD. In tissue cultured urothelium, NO alters the barrier function of the bladder [277], however in vitro studies have indicated no long-term effects [278]. The involvement of NO in altering urothelial barrier function remains to be determined.

Interstitial cystitis (IC) is a bladder inflammation of unknown aetiology, but manifests itself as diminished bladder capacity, and frequent, painful urination, and in some patients there is a loss of barrier function [231]. Furthermore, in urine from IC patients there is a decreased level of heparin binding epidermal growth factor and greater than average levels of antiproliferative factor [279]. Whether these are causal of, or secondary to, IC requires fur-

ther study, however they may be useful as markers for the diagnosis of the condition.

A common theme in cystitis is a loss of barrier function, which allows movement of urinary constituents to the basolateral surface. When added to the blood-side surface, 500 mM urea causes an initial rapid and saturating increase of transepithelial conductance, after a delay there is a rapid and irreversible increase. The initial increase was at the apical membrane while the irreversible phase was paracellular, due to loss of tight junction integrity or loss of surface cells [280]. 500 mM urea added to both sides of the membrane slows the initial increase in conductance and delays the onset of the irreversible loss of barrier function. Preliminary observations suggest that a high serosal potassium level also degrades barrier function.

## V. MOLECULAR TARGETS IN REGULATING LOWER URINARY TRACT (LUT) FUNCTION

One way to think about potential molecular targets for the causes and treatment of incontinence is to identify the dysfunctional and failing elements and then examine the gene products involved. For example, if incontinence depends upon failure of an effective muscle contraction, then the elements responsible would be a rational focus. Beyond the muscle itself, neural regulation needed might be compromised, with a similar list of gene products.

While this could lead to an impossibly long list of potential molecular targets, regulation of gene expression serving cellular functions is a recurring theme. Thus, events affecting the regulation of one gene product will often affect the regulation of other products serving related functions, even in different tissues [281]. Thus, in the physiological responses to exercise, immediate early gene responses (c-jun, c-fos), CREB activation and serum responses elements function in multiple systems and imply parallel or shared mechanisms in the regulation of gene expression by external stimuli. Incontinence per se is much more prevalent in the ageing or aged population, thus specific molecular targets that are affected by the ageing process may be elevated in potential significance. A particularly important aspect in this regard may be oxidative stress and the consequences to cell and tissue function. Perhaps the most useful targets are those with specificity to the lower urinary tract and bowel. Knowledge concerning specific expression in these systems is only now being developed, but the diversity of cell types and their relative uniqueness make probable that targets can be exploited.

### 1. Biomechanical phenotypes

Normal LUT and bowel function occurs against a background of tissue mechanics and structural integrity. Failure to maintain these properties is a prominent aspect of ageing; skin elasticity is replaced by sagging and wrinkling in older age. Similar events occur in the LUT and bowel (vide infra) in that the regulation of extracellular matrix and intracellular mechanical integrity, degrade with age. This may partly be related to reduced cellular density, via cell death and/or disordered cell and tissue neogenesis. A general set of targets would include both the processes of structural deterioration and of reduced regeneration or replacement.

Molecular events could counter this decline, in particular responses to mechanical forces or stress. Thus, muscle normally adapts to increased mechanical demands with hyperplastic and hypertrophic growth, thus increasing force generation. Extracellular matrix elements and tissue mechanical integrity are also reinforced by non-destructive mechanical stresses. For example, in lung tissue, changes to gene expression induced by mechanical forces are communicated to other cell types that have not experienced mechanical stimuli, thus demonstrating a potentially important pathway for tissue re-modeling [282]. Endothelial cells, and possibly by analogy urothelial cells, are particularly responsive to mechanical forces [283].

In endothelium, flow-responsive genes fall into functional clusters including those for transcription factors, antioxidants, signaling molecules, cell cycle regulators, and genes involved in cellular differentiation. These events depend upon a cellular signaling network that controls expression of the gene products involved. At the molecular level, response elements in the promoter regions of involved genes have been described, the activity of which are regulated by stress and/or stretch [284, 285]. In some organs, the response to mechanical force is integrated with those to reactive oxygen species and oxidative stress [286]. It is possible that some of the age-related degradation of structural gene expression, and thus events that may predispose to incontinence, involves dysfunction or deterioration in these regulatory signaling pathways. Very little is known about bladder, urethral or bowel specific signaling pathways analogous to those of endothelium and bone. However, interesting possibilities include:

### *a) TGF-β pathway*

The transforming growth factor-β signaling pathway is necessary for tubulo-interstitial fibrosis after ureteral obstruction [287]. In this case, damage related to cell phenotypic change and collagen deposition is prevented by interruption of TGF-β signaling.

### *b) Hypertrophy signaling*

Pathways responsible for inducing hypertrophy in response to increased mechanical load or activity are logical molecular targets. Ageing and functional deterioration may reflect dysfunction in these pathways. The molecular details serving this form of signaling have not been fully elucidated in the LUT. However, loading or stretch is transduced, sometimes by cytoskeletal deformation and/or via stretch-activated ion channels, into altered gene expression. Intracellular signaling pathways initiated by the transducing event(s) mediate the coordinated response in several tissues [288-292].

### *c) Ischaemia signaling*

Structural derangement with time in the LUT may depend in part upon repeated ischaemic insults. Cellular responses to ischaemia include alterations in gene expression that to some extent overlap those outlined above [293-295]. **Figure 15** shows examples of upregulated markers in ganglia located in the guinea-pig bladder wall. These include those associated with cell growth and division (c-jun and c-fos), as well as those associated with cell death (Bax and caspase) and demonstrates the increased cell turnover that accompanies this situation.

## 2. EC-COUPLING SPECIFIC TO LUT AND/OR BOWEL & RECTUM

EC-coupling in LUT muscles contributes an important aspect to the maintenance of continence. The gene products centre upon the signal transduction pathways that regulate and modulate intracellular $Ca^{2+}$ transients, the central operators and modulatory proteins of the contractile apparatus, and the appropriate spread of activation between cells. Some details of the latter process in EC-coupling regulatory signaling through this appproach are being revealed [35, 73, 296]. The demonstration that contractile protein expression and function may be altered along with hypertrophy suggests that incontinence related dysfunction in smooth muscle could also involve changes to the contractile machinery. For example an alteration to the $Ca^{2+}$-sensitivity of the contractile proteins could have profound effects on contractile functions, without alteration to the properties of upstream receptor properties or secnd messenger signalling [216, 297, 298]. Potential molecular targets relate to the cellular signal transduction elements responsible for setting the functional status of each of these components. The troponin superfamily presents suggestive possibilities, as troponins modulate the contractile machinery in striated and cardiac muscle, but also are expressed in the LUT [299]. Control of troponin expression may be via transcriptional enhancers [300]. A potential approach would be to create molecules with interactive domains designed for specific tissues. For example, a peptide $Ca^{2+}$ mimetic has been created from the $Ca^{2+}$ binding domains of the troponin family [301]. A similar approach could be used in the design of small molecules or peptides that affect the unique elements of the continence contractile machinery. Because EC-coupling involves the regulation of intracellular $Ca^{2+}$, the gene products involved in this complex system are also potential targets, including those involved with mitochondrial regulation (see below).

## 3. NEUROMUSCULAR SIGNALING AND TRANSMISSION

### *a) Neurotransmitter receptors*

The molecular regulation of neurotransmitter receptor phenotype is not well understood in the LUT and bowel. Perhaps most rewarding would be to define the regulatory pathways affecting expression of receptor subtypes and age-related changes associated with incontinence. Most effort is directed towards understanding the expression of muscarinic receptors [302-304] ; there is little work with other modulatory receptors, such as the purinergic system.

### *b) Neurotrophic interactions - the role of nerve growth factor (NGF)*

A growing body of information has accumulated on cell-cell interactions in the maintenance of normal LUT and bowel function. Specifically, NGF has been implicated as an important signalling protein or cytokine involved in a range of problems with LUT function [305]. This paradigm has particular interest because it connects intrinsic muscle function with that of innervating sensory and motor nerves, as well as central nervous system pathways responsible for normal bladder filling and emptying. Muscle, and perhaps urothelial cells, produces NGF in response to functional stimuli (e.g. stretch, contractile activity, inflammation). NGF is secreted and taken up by the innervating nerves, activating surface tyrosine kina-

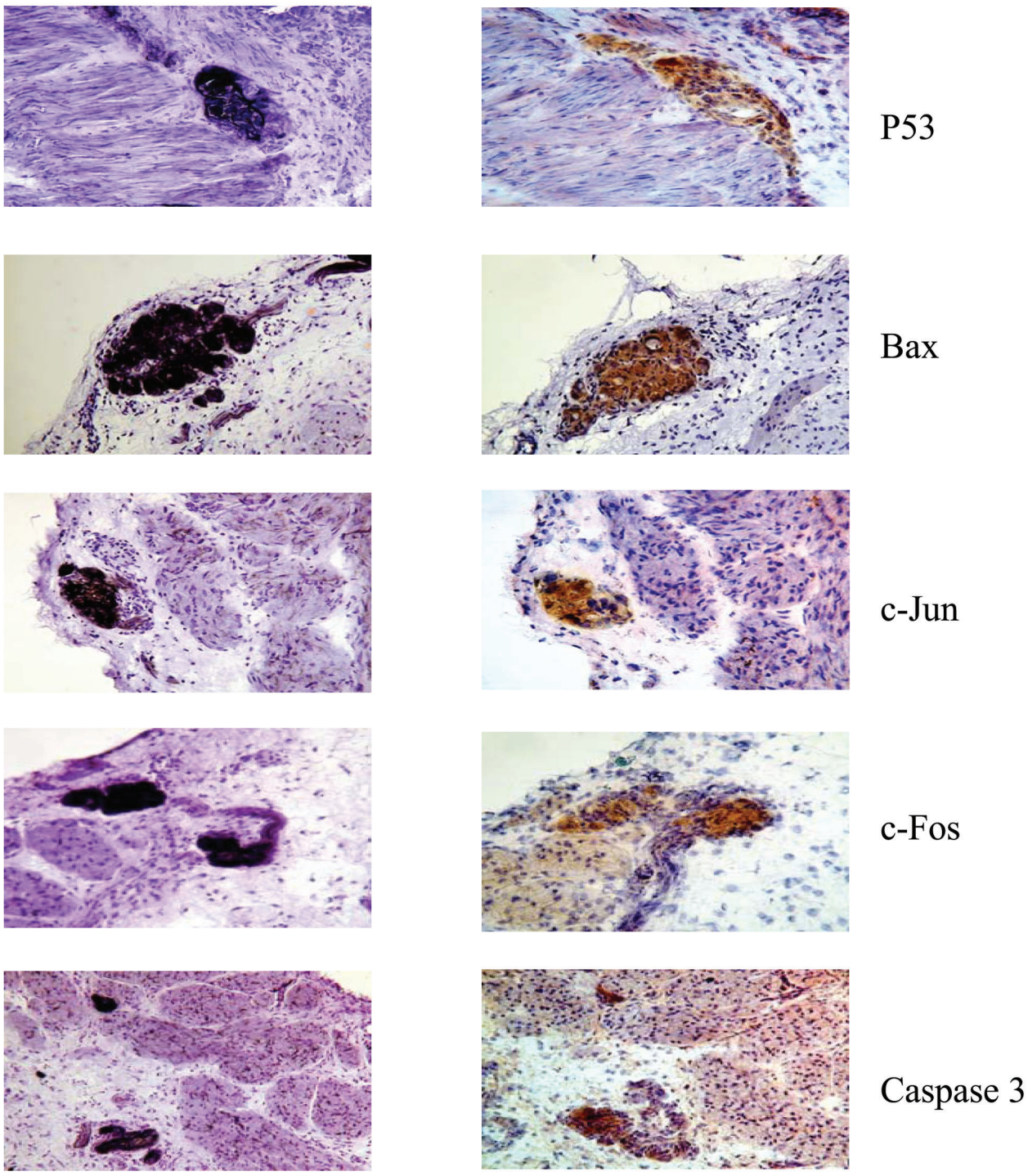


***Figure 15. Cell growth and death markers in response to ischaemia***
***Examples of cell markers altered in ganglia from the bladder wall of guinea-pigs. The animals had been subjected to occlusion of the terminal aorta for one hour, followed by reperfusion for one hour. Each pair are adjacent sections from one bladder. Sections in the right hand column were stained to show the presence of acetylcholine esterase, a reliable marker for ganglia. The adjacent sections show immunohistochemical localisation of the various markers.***

se receptors in passing [306]. NGF is retrogradely transported to the neuronal nucleus, and initiates a spectrum of responses in neurons, including altered gene expression. These responses allow the neurons to withstand apoptotic induction, prevent atrophy and maintain normal expression of functionally important gene products, including ion channels [307]. Remarkably, production of excess NGF is just as damaging as its removal, albeit for different reasons [308]. NGF production is regulated via a variety of mechanisms, including mechanical stretch of bladder muscle. This induces NGF production in vivo and in vitro via the transcription factors AP-1 and NF-κB [309]. Many aspects remain unclear, however, about how NGF affects the performance of organs, including: the mechanisms of secretion; the active form of the secreted molecule and its mode of action. Whether controls unique to the LUT or bowel exist is unknown.

Manipulation of NGF expression or signalling in the LUT and bowel is potentially an attractive molecular target for treatment of dysfunction. Both diabetes and age disturb the proper balance of NGF production and effects. In a cellular model of age-related disease, NGF and trk-A expression were altered in a detrimental fashion [310]. A significant literature describes age-related changes in NGF protein [311-313] but studies of the specific role NGF may play in incontinence are lacking. Attempts to manipulate NGF expression in diabetes via gene delivery have been conducted [314] and have been experiments to block NGF action [315]. It is also unclear if other protein and/or cytokine signals are important to the maintenance of normal function LUT and bowel function, but likely candidates include GDNF, CNTF and FGF

## 4. Mitochondria, $Ca^{2+}$ and the endoplasmic reticulum

Studies of the roles of mitochondria and endoplasmic reticulum (ER) in $Ca^{2+}$ homeostasis and smooth muscle contraction suggest that they present significant molecular targets. The $Ca^{2+}$ signaling network is particularly attractive because of its position astride the important cellular pathways serving regulation of ATP production, control of gene expression and maintenance of ER protein synthesis. It is clear that mitochondria and the ER work in concert with signals to the nuclear expression machinery to accomplish these tasks. It is also evident that mitochondrial function and integrity degrade with ageing and in certain diseases, and that this loss of normal participation in $Ca^{2+}$ signaling contributes to the problems [316].

Mitochondrial function, and possibly ER function, is damaged by reactive oxygen species (ROS), free radicals and other consequences of ischaemia-reperfusion injury [317, 318]. Thus, a potential general target is the process of damage and oxidation caused by these events. Cellular mechanisms that protect against such damage might be induced and enhanced by therapeutic agents that capitalize upon the endogenous capacity for repair. Some studies have been described in other tissues and organs but there is little work that is specific to the LUT or bowel. One report suggests a plant derivative has protective activity following experimental outlet obstruction [319], reinforcing the possibility that effective molecular targets do exist. Additional possibilities may be in the hypoxia-inducible factor-1 (HIF-1) signaling pathways. Multiple stimuli induce activation of HIF-1α, which is followed by changes in gene expression geared toward repair and restoration [320].

Anomalous spontaneous muscle activity may play a significant role in various forms of incontinence, and mitochondria participate in the generation and maintenance of spontaneous contractile activity in bladder smooth muscle [321]. Decay of mitochondrial function and damage to mitochondrial DNA (mtDNA) may predispose muscles and other cells in the LUT and bowel to abnormal contractile events. Damage to mtDNA is a prominent feature in organ failure in the cardiovascular system [318] but it has not been examined in the aged LUT. The molecular elements involved in $Ca^{2+}$ signaling are also not fully understood. Additional potential molecular targets in this area include the SERCA $Ca^{2+}$ pumps responsible for maintaining and recharging the ER with $Ca^{2+}$, without which contractile failure and protein synthesis are affected.

The mitochondrial repair machinery and molecules involved in protection of mtDNA are interesting targets. There is an age-dependent decline in the importation of DNA repair machinery [322], and failure of replication fidelity and decline in repair capability would accelerate mtDNA deterioration and increase free radical generation. This series of events leads to contractile failure in the heart and could cause similar failure in the LUT. Because the importation of proteins into mitochondria, and their appropriate assembly, depends upon chaperones, these are also likely targets. The heat shock protein (HSP) families stand out. HSP 70 and HSP 90 family members are directly involved in mitochondrial protein importation. Expression of these gene products is also reversibly down-regulated with ageing [323]. In addition, the HSPs prevent apoptosis in a number of experi-

mental systems [324]. Furthermore, the role played by mitochondria in controlling cellular death via apoptosis is vital. It is reasonable to assume that progressive deterioration of mitochondrial function in the LUT would eventually lead to death of some of the cellular elements and a worsening of normal operation.

## 5. Urothelium and DEG/ENaC ion channels

ENaC channels have already been mentioned in the context of urothelial ion transport (section 5). The DEG/ENaC family was named for the first two subfamilies identified : degenerins, *C. elegans* proteins that mutate to cause cell swelling and degeneration, and epithelial $Na^+$ channels (ENaC) [325]. All of these ion channels are susceptible to blockade by the diuretic, amiloride. ENaC are composed of four different units (α, β, γ, δ), the first three of which are found in the human urinary bladder [326]. **Figure 16** shows that the expression of different units is increased in tissue from obstructed bladders. If ion transport does play a role in urothelial functions such as sensations of bladder fullness, then over expression of transport proteins will increase the gain of these functions.

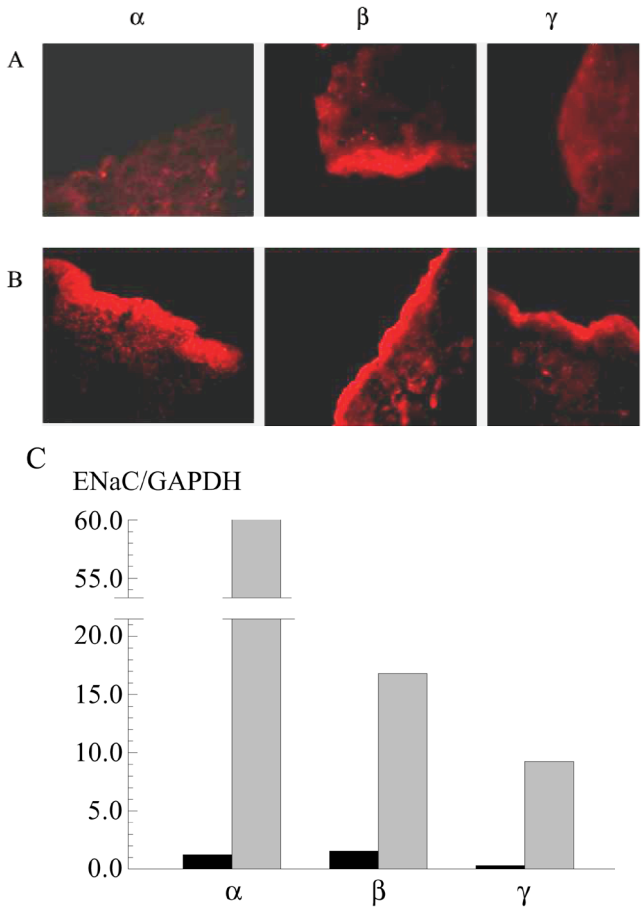


***Figure 16. ENaC protein expression in the bladder urothelium A,B: Immunofluorescence study of ENaC subunits (a, b, g) protein in the urothelium of the human urinary bladder. Normal unobstructed bladder (A) and obstructed bladders due to benign prostatic hypertrophy (B). Expressed protein was much greater in samples from obstructed bladders, as seen by the more distinct red fluorescent label concentrated at the urothelial border. C: Semi-quantitative RT-PCR analysis of the protein translation in samples from stable bladders (black bars) and obstructed bladders (grey bars). Values are expressed as a ratio of the ubiquitous house-keeping gene product GAPDH.***

# VI. ARTIFICIAL TISSUE CONSTRUCT FOR THE LOWER URINARY TRACT

## 1. The current need

There are over 400 million people worldwide who have bladder disease and it is probable that there will always be a sub-set who will require primary surgical intervention, as well as those who fail to respond to non-operative measures. Bladder replacement and augmentation is a well-documented procedure [327] but they are not without attendant complications. Patients having an ileal conduit can expect an overall complication rate of 23% [328].

The figure is similar for patients having a cystoplasty, orthotopic bladder or neobladder. In addition, metabolic disturbance, altered renal function, bone disease, mucous production, urinary infection, urolithiasis and possibly tumours are all well-documented side-effects [329]. These procedures are used because they offer an overall improvement in quality of life, however they do not offer an ideal solution.

With respect to the urethra, several grafts can be used when an end-to-end anastomosis is inappropriate, and include pedicled skin flaps or free skin grafts [330] e.g. preputial or post auricular Wolfe graft and buccal mucosa [331].

In expert hands these grafts have high success rates. However, in patients who have pan-urethral balanitis xerotica obliterans, where one might most preferably use buccal mucosal graft, any failure or re-stricture may lead to a re-operation without the first choice of graft tissue being available - since there is only a finite supply of buccal mucosal graft.

## 2. Basic requirements

At present there is no successful artificial implant that has achieved acceptance as a clinically applicable device [332]. There have been many attempts with many different approaches and these will be discussed as part of this section. The aim would be to allow the successful application of biotechnology to create devices that can be used for the expansion and replacement of absent or damaged tissue in the urological tract. To produce such an implant one must first understand the clinical problems for which such an implant represents a realistic treatment. Clearly, an implant must be better than the existing alternatives such as segments of bowel in the bladder or buccal mucosa and skin in the urethra. This already represents a significant criterion, because existing implants have been extensively applied with long-term clinical success. Why would the generation of such implants be useful ?

- To avoid complications with existing implants.
- To offer improved function compared to existing implants. The long-term aim is to produce a graft that would be for organ augmentation, but also for restoration of function.

In principle several approaches are possible, and the ideal would be use a small sample of the patient's tissue as a starting material to minimise the possibility of rejection. At present research has been dircted to enhancing the reservoir capacity or conduit functions of the lower urinary tract. A more difficult problem will be to generate artificial implants that also contract and thereby fulfill the need to regulate flow of urine. This latter approach ha this far not been successfully undertaken, although several groups (below) are attempting to measure the functional properte sof potential artificial implants.

In some circumstances, isolated cells embedded into a suitable medium may be injected directly; such as myocytes into the sphincter to improve continence, or with chondrocytes to improve the vesico-ureteric junction [333, 334] or implantation of cells into the healthy urethra [335] In this case tissue regeneration occurs without the aid of a scaffold. Alternatively, a scaffold may be implanted over which tissues can expand or regenerate [336]. Finally, as for example with the generation of neobladders, a specifically engineered implant will be required [337]. As with other newly-developing fields, the need for continued development will only be evident after the clinical application of grafts, and with extensive testing on animal models.

**Figure 17** outlines a strategy for the generation of, for example, a bladder implant. It has the goals of producing in the first instance a structure that will act as an effective storage organ, but secondly one that has regulated contractile function that can void accumulated urine.

In general the particular technical aspects that require consideration are to:

- devise methods for the mass production of cells with a well-defined phenotype
- use a scaffold that does not adversely affect cells or its environment
- characterise cell function and measure physiological functions of the implant
- generate a nutrient supply for cells
- manufacture a cost-effective implant

In addition future developments may need to consider the possibility of altering the cell phenotype, possibility through genetic manipulation, to tailor grafts to particular circumstances.

## 3. Scaffolds for implant structure

Scaffolds give cells a three-dimensional support structure in which to grow but do not damage the cellular phenotype. In general terms these are made from three types of material [338] :

- Naturally derived materials - e.g. collagen and alginate
- Acellular tissue matrices - e.g. bladder submucosa and small intestinal submucosa [339]
- Synthetic polymers - e.g. polyglycolic acid (PGA) and polylactic acid (PLA) and their combination poly(lactic-co-glycolic) acid (PLGA) [340]. **Figure 18** shows examples of the above three types of inert material.
- Decellularised tissue upon which more compatible cells may be layered, e.g. de-mucosalised gut segments [299].

Scaffolds may be modified to enhance cell adhesion [341], or the promotion of particular cells or cellular properties [342]. The decellularisation of tissue matrices is an advance in the provision of a tissue engineered graft material. However, the agents used to perform this task may adversely affect cell compatibility and subsequent graft remodeling [343]. There is a point of view that materials should not stimulate an inflammatory response [343]. It is impor-

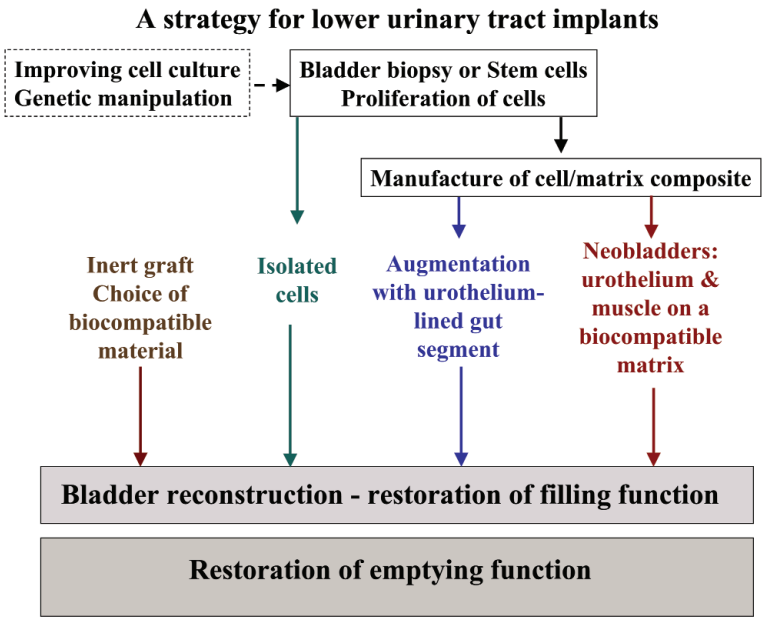


*Figure 17. Strategy for the development of tissue engineered components for the lower urinary tract.*
*A strategy to show the potential stages in the formation of functional implants suitable for the lower urinary tract. The aim is to generate implants that can store urine and contract to empty. The first stage is the development of implants that can fulfil the storage criterion. Biological implants rely on the development of biocompatible materials that can be used alone or seeded with urothelial cells or co-cultures of urothelial and smooth muscle cells. Cells can originate from the patient either as differentiated or stem cells, and may also be genetically manipulated to optimise the cellular genome.*

tant to define this more carefully, for a graft that produced no response could not heal into place and may not be able to generate a nutrient bond with the host. Hence there must be some form of inflammatory response but this must not be of the sort that would lead to graft destruction or rejection.

In principle support scaffolds may be used for three purposes :

- To provide an implant that is purely structural and provide a framework over which the host tissues would generate their own cellular replacement [336]. Bladder allowed to regenerate in this way maintained some functional characteristics. Other work has not been able to duplicate the same results with such clarity however, with reports of graft shrinkage and reduced muscle content [344, 345].
- To use native tissue such as bowel and replace its epithelium with urothelium. This would remove many of the secondary complications that arise with current bowel replacement procedures that arise from contact of urine with gut mucosa. Culture of urothelium is relatively straightforward [346] and data suggests that it may be possible to transfer these cells on a scaffold and use them to form a urothelial layer on de-epithelialised bowel segments [340]. This practical procedure overcomes the previous difficulties encountered with attempts at monolayer transfer [347].
- These first two procedures generate essentially acontractile implants. The most desirable structure would be to use a scaffold to support an implant with both cultured urothelium and smooth muscle cells, to provide respectively both a barrier function and contractile function [397].

## 4. Characterisation of cell function and graft function

The phenotype of cultured cells that could be implanted as part of a tissue-engineered graft has been measured [6, 348-350]. Whilst there are changes to some cell functions, those that appear to be most involved in physiological control of the bladder remain relatively intact. With cultured detrusor cells intracellular $Ca^{2+}$ signalling pathways and electrophysiological activity are retained, as well as their contractile response to contractile agonists (**Figure 19**). Cultured urothelial cells are able to maintain a barrier function between the apical (urine) and baso-lateral faces, although their transport characteristics remain to be fully evaluated.

Whilst these phenotypic characterizations have been carried out using isolated cells or monocultures, it remains to be established if such functions persist in more complex co-cultures and when immobilized on a support scaffold. In addition, other cells are found in the bladder wall, including fibroblasts and myofibroblasts. These may be involved in collagen deposition and even afferent signalling of bladder fullness [73]. Whether such cells should be included in the fabrication of a complex graft, or whether invasion from neighbouring tissue will be adequate also remains to be established.

The physiological properties of cells cultured in an environment similar to an engineered graft have been less intensively investigated. A few studies have measured the ability of cultured detrusor cells to contract in response to physiological interventions [351, 352] (**Figure 20**).

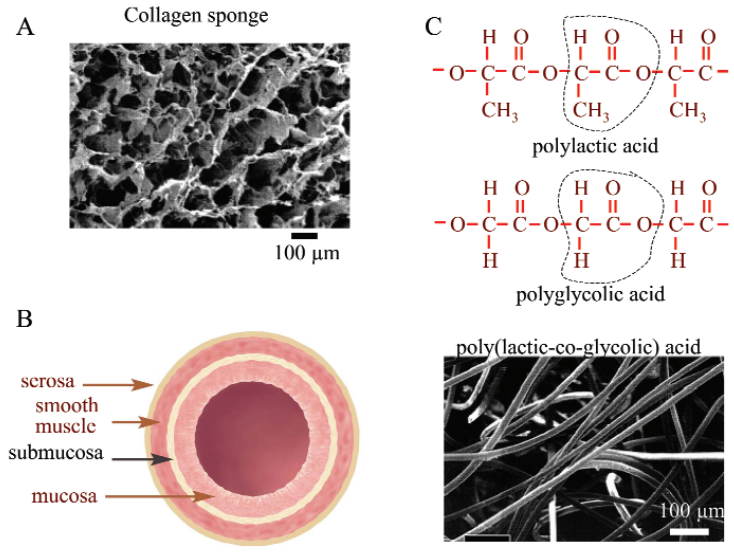


*Figure 18. Materials used in the construction of tissue-engineered implants*
*A. Natural materials such as collagen to form a porous sponge. B: biological material used to form an acellular matrix of submucosal material (black arrow) after chemical removal of the inner and outer layers. C: synthetic polymers of lactic or glycolic acid to form long strands of intermeshing fibres.*

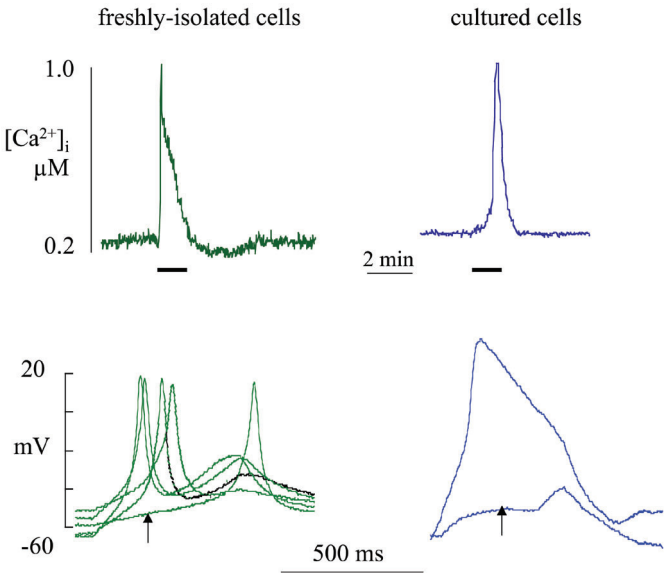


*Figure 19. Intracellular $Ca^{2+}$ and electrophysiological responses in freshly-isolated and cultured human detrusor smooth muscle cells*
*A: top - results from freshly-isolated cells showing the response to 10 µM carbachol on the intracellular $[Ca^{2+}]$, $[Ca^{2+}]_i$,; bottom – action potentials elicited by passing current (600 ms – 5-25 pA) into isolated cells using a CsCl-filled patch pipette. B: top – changes of $[Ca^{2+}]_i$, to 10 µM carbachol; bottom – action potential elicited by 15 pA current. In the two bottom panels the arrows point to traces where the passage of intracellular current failed to elicit an action potential.*

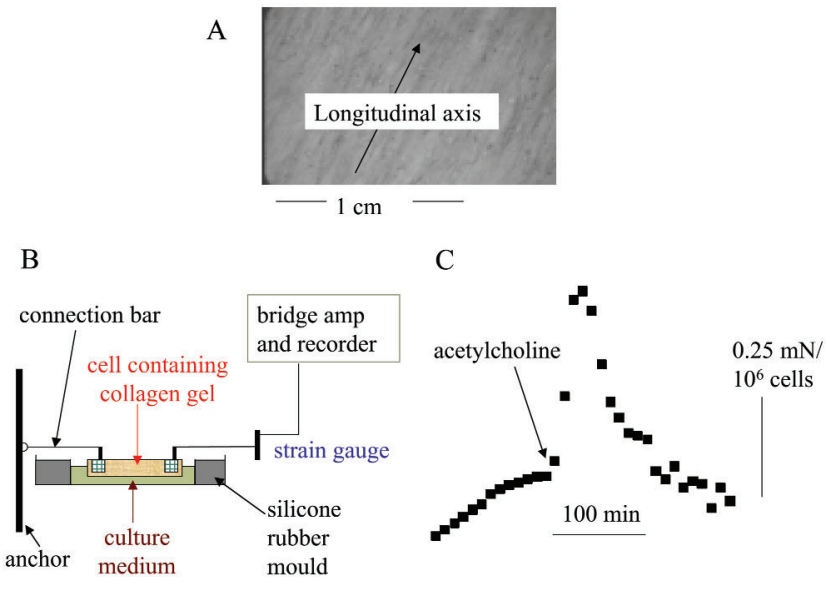


*Figure 20. Contractile responses from cultured human detrusor smooth muscle cells*
*A: a collagen gel with cultured human detrusor cells. The longitudinal axis is shown along which cells preferentially orientate. B: block diagram of the experimental apparatus to measure contractile force of a cell-containing collagen gel. The gel is floated on a culture medium base and has embedded in the gel to mounts for attachment to a solid base and isometric force transducer. Contraction of the gel generates a signal in the transducer which is recorded with a bridge amplifier and the signal recorded. C: Example of a recording where tension was noted at 10 minute intervals. At the arrow a 200 µM solution of acetylcholine was added to the culture medium to give a finally dilute the acetylcholine approximately 10-fold. A transient increase of tension in the gel was observed when acetylcholine was added.*

The generation of nerve growth into an implant is also important. Current work is concerned with regenerating nerves that innervate existing urological tract tissue and how this may represent an avenue for treatment [353, 354]. Work with foetal mice has shown how by growing detrusor cells and stretching them, their production of growth factors alters [355].

Alternative approaches using stem cell technology have been suggested and the strategies employed have been reviewed [299]. Whilst they represent an exciting prospect for the future, the current understanding of their control and differentiation is not yet sufficient to merit application in reconstruction of the lower urinary tract.

### 5. Generating a nutrient supply for grafts

The development of a new blood supply to free grafts for urethral replacement takes around 96 hours [356]. During the first half of this period, the graft is at a lower temperature than the surrounding tissue and draws nutrients directly from the host tissue bed. This is a critical stage and is successful for thin grafts, as critical diffusion distances are only about 150-200 $\mu$m from a blood supply [357]. For the second phase the graft acquires a microcirculation and its temperature rises to that of core body temperature. Clinical results demonstrate that this is a successful process, but these grafts have been harvested from well-oxygenated tissue with an active nutrient supply and thus initially are in a good metabolic condition. A tissue-engineered graft, developed in tissue culture, may have a much lower state of oxygenation, owing to reliance on diffusion across a culture medium. Potentially they may also be exposed to toxic effects of reactive oxygen species if are oxygenated at excessive levels of $PO_2$.

The promotion of angiogenesis for the vascularisation of tissue-engineered grafts remains the most significant hurdle to overcome in the generation of large constructs [357]. Neo-vascularisation consists of two processes -the in situ assembly of capillaries from undifferentiated endothelial cells and angiogenesis, the sprouting of capillaries from preexisting blood vessels [358]. Several factors stimulate the growth of vessels: hypoxia stimulates angiogenesis, with direct effects on component cells, and indirectly by increasing their sensitivity to angiogenic factors, most particularly vascular endothelial growth factor (VEGF) [357, 358].

As part of a host tissue reaction to an implant, factors are produced that increase endothelial cell activation and proliferation. These include VEGF and platelet derived growth factor (PDGF), both of which have direct effects on endothelial cells. In addition, acidic and basic fibroblast growth factors as well as angiogenin exert more indirect effects [357]. Thus, the inclusion of endothelial cells in co-cultures, or in bioreactors for implants could be an advantage [342, 359].

## VII. PHYSIOLOGY OF THE LOWER GASTRO-INTESTINAL TRACT - THE RECTUM AND ANAL SPHINCTER

### 1. Functions of the G-I tract

The functions of the lower GI tract, namely the colon, rectum and anal canal (**Figure 21**) are basically the same as that of the bladder and urethra: i.e. continent storage of waste material and a mechanism for voluntary elimination. The difference is that the waste is usually either solid or semi-solid faeces or flatus (gas). Continuing the analogy with the urinary tract, the distal colon and rectum are the main storage organs equivalent to the bladder, the anal sphincter provides continence and is equivalent to the external urethral sphincter. Functional differences are due to the fact that faecal material can be returned to the colon from the rectum, and that a detection mechanism is present to allow assessment of the rec-

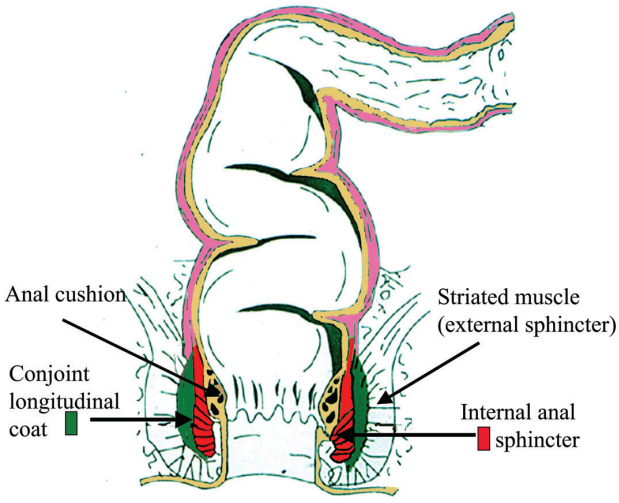


***Figure 21. Diagram of the anatomy of the human rectum and anal canal.***
***The internal anal sphincter and conjoint longitudinal coat are shown as separate regions from which muscle samples were dissected***

tal content, so that flatus can be passed at times when passing faeces would not be appropriate.

The sequence of events that occurs in storage and defecation is as follows. The rectum remains empty for most of the time, but fills as faecal matter accumulates in the descending and sigmoid colon, and is pushed forward through by occasional peristaltic waves (mass movements). As faecal matter enters the rectum the walls relax, and filling can occur with little increase in rectal pressure. Distension of the rectum triggers a recto-anal inhibitory reflex, which lowers the pressure in the anal canal, allowing the rectal contents to enter the anus. Periodic relaxations of the internal anal sphincter allow anorectal sampling of the contents [360, 361] using the rich sensory innervation of the anal canal, which allows us to discriminate between gas, liquid and solid. During these phases the external striated sphincter is contracted to maintain continence. As filling continues, sensory information ascending to the brain leads to the sensation of rectal fullness. If defecation is deemed appropriate, voluntary relaxation of the external sphincter occurs and peristalsis in the colon and rectum is initiated, usually by abdominal straining resulting in relaxation of the internal sphincter and expulsion of the rectal contents. If defecation is inappropriate, rectal contents may return to the colon.

Unlike the situation in the urinary tract, however, the gut wall contains all the machinery (intrinsic pacemakers and neural networks) to programme the activity of the smooth muscle that is necessary to expel the waste material (relaxation of the internal anal sphincter, initiation and co-ordination of peristalsis, see **Figure 22**). It is only the external striated sphincter that absolutely requires extrinsic innervation to contract, through activation of somatic motor neurones whose axons run in the pudendal nerves. The distal gut does, however, receive extrinsic innervation via the autonomic nervous system, with parasympathetic input to the distal colon and anorectum from the sacral roots through the pelvic nerves, and sympathetic input from the lumbar cord running through the mesenteric and pelvic plexuses (**Figure 23**). A spinal reflex centre uses this autonomic extrinsic pathway to maintain rectal compliance during filling, and to help switch on peristaltic activity necessary to produce defecation, as well as to initiate the correct pattern of activity in the somatic nerves to the extrinsic sphincter.

The properties of the various components will be described in more detail. Most of the work on the properties of the smooth muscles and their intrinsic

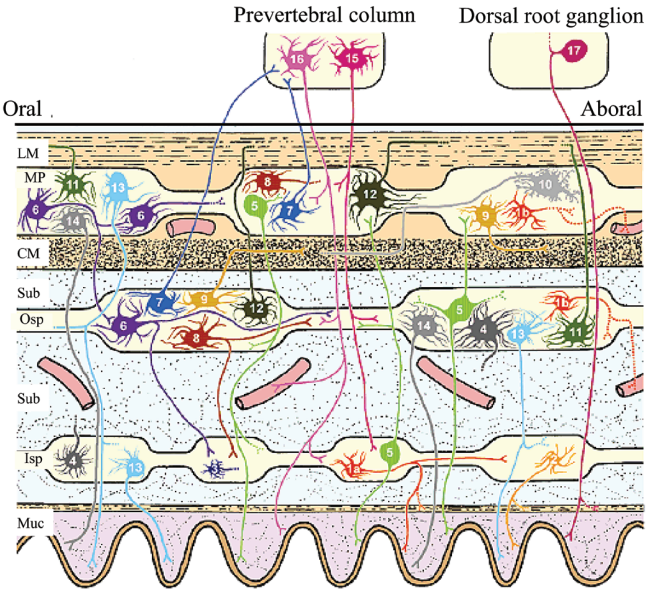


***Figure 22. The enteric nervous system in the pig small intestine***
***A diagram showing the different classes of neurone present in the various plexuses of the enteric nervous system in the pig small intestine. Each number refers to a type of neurone with a distinctive neuro-chemical profile. Modified from [370].***

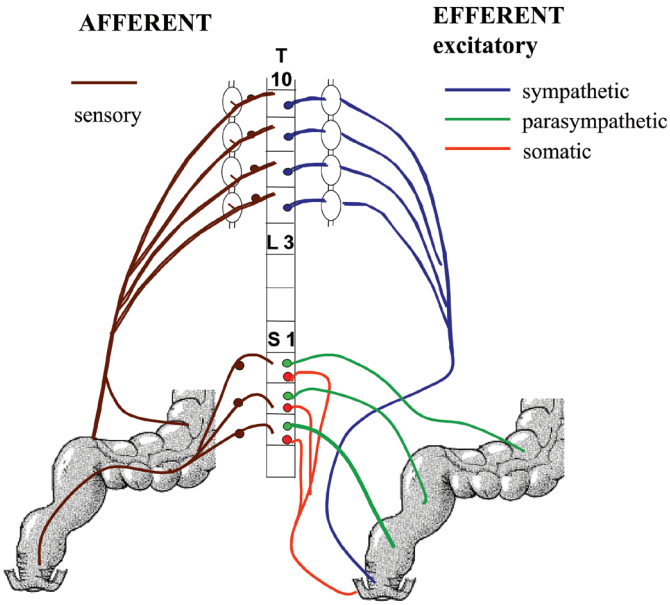


***Figure 23. The extrinsic innervation of the distal gut.***
***The afferent and efferent supplies to the distal gut are shown as separate diagrams. Neurones originate from thoraco-lumbar and sacral sections,***

innervation comes from in vitro research using human tissue : studies on pig tissue yield similar results.

## 2. Smooth muscle properties

### *a) The rectum*

A complete layer of outer longitudinal and inner circular smooth muscle surrounds the rectum. The properties of the two layers are probably similar to those in other longitudinal elements of the gut. In the human both the circular and longitudinal smooth muscle layers generate phasic contractions at a frequency of about 3-4 per minute [362]. In the circular smooth muscle there is little if any intrinsic tone. In contrast the longitudinal smooth muscle develops a basal tension of some 0.2 $g.mg^{-1}$ tissue, and phasic contractions rise from this. These spontaneous contractions are unaffected by tetrodotoxin, and are probably evoked by slow waves generated in the interstitial cells of Cajal (see below). Catecholamines abolish the spontaneous contractions in both muscle layers and reduce the baseline tension in the longitudinal smooth muscle. Both muscle layers respond to activation of muscarinic receptors by contracting (**Figure 24**). Activation of either α- or β-adrenoreceptors can induce relaxation, exemplified by the fact that the phenylephrine and isoprenaline produce the same relaxant responses as noradrenaline. α-adrenoreceptors are known to induce relaxation of the longitudinal elements of the gut in many mammalian species through opening of $Ca^{2+}$-activated $K^+$ channels [363]

### *b) The anal canal*

The longitudinal layer of smooth muscle in the rectum extends into the anal canal, where it forms the conjoint longitudinal coat, and the circular layer forms the internal anal sphincter (**Figure 21**). In contrast to the rectum, the circular smooth muscle of the internal anal sphincter generates significant spontaneous tone [364]. The smooth muscle also relaxes in response to muscarinic receptor stimulation, contracts in response to activation of α-adrenoreceptors (Figure 24), and relaxes in response to stimulation of β-adrenoreceptors [365]. Muscle from the conjoint longitudinal coat generates a small amount of basic tone but shows little spontaneous phasic activity. They respond to activation of both α-adrenoreceptors and muscarinic receptors by contracting (**Figure 24**).

**Table 6** compares the basic properties of the four smooth muscles, emphasising their heterogeneity. The responsiveness of these smooth muscles to stimulation of their adrenergic receptors reflects their overall response to circulating adrenaline and activation of sympathetic nerves, which will ensure that in 'flight and fight' conditions, activity will be reduced in the longitudinal parts of the gut, whilst the smooth muscle sphincters will be closed, ensuring continence whilst reducing overall energy expenditure. Under extreme conditions of stress, if circulating levels of adrenaline become too high, the relaxant responses to β-adrenoreceptor stimulation on the sphincteric smooth muscle may underlie the anecdotal accounts of involuntary loss of faeces and urine.

## 3. Pacemaker activity

Spontaneous, or more accurately, non-neurogenic contractile activity in gastrointestinal smooth muscles is now known to be generated by interstitial cells of Cajal (ICCs). These cells are named after Santiago Ramon y Cajal, a Spanish histologist who described them at the end of the 19th century [67]. Recent studies of these cells has been facilitated by the discovery that they express on their surface membrane a receptor tyrosine kinase that is the gene product of c-kit, [366, 367]. ICCs are arranged in distinct ways in different parts of the gut. In the longitudinal elements there is an extensive plexus of ICCs running with the neurones in the myenteric plexus (IC-MY), and often another plexus at the submucosal surface of the circular smooth muscle (IC-SM). In these the interstitial cells are linked to each other,

***Table 6. Properties of smooth muscle from human ano-rectum.***
§ indicates relaxation, * contraction

| | Tone | response to muscarinic agonist | response to noradrenaline |
|---|---|---|---|
| Rectal circular | * | * | § |
| Rectal longitudinal | + | * | § |
| Internal anal sphincter | +++ | § | * |
| Conjoint longitudinal coat | + | * | * |

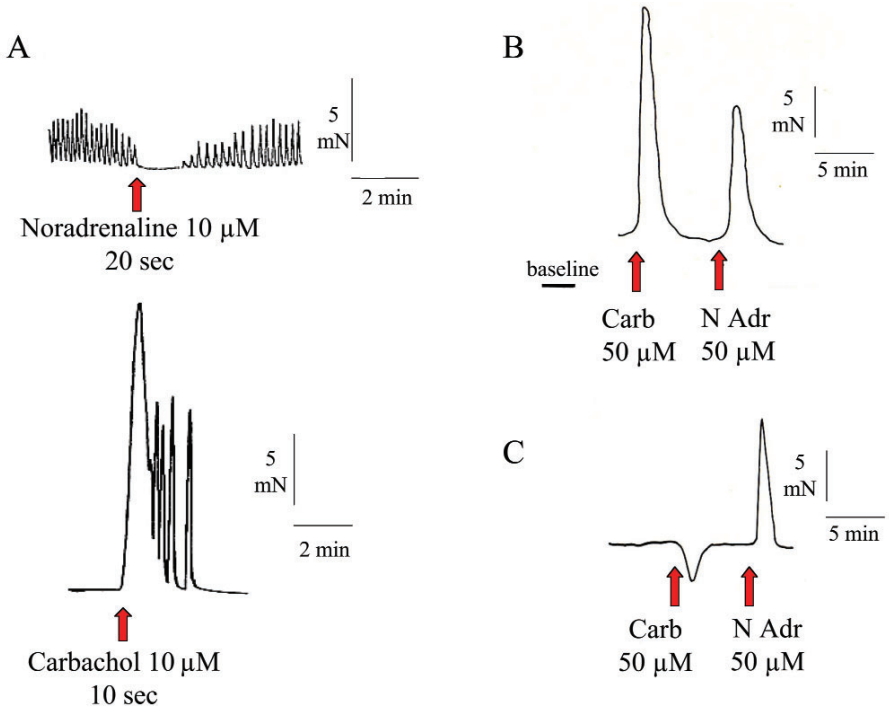


***Figure 24. Effects of noradrenaline and carbachol on smooth muscles of the lower G-I tract***
***A: Effects of noradrenaline and carbachol on a strip of human rectum circular smooth muscle. B: Responses from the conjoint longitudinal coat of the human anal canal. C: Responses from the internal anal sphincter. In B and C the responses to carbachol were obtained in the presence of the nicotinic receptor antagonist hexamethonium (1 µM). Modified from [365]***

and to the adjacent smooth muscle cells through gap-junctions. Interstitial cells also run along the outside of smooth muscle bundles within the muscle layers (IC-IM). IC-MY and IC-SM are thought to be pacemakers in the human colon and rectum. IC-IM maybe involved in helping conduct the activity within the muscle layers. Isolated ICCs undergo rhythmic large depolarisations at frequencies similar to the phasic contractions seen in intact smooth muscles. The underlying mechanism has not been completely resolved, and may vary in different parts of the gut. It is thought that changes to $[Ca^{2+}]_i$ are determined by phasic release of $Ca^{2+}$ from endoplasmic reticulum through an $IP_3$-dependent mechanism and also uptake of $Ca^{2+}$ by mitochondria. This leads to activation of $Ca^{2+}$-dependent conductances (possibly $Cl^-$ channels) that can generate depolarising (inward) currents [368]. When linked together in networks and activated synchronously, the ICCs can inject sufficient current into adjacent smooth muscle cells to produce the characteristic slow waves of depolarisation. If these slow waves are of sufficient magnitude, they will trigger activation of voltage sensitive $Ca^{2+}$ currents in the smooth muscle cells, and may initiate action potentials and contraction [369].

## 4. Innervation

The intrinsic innervation of the gut is immensely complicated. As an example, Figure 22 [370] shows a schematic diagram with the various identified neurones in the pig small intestine. What are not shown in this figure are the ICCs. Evidence is rapidly accumulating that ICCs may be the main target for the excitatory and inhibitory motor neurones that alter the contractile activity of the smooth muscle layers [371]. The cell bodies of the neurones lie within the various plexuses, and the morphology and neuronal content of each varies along the gut.

**Figure 25** shows whole mount preparations of the myenteric and submucosal plexuses at various points in the distal gut. The density of the neurones in the myenteric plexus declines towards the distal rectum, and few ganglia are present in the anal canal. Ganglia will not normally be present in the strips of smooth muscle dissected for organ bath experiments. These will, however, contain various axons running within the smooth muscle strips, and these can be activated by electrical field stimulation. The density of the smooth muscle innervation has not been examined in the human ano-rectum, although an elegant study of the taenia of the guinea-pig caecum [372] shows nerves are relatively sparse within the smooth muscle bundles, and are frequently associated with ICCs. This is in contrast to mammalian urinary bladder in which the innervation is very dense, with every smooth muscle receiving at least one close contact with a parasympathetic nerve varicosity [373]. The ease with which the intrinsic axons can be

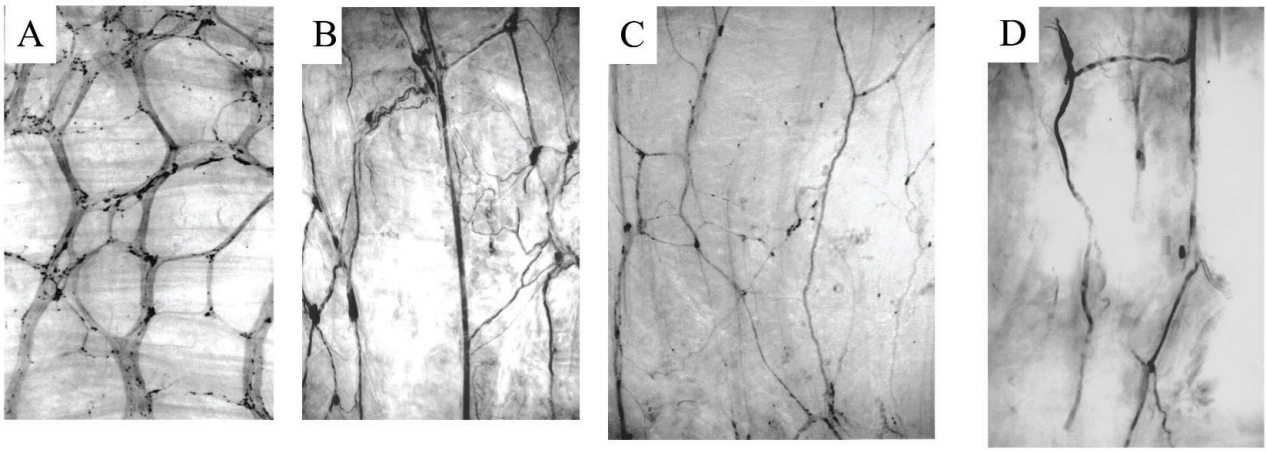


***Figure 25. Nerve plexuses in the human distal gut***
***Photomicrographs of nerve plexuses in the human distal gut, stained with NADPH diaphorase histochemistry to show NOS-containing neurones. A: Myenteric plexus in proximal two-thirds of the rectum. B: Myenteric plexus in the distal rectum. C: Submucosal plexus in the proximal rectum. D: Myenteric plexus in the upper anal canal. Modified from [377]***

activated depends on their size, with the larger axons having a lower threshold for activation. The release of transmitters and any co-transmitters or modulatory peptides may depend on the pattern of stimulation. Thus for any isolated strip the pattern of response will vary with different stimulation parameters, and also with the tone of the preparation. Judicious use of specific receptor antagonists or chemicals inhibiting synthesis of release of transmitters can be used to attempt identification of nerves present. Alternatively histochemical and immunohistochemical identification of intrinsic nerves in the strips can be attempted. Thus it is not surprising that the evoked responses of strips of rectal smooth muscle are normally quite small and varied - where there is initial tone, the normal response is a transient small relaxation followed by a small contraction.

The response of the internal anal sphincter is normally one of pure relaxation, although some isolated preparations show a mixed response consisting of an initial relaxation followed by a contraction (**Figure 26**). With pure relaxation responses there is little if any effect of blocking adrenergic nerves with guanethidine, and any muscarinic response with atropine. The relaxation of both rectum [374] and internal anal sphincter [375] is however attenuated by nitric oxide synthase inhibitors, and there is good evidence for NO being a major inhibitory transmitter in both. In addition the relaxant effect of muscarinic receptor stimulation in the internal anal sphincter is indirect [376] and also attenuated by NOS inhibitors [377].

The relaxation of the internal anal sphincter in response to a stretch of the rectum (the recto-anal reflex) is of fundamental importance in anorectal behaviour [377]. The inhibitory transmitter mediating this response is also NO [375], and there are NOS-containing neurones in the rectal myenteric plexus, as well as nitrergic nerve fasicles penetrating the internal anal sphincter [378] (**Figure 27**).

## 5. Abnormalities of innervation

Hirschsprung's disease is characterised by the presence of a non-propulsive, non-relaxing aganglionic segment of the gut that extends proximally for a variable distance from the distal rectum. The distribution of NOS-positive ganglia and nerves in resected bowel segments from infants with and without the disease was investigated [379].

In the transition zone between the ganglionic and aganglionic segment there was an alteration in the pattern of the neurones, with the ganglia and internodal axons lining up along the cranio-caudal axis of the gut, and a progressive decrease in the number of both, leaving the aganglionic area devoid of both ganglia and NOS containing nerves. Other nerves were however still present. Since inhibitory nerves are proposed to have their effects predominantly through the ICCs, the lack of inhibitory innervation may allow unrestrained excitation of the circular muscle through slow waves, accounting for the non-contracting activity, as well as the absence of the ano-rectal inhibitory reflex.

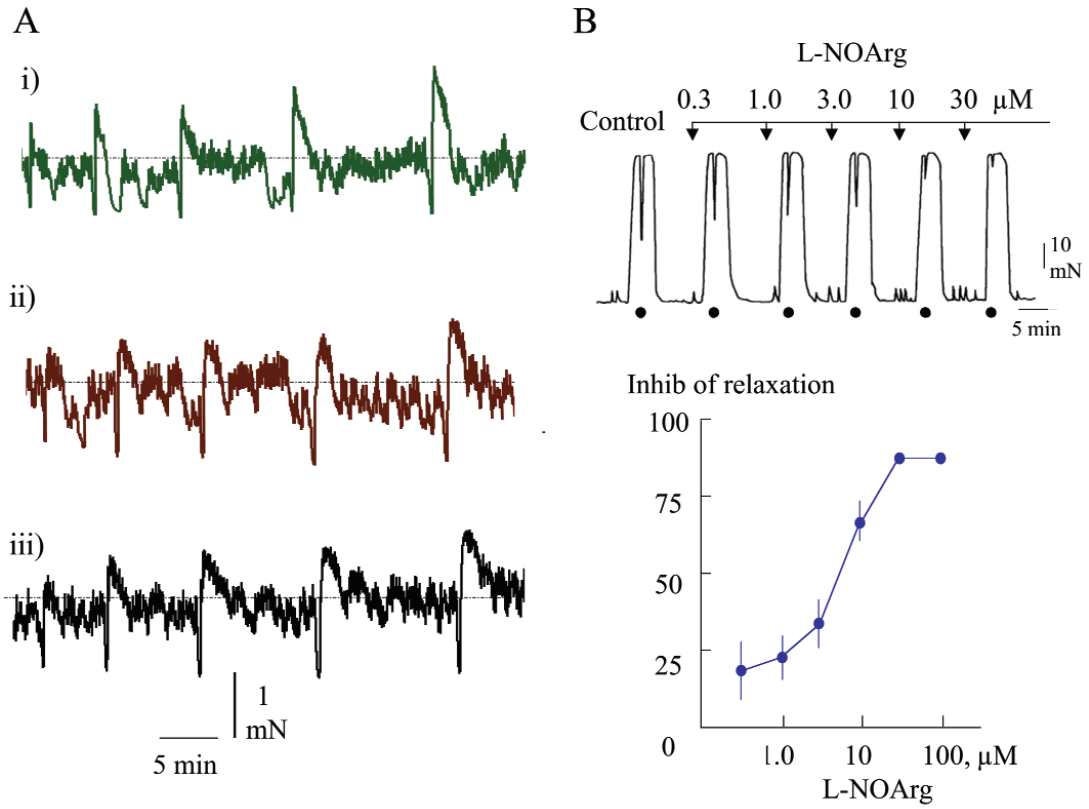


*Figure 26. Pig internal anal smooth muscle: evidence for nitrergic innervation*
*A: EFS at increasing frequencies from 1-40 Hz (0.2 ms, 30 V, 1 s trains). i) control, ii) after addition of 1 µM guanethidine, iii) after additional application of 1 µM atropine. This strip was unusual in showing a pronounced after-contraction after the initial relaxation, and spontaneous relaxations. Guanethidine slightly reduced the contractions, and atropine abolished the larger spontaneous relaxations. At least 5 min was left between each stimulation. The dotted lines show the base-line in each trace. B: Effects of the NOS inhibitor L-NOArg on the responses to electrical field stimulation (10 V, 0.5 ms, 8 Hz, 1 second trains) applied during the contractile response to 5 seconds of 3 µM histamine. The L-NOArg was increased from 0.3 to 30 µM. Below the tracing is a plot of the extent to which L-NOArg inhibited the relaxation due to electrical stimulation of the pre-contracted preparation. Mean data ± s.d*

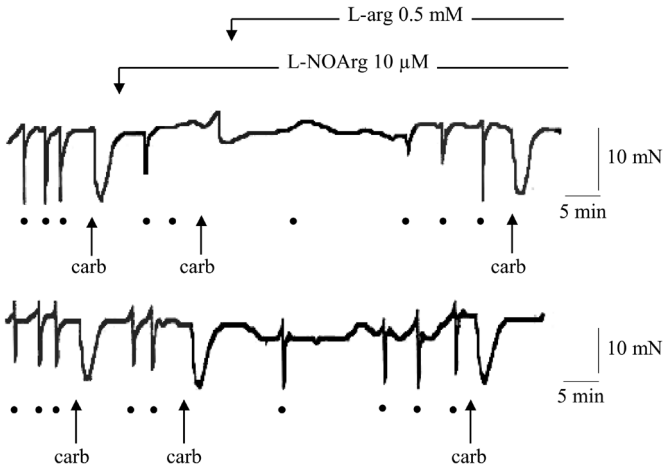


*Figure 27. Human internal sphincter: nitrergic responses*
*Responses to electrical field stimulation at spots (10 V, 0.5 ms, 10 Hz, 1 s train) and carbachol (100 µM at arrows) – both interventions generated relaxatory responses. Bottom tracing is control. Top tracing shows the effects of NOS inhibition with a competitive substrate L-NOArg and eventual reversal with excess L-arginine. Modified from [364].*

## VIII. RECOMMENDATIONS FOR BASIC RESEARCH

1. To place a greater emphasis on the integrated systems physiology and systems pharmacology of the lower urinary tract (LUT), lower gastro-intestinal tract (LGIT) and genital tract (GT). "Systems physiology" refers to the whole system as an integrated unit, such as LUT, rather than a part such as the urethra or bladder.
2. To generate and characterise good animal models to study the pathophysiology of the LUT, LGIT and GT.
3. To identify targeted drug models, using human tissue from well-characterised patient groups and tissue from animal models.
4. To generate a greater understanding of structure-function relationships of all the tissues of the LUT, LGIT and GT.

   These should include :

   - Smooth muscle function from: bladder dome, trigone, bladder neck and vesico-urethral junction; urethra prostate ; rectum and anus ; genital tract such as vaginal wall.

   Striated muscle of intraurethral sphincter, external anal sphincter and pelvic floor

   - Tissue interactions such as between epithelium and stroma.

   - Their functional innervation.
5. There should be a greater promotion of basic research into LUT, LGIT and GT function through:

   - increased collaboration between basic, medical and surgical sciences

   - greater representation of medical and surgical scientists on research advisory boards of major funding agencies.

   - identification of multidisciplinary research strategies to investigate LUT, LGIT and GT pathophysiology

   - organisation of structured, multidisciplinary research meetings on topics relevant to understanding the pathophysiology of the LUT, LGIT and GT.

   - the establishment of research centres of excellence.

## IX. GLOSSARY AND NOTES ON CONVENTION

| | |
|---|---|
| AP-1 | a transcription factor, formed as a heterodimer of c-jun and c-fos |
| ATP | adenosine triphosphate |
| BK channel | large conductance $Ca^{2+}$-activated $K^+$ channel |
| BOO | bladder outlet obstruction |
| BPH | benign prostatic hyperplasia |
| C | bladder compliance |
| cAMP | cyclic adenosine monophosphate |
| cGMP | cyclic guanosine monophosphate |
| c-fos, c-jun | cellular oncogenes, or proto-oncogenes |
| CGRP | calcitonin gene-related peptide |
| c-kit | a protein-tyrosine kinase receptor that is specific for Stem Cell Factor |
| CNS | central nervous system |
| CNTF | ciliary neurotrophic factor |
| CO | carbon monoxide |
| CREB | cAMP responsive element binding protein |
| Da | Dalton - unit of molecular weight |
| DEG/ENaC | degenerin/ENaC superfamily of ion channels |
| EC-coupling | excitation-contraction coupling |
| emg | electromyogram |
| ENaC | epithelial $Na^+$ channel |
| ER | endoplasmic reticulum |
| FGF | fibroblastic growth factor |
| GAG | glycosaminoglycan |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GDNF | glial derived neurotrophic factor |
| G-I tract | gastro-intestinal tract |
| G-protein | guanosine phosphate binding protein |
| GRK | G-protein coupled receptor kinase |
| GT | genitary tract |
| HIF-1 | hypoxia-inducible factor-1 |
| HSP | heat shock protein |
| 5-HT | 5-hydroxytryptamine (serotonin) |
| IC | interstitial cystitis |
| ICC | interstitial cell of Cajal |
| IC-MY | interstitial cells of myenetric plexus |
| IC-SM | interstitial cells of smooth muscle |
| $IP_3$ | inositol trisphosphate |
| KO mouse | (gene) knock-out mouse |
| LC17 | myosin light chain 17 kDa isoforms |
| LGIT | lower gastro-intestinal tract |
| L-NAME | Nω-nitro-L-arginine methyl ester |
| L-NOArg | $N_G$-nitro-L-arginine |
| LUT | lower urinary tract |
| M-receptor | muscarinic receptor (protein level) |
| m-receptor | muscarinic receptor (gene level) |
| MnSOD | manganese superoxide dismutase |
| mRNA | messenger RNA |
| MtDNA | mitochondrial DNA |
| NF-κB | nuclear factor κB - a transcription factor |

| | |
|---|---|
| NGF | nerve groth factor |
| NK | neurokinin |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| nNOS | neuronal nitric oxide synthase |
| ODQ | 1H-[1,2,4]oxxadiazolo[4,3-a] quinoxaline-1-one |
| Osm | osmoles |
| P1 receptor | purine receptor of type 1 family |
| P2X receptor | purine receptor of type 2X family |
| P2Y receptor | purine receptor of type 2Y family |
| PDGF | platelet-derived growth factor |
| PGA | polyglycolic acid |
| PKC | protein kinase C |
| PLA | polylactic acid |
| PLGA | poly(lactic-co-glycolic) acid |
| $PO_2$ | partial pressure of $O_2$ |
| Rho kinase | a serine/threonine kinase |
| ROS | reactive oxygen species |
| RT-PCR | reverse transcriptase - polymerase chain reaction |
| S | Siemen - unit of electrical conductance |
| SERCA pump | sarco/endoplasmic reticulum $Ca^{2+}$ pump |
| SM1,2 | smooth muscle myosin heavy chain (SMMHC) isoforms, SM isoforms |
| SNAP | synaptosomal associated protein |
| SNARE | soluble N-ethylmaleimide sensitive factor attachment receptor |
| SR | sarcoplasmic reticulum |
| TEA | tetraethyl ammonium (ion) |
| TGF-β | transforming growth factor-β |
| trk-A | trk proto-oncogene family member |
| TRP | transient receptor potential |
| UDIF | urothelial-derived inhoibitory factor |
| UP | uroplakin |
| UT-B | urea transporter-B |
| v | (smooth muscle) velocity of shortening |
| vmax | maximum velocity of shortening |
| VEGF | vascular endothelial growth factor |
| VIP | vasoactive intestinal peptide |

## NOTES ON CONVENTION

Throughout units of parameters and variables are expressed in SI units and in particular are based on the SI units of: length, metre (m) ; mass, kilogramme (kg) ; time, second (s) ; electric current, ampere (A); amount of substance (mol, M). SI unit prefixes are also used, in particular k ($10^3$), m ($10^{-3}$), $\mu$ ($10^{-6}$), n ($10^{-9}$), p ($10^{-12}$).

Derived units are combinations of the SI units and include those for : voltage (V, = kg $m^2$ $s^{-3}$ $A^{-1}$) ; resistance ($\Omega$,= $V.A^{-1}$) ; conductance (S = $\Omega^{-1}$) ; force (Newton, N = $kg.m.s^{-2}$) ; frequency (Hz, $s^{-1}$).

When the meaning is clear some non-SI units, but with derivation from SI units, are used; these include gram (g), minute (min), hour (hr).

Some non-SI units that do not have a precise definition are also used on occasion when their inclusion harmonises with conventional use: these include litre (l, approximating to $dm^3$) and $cm.H_2O$ as a unit of pressure. The term Osm refers to the effective number of moles in a solution (per unit kg solvent) that exert an osmotic pressure. The term Da (Dalton) is a unit of molecular or atomic weight.

Note that the molar unit of concentration (moles per $dm^3$ solvent), is used throughout and is donated by the letter M. The non-standard form of concentration mol/L is avoided, as it has no meaning in the SI system of units (L is the SI unit symbol for electrical self inductance).

Symbols for ions in solution, e.g. $Ca^{2+}$, $Na^+$, etc, refer to the species that are presumed to take part in chemical reactions. No assumptions are made about the activity coefficient of the species in solution.

Symbols for metals, Ca, Na, etc, refer to chemical moieties and this makes no statement as to the subfraction that will take part in a biological process, eg Na-pump.

When experimental data are given that use techniques to measure quantities that cannot measure exclusively the ionic sub-fraction, the name is either written in full, or the chemical symbol is quoted without any valence charge.

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