INVESTIGATING THE ROLE OF PDGFRα+ INTERESTITIAL CELLS IN BLADDER DETRUSOR CONTRACTIONS – COMPUTATIONAL STUDIES

M-Tech Stage-II Report

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by

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TABLE OF CONTENTS

ABSTRACT

PDGFRα+ cells are a specialised population of cells found lying in the interstitial space of visceral organs of the GI Tract and the urinary bladder. They form a network of cells with smooth muscle cells and interstitial cells of Cajal, and this heterogeneous syncytium is known as the SIP syncytium in literature. Cells in these networks interact with one another electrically via gap junctions. As well, the ICC and PDGFRα+ cells have the mechanisms to integrate external stimuli which are both neural and mechanical in origin. More specifically, PDGFRα+ cells are known to mediate inhibitory purinergic neurotransmission, which is a unique feature of this cell type. Here, as part of this thesis, we aim to explore two pathways, (i) coupling of P2Y receptors with SK3 channels and (ii) and coupling stretch sensitive Piezo1 and TRPV4 channels with SK3 channels, towards generation of inhibitory signals when presented with purinergic and mechanical stimuli. This report details a detailed literature survey for the two aforementioned signaling pathways, and also included computational studies of the first pathway using the framework described by Yeoh et al., 2016. The model was implemented on MATLAB and a detailed analysis of this model was performed, its advantages and its limitations were ascertained, and as well of the mechanisms present on PDGFRα+ cells not captured by this model was studied from literature. Finally, a conceptual framework including new mechanisms which include the TRPV4-SK3 coupling. Piezo1-SK3 coupling and other ionic conductances has been proposed as part of future work.

Keywords: PDGFRα+ cells; Purinergic Signaling, TRPV4; Piezo1; Bladder Electrophysiology

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

The urinary system is a visceral organ system located in the lower abdominal portion of the body. Its primary function is to collect, transport, store, and expel urine in a highly coordinated fashion. The urinary tract ensures the elimination of metabolic waste products and toxic waste products generated by the kidney. It extends from the proximal end of the renal papillae to the urethra via the distal-end of renal papillae, renal pelvis, ureters and the urinary bladder. Each of these components of the urinary tract has distinct anatomical features, distinct cell types and each part plays a unique and critical function. The principal function of the bladder and its associated organs is the storage and coordinated release of urine. The mechanism of storage and expulsion of urine is brought about by the combined action of neural networks present in the urinary system and the association of these neural networks with the various cell types present in the bladder wall. However, many aspects of the functioning of the bladder at the cellular level is still poorly understood. In this study we are investigating the role of a specific cell type called the platelet derived growth factor receptor alpha positive (PDGFRα+) cells (Fowler et al., 2008) which are found occupying interstitial spaces in the bladder wall using computational modeling and methods.

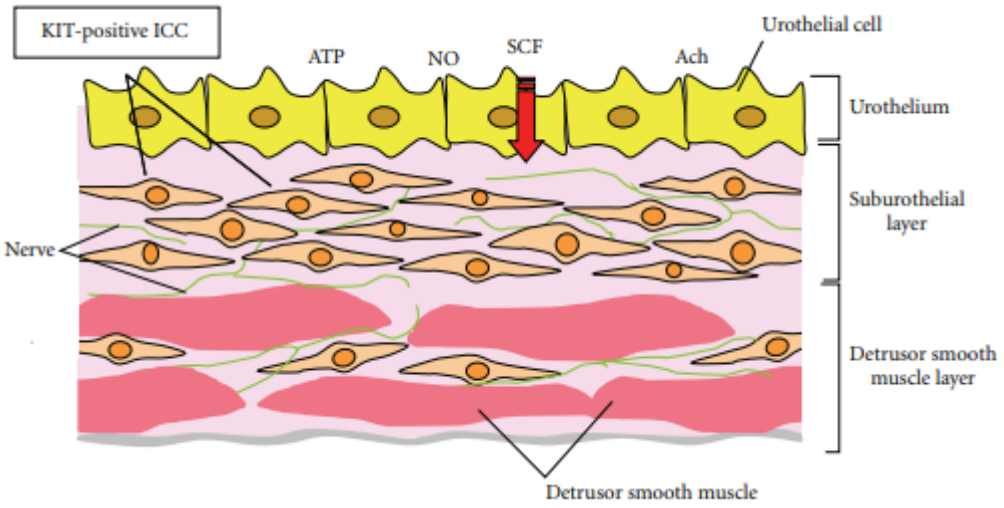


Figure 1.1: A schematic of the layers in the urinary tract wall (Kubota et al., 2011)

PDGFRα+ cells are a subtype of interstitial cells (ICs). They were first reported in the gastro-intestinal (GI) tract and they are known to be morphologically similar to fibroblast cells, hence they are also called fibroblast like cells (FLC). Extensive studies conducted on this cell population revealed that unlike fibroblast cells, these cells have more crucial role in purinergic inhibitory neurotransmission (Kurahashi et al., 2014; Lee et al., 2014). PDGFRα+ cells in the bladder as well as in the GI tract are distributed along the processes of enteric motor neurons and between longitudinal and circular muscle bundles. They are located in close proximity to nerve cells and form gap junctions with smooth muscle cells (SMCs), forming SIP syncytium (explained in chapter 2).

These cells abundantly express P2Y1 receptor, one of the major purino receptor responsible for the Ca^{2+} transients in the GI tract and small conductance Ca^{2+} activated K^{+} channels, responsible for the hyperpolarization, leading to inhibitory responses (Kurahashi et al., 2011; Kurahashi et al., 2014). Evidences of these cells present in close proximity to SMCs and nerve cells, along with electrophysiological studies investigating its functional role suggest a likely role of these PDGFRα+ cells in mediating inhibitory neurotransmission which helps in the relaxation of bladder detrusor smooth muscle (Baker et al., 2013; Kurahashi et al., 2014). However, the underlying cellular processes which bring about the function of these cells are still not clearly understood.

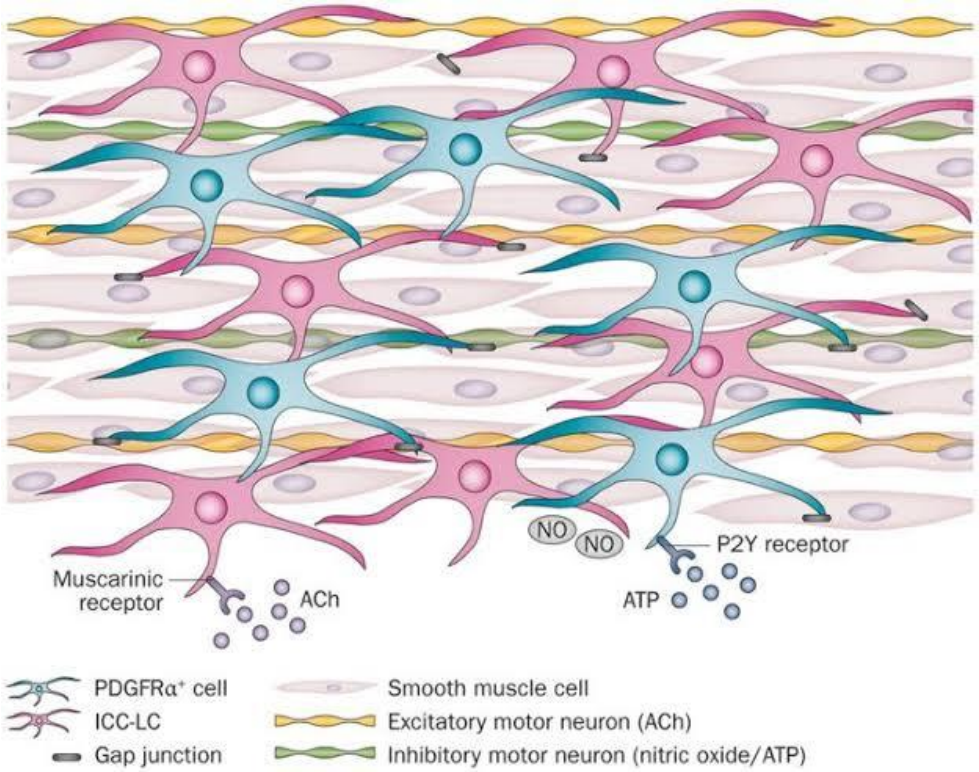


Figure 1.2: A schematic of PDGFRα+ cells in the Ca^{2+} signalling (Drumm et al., 2014).

As part of studies carried out in my MTP stage II, we have done an extended literature survey focussed on the functional role of PDGFRα+ cells with special emphasis on the ion transport mechanisms involved in this cell population; Also, included in this report is the implementation and analysis of an existing computational model by Yeoh et al., (2016), which investigates the cellular pathways for purinergic signal transduction in PDGFRα+ cells. The following section summarises the objectives of the present study.

1.2.OBJECTIVES OF THIS STUDY

- To develop a biophysically detailed computational model of PDGFRα+ cells for the urinary bladder which captures two essential features of PDGFRα+ cell physiology, namely, (i) purinergic inhibitory neurotransmission; (ii) stretch mediated inhibitory responses
- To understand the functional relationship between ATP activated P2Y1 receptor and SK3 channels. The coupling of this pathway involves the role calcium dynamics via the Endoplasmic Reticulum (ER).
- Study the coupling mechanisms between TRPV4 and SK3 in suppressing bladder contractions

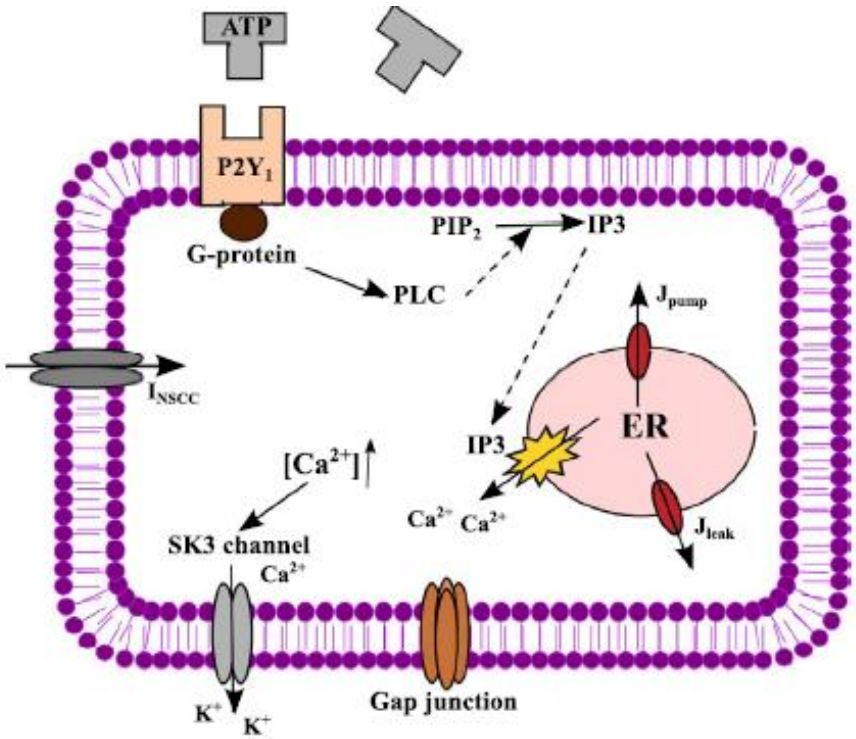


Figure 1.3: A schematic representation of the PDGFRα+ cell model (Source: Yeoh et al., 2016)

1.3. WORK DONE IN MTP STAGE I

a. Learnt MATLAB (Introduction to programming with MATLAB, MOOC- Coursera, Vanderbilt University) | MATLAB is the platform on which all modeling work for these studies is being carried out in the lab.

b. Conducted a first round of literature survey towards studying the biophysical properties and the electrophysiology of PDGFRα+ cells.

c. Implemented and analysed a computational model reported by Yeoh et al., (2016), see figure 1.3 for the schematic of the existing model of PDGFRα+ cell. Verified different components of the model, identified shortcomings of certain model components and rectified a few of them to reproduce the results as reported by Yeoh et al., (2016).

1.4. WORK DONE IN MTP STAGE II

i. Conducted an extended literature survey focussing on PDGFRα+ cells in the Bladder. The focus of the extended literature survey included understanding Purinergic inhibitory regulation and the effect of UTP on PDGFRα+ cells in murine bladder (Lee et al., 2014; Lee et al., 2017; Koh et al., 2018); Effect of UTP on Ca^{2+} activated K^{+} channels in PDGFRα+ cells (Lee et al., 2015); Expression of mechanosensitve Piezo1 channels in PDGFRα+ cells (Dalghi et al., 2019); Properties of SK3 channel in PDGFRα+ cells (Hayashi et al., 2019); Rhythmic Ca^{2+} events and signaling in lamina propria network which consist of PDGFRα+ cell populations in the bladder (Baker et al., 2013; Heppner et al., 2017)

ii. Based on the extended literature survey, conceptualised and proposed a broader framework for the development of a bladder specific mathematical model of PDGFRα+ cells as part of future work

1.5.ORGANISATION OF THE REPORT

The report consists of 5 chapters. Chapter 1 gives an introduction to the work. Chapter 2 provides a survey of some important findings regarding PDGFRα+ cell physiology from the literature and includes the extended literature survey. In chapter 3, we detail the methods describing the model components and important equations for the same. Chapter 4 contains the results of the simulations performed and discussion/analysis of the key results. Finally, chapter 5 details mechanisms to be modelled for developing a bladder specific PDGFRα+ cell.

CHAPTER 2

LITERATURE REVIEW

2.1. INTERSTITIAL CELLS IN THE BLADDER

Interstitial cells (ICs) are the cells present in an unspecific number in between any specific cell type. The urinary tract ICs are found in between the smooth muscle bundles below the epithelial layer that face the lumen of the urinary tract, or they are found between glandular and stromal cells. Interstitial cells in the urinary bladder were considered to be similar to the interstitial cells of Cajal (ICCs) of gastro-intestinal (GI) tract. These cells in GI-tract have pacemaker roles, and they are the reason for the generation of contractions such as the peristaltic movements of the GI-tract. In urinary tract also the ICs were first identified to have similar structure and functions that of the ICCs. Further studies in this field has shown that the bladder ICs are much more complex and have a lot of variance of cell types in it. The bladder ICs are broadly grouped into many types. This difference in cell types may occur due to the extensive spreading of these ICs, which may lead them to differentiate into several phenotypes. Majorly they are divided into, interstitial cells of cajal like cells (ICC-L), fibroblast like cells, mast cells, and telocytes. This grouping was made based on the Immunohistochemical studies. The reason for these ICs to differentiate into different phenotypic cells are not known, literature suggest it may be due to some diseased state or any other factor which lead to the differentiation of one type of IC to another (Koh et al., 2018).

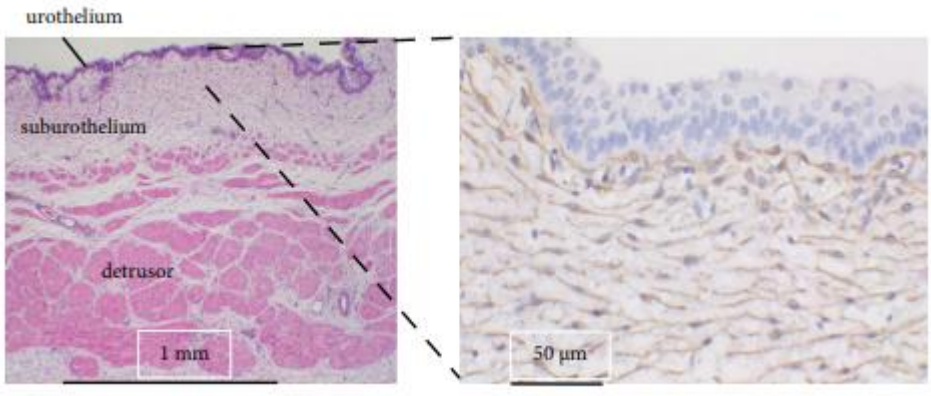


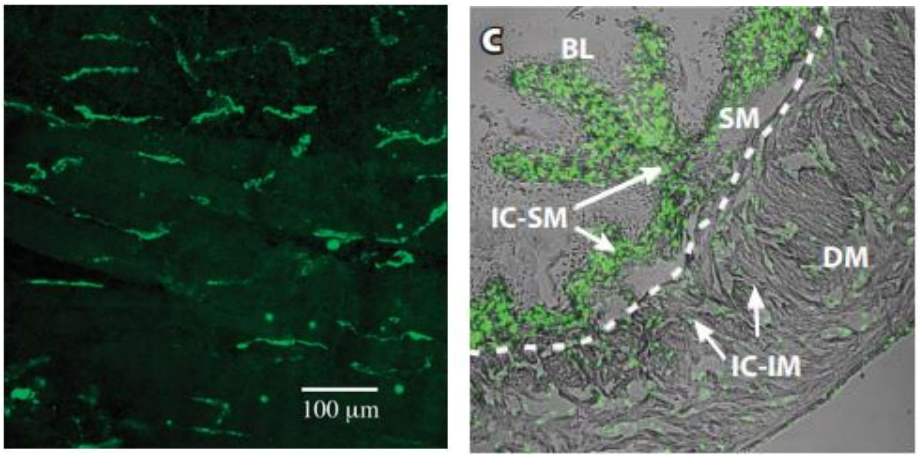
Figure 2.1: Sheep bladder wall stained with haematoxylin and eosin showing a network of ICs in the urothelium and suburothelium (Birder et al., 2011)

Bladder is an organ that expands significantly in volume without increasing the pressure associated with it. The bladder smooth muscle cells (SMCs) have calcium channels activated by the elongation of cells. Activation of these calcium channels initiates inward currents which in turn depolarizes the SMCs and increases the probability of voltage-dependent Ca2+ channel openings. Which leads to generation of action potentials and contractions. The interstitial cells (ICs) which are present in a large number in detrusor muscles helps in stabilizing the detrusor membrane potential. The lamina propria and submucosa of the bladder wall also consists a large number of interstitial cell populations, but the role of them in physiology and during pathological conditions of bladder are not well known (Wellner et al., 1993).

2.1.1. ICs in the Detrusor

Microscopic studies show that the cells with same properties as that of ICs of GI tract are also present in the detrusors of humans. These cells were located on the edges of detrusor muscle bundles and within them. These cells were bipolar and have long, thin sections which lies in close association with SMCs. Also, they have incomplete basal laminae, caveolae and organelles such as rough endoplasmic reticulum, mitochondria, and Golgi apparatus. Many of the properties of these cells are similar to the ICCs of GI tract, so they are referred as interstitial cells of cajal like cells (ICC-L). There is another group of bipolar cells without basal laminae, caveolae, or dense bands, these were called as fibroblast like cells (Koh et al., 2018).

Other than the ultrastructural similarity ICC-Ls vary in all other aspects from the ICCs of GI tract (majorly not being immunoreactive to CD117 (c-Kit)), and they show similarities to the PDGFRα+ ICs in the GI tract. A large number of PDGFRα+ cells are found in the detrusor muscles and submucosa. The PDGFRα+ cells in GI tract and bladder have spindle and stellate morphology. They appear to be organized into an interconnecting network between muscle bundles. Also, the PDGFRα+ cells are visibly different from the SMCs and they are distributed along the edges of the detrusor muscle bundles (figure 2.2) (Koh et al., 2012).



(a) (b)

Figure 2.2: (a) Urethra labelled with anti-Kit antibody, the fluorescence was shown by the cells expected to produce electrical signals (initially expected to be ICC or ICC-L), (b) PDGFRα+ cells distributed around and within the smooth muscle bundles of the detrusor muscle (Koh et al., 2012)

Another cell type present in ICs of the bladder are telocytes, but their physiological functions are unknown only means of understanding these cell types are the ultrastructural features. There are no specific immunological markers are present for these cells. They can be marked using the CD34 antigen and are expressed in many other cell types such as endothelial cells, suggesting that these cells have a wide variety of phenotypes (Vannucchi et al., 2014).

2.1.2. ICs in the Submucosa

In submucosal region, there are abundant number of interstitial cells (ICs), complex nerves and blood vessels present. Also, studies show that there is no much difference in the cells present in the lamina propria and submucosa. This suggests that the submucosal region is actively involved in the regulation of neural signals to the detrusor muscle. The ICs present in the submucosa are divided as c-Kit+ cells, ICC-L, fibroblasts, and myofibroblasts. The ICs in submucosa are identified using the markers c-Kit, vimentin, and PDGFRα. Majority of submucosal cells are positive to PDGFRα, also these PDGFRα+ cells are vimentin+ and c-Kit− in humans. These cells are located near nerve varicosities. The ICs of submucosa also contains fibroblasts, and myofibroblasts like cells, which are involved in the contractile mechanisms. When studied using immune-labelling they found to be positive to α-actin, vimentin, desmin, and fibro nexus. Also, they show a stellate morphology like PDGFRα+ cells with interconnecting networks. This suggests a possibility of PDGFRα+ cells converting in to myofibroblast phenotypes in diseased conditions (McCloskey et al., 2010).

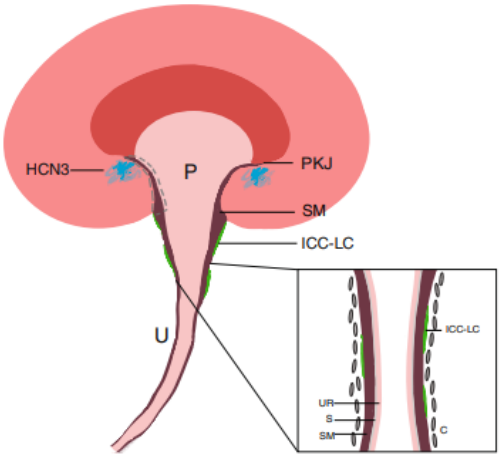


Figure 2.3: Location of different cell types in ureters (Feeney et al., 2014)

The ICC-L cells identified to have cholinergic properties have close association with the varicose nerves. But it is clear from the studies that ICC-L cells in the bladder do not have any direct or specialized contact with any nerve fibre, but the ICCs of GI tract are in direct contact with nerve fibres. Also, some literature says that the pre-contracted detrusor muscles are relaxed by the nitrergic effects of the bladder muscles (by nitric oxide (NO)). Receptor for the NO is soluble guanylyl cyclase, and these soluble guanylyl cyclase in detrusor muscles are found to be localized in PDGFRα+ cells by immune-histochemical studies. But their function is not well known. Ectonucleoside triphosphate di-phosphohydrolase 2, is a membrane ectonucleosides that hydrolyses 5 -triphosphates (E.g. ATP to AMP), found in the cells whose anatomical location is similar to that of ICs, but they are not found in SMCs. Co-labelling of ectonucleoside triphosphate di-phosphohydrolase 2 along with CD34, vimentin and CX43 suggested that, cells which express ectonucleoside triphosphate di-phosphohydrolase 2 can be PDGFRα+ cells. These concludes that coexistence of ICs with SMCs, which express NTPDase1 (CD39) and 5 -nucleosidase (NT5E or CD73), contribute to deactivation of purines (Yu et al., 2011; Koh et al., 2018)

2.2. PLATELET DERIVED GROWTH FACTOR RECEPTOR ALPHA POSITIVE CELLS

The platelet derived growth factor receptor alpha positive (PDGFRα+) cells or the fibroblast like cells were discovered in 1974 as a serum factor which stimulate the vascular smooth muscle cell proliferation. This growth factor has various functions in different physiological and pathophysiological conditions, and also it acts as mitogen and chemotactic factors. The role of PDGFR is known for its role in embryonic development and wound healing. The role

of this growth factor in normal physiological conditions such as maintenance of vascular tone, platelet aggregation, and homeostatic regulation of interstitial tissue pressure are less known. cells of vascular smooth muscle, fibroblast, neurons, macrophages, and platelets show PDGFR expressions. PDGF has five isoforms, and these ligands react on their specific receptor tyrosine kinases PDGFRα and PDGFRβ, these are seen to be expressed on different cell types mentioned above (Monaghan et al., 2012).

2.3. ULTRASTRUCTURAL PROPERTIES

2.3.1. Distribution of PDGFRα+ cells

The platelet derived growth factor receptor alpha positive (PDGFRα+) cells are found in the gastro-intestinal tract and in the bladder. In the GI tract the antibodies against PDGFRα+ cells are known as fibroblast like cells, these cells are reported to be involved in the inhibitory purinergic neurotransmission. Distribution and identification of PDGFRα+cells in the murine bladder, human bladder and guinea pig bladder are extensively studied to know their ultrastructural properties. Immunohistochemical studies, confocal microscopy, fluorescence confocal microscopy, DAB immunohistochemistry, and western blotting are the methods widely used to study the cell structures and morphology (Koh et al., 2012, Monaghan et al., 2012).

PDGFRα+ cells in the ascending (A), transverse (T), and sigmoid (S) colons of human tunica muscularis has reported to have multi-polar shapes and they were seen to be interconnected with each other to form discrete network. When the muscle layers are circular and longitudinal, then the cell bodies and the processes of PDGFRα+ cells run parallel to the SMC bundles. The PDGFRα+ cells in the myenteric region are more stellate in shape, and also they formed extensively fine interconnecting networks within themselves resembling the ICCs. Even though the shape and the distribution of these cells vary in different regions, the morphology of the cells in all the regions are of the same (Kurahashi et al., 2012).

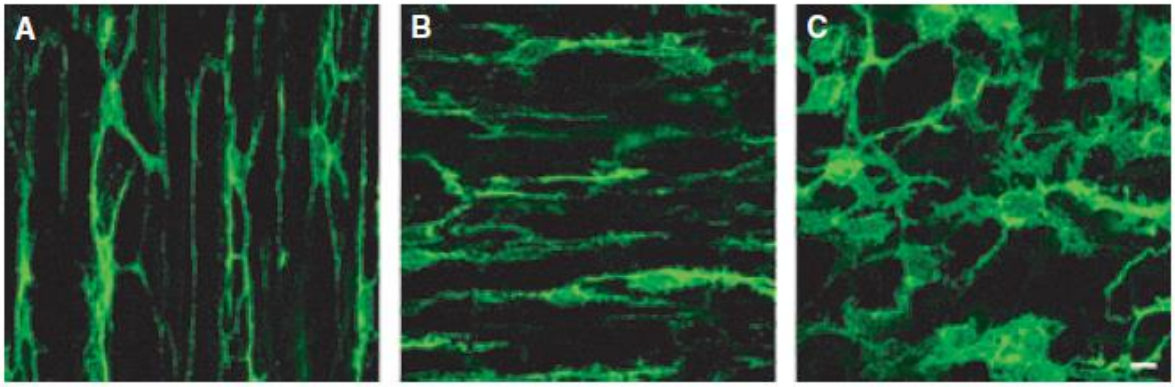


Figure 2.4: PDGFRα+ cells (green) in (A) circular muscle layer, (B) longitudinal muscle layer, (C) myenteric region. (scale bar = 10 µm) (Kurahashi et al., 2011).

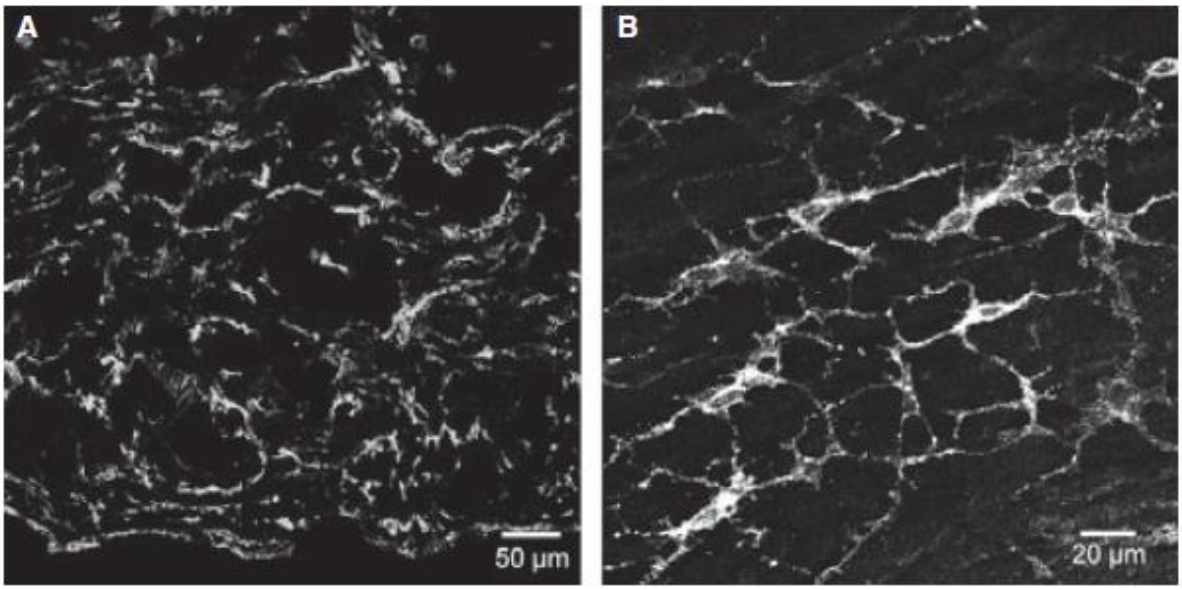


Figure 2.5: PDGFRα+ cells in the bladder detrusor cells in (A) entire muscularis. (B) whole mount of the bladder (Koh et al., 2012).

In bladder PDGFRα+ cells are widely distributed and possesses spindle- and stellate-shaped morphologies similar to that of the GI tract. These cells also have interconnecting network within themselves with cell body and multiple cell processes branching towards and making contact with the neighbouring cells. PDGFRα cells lie along the borders of smooth muscle bundles and within the detrusor muscle bundles, and also found between individual smooth muscle cells in smaller bundles of SMCs. A dense population of PDGFRα+ cells are present within the lamina propria of the bladder with the cellular network closely packed in the sub-urothelial region, and these cells are not found in the urothelial region. The PDGFRα + cells and SMCs are seen to be arranged in a complicated fashion throughout the detrusor muscles (Monaghan et al., 2012).

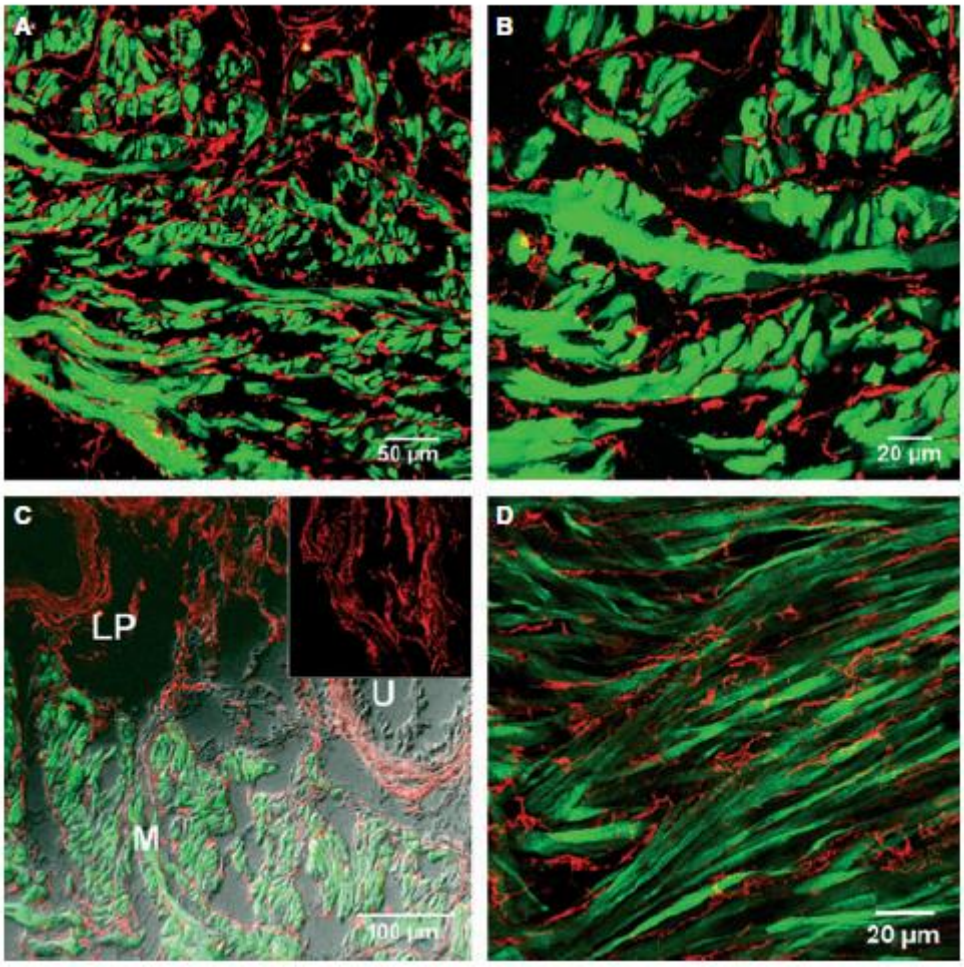


Figure 2.6: Double labelling of PDGFRα + cells and SMCs in the bladder detrusor (PDGFRα – red, smMHC/Cre/eGFP-green) (A) within the detrusor muscle. (B) lying interstitially along and in between the SM bundles. (C) in the muscularis(M), lamina propria (LP), and in urothelium(U). (D)Arrangement of PDGFRα + cells and SMCs in the whole mount (confocal image) (Koh et al., 2012).

2.3.2. Localisation of PDGFRα + cells

The networks formed by PDGFRα + cells are seen to be very similar to that of the ICC networks. This makes it necessary to investigate the cell types labelled with anti-PDGFRα and anti-C kit antibodies are similar or are they different subpopulation of interstitial cells, whether they have any close association or not. The cells when studied by collabelling it was observed that the PDGFRα + cells are different from the ICCs, but were located in the same region. Study of the localization with the neuronal cells are also important because of their reported role in the inhibitory neurotransmissions. These cells are reported to regulate the purinergic neuro transmissions by which inhibitory responses are achieved is via the small conductance

Ca^{2+} activated K^+ channels (SK3). The expression of these channels near the PDGFRα + cells are also confirmed by the Immunolabelling studies suggesting there might be a role of these cells in mediating the purinergic inputs (Figure 2.7) (Monaghan et al., 2012, Kurahashi et al., 2011).

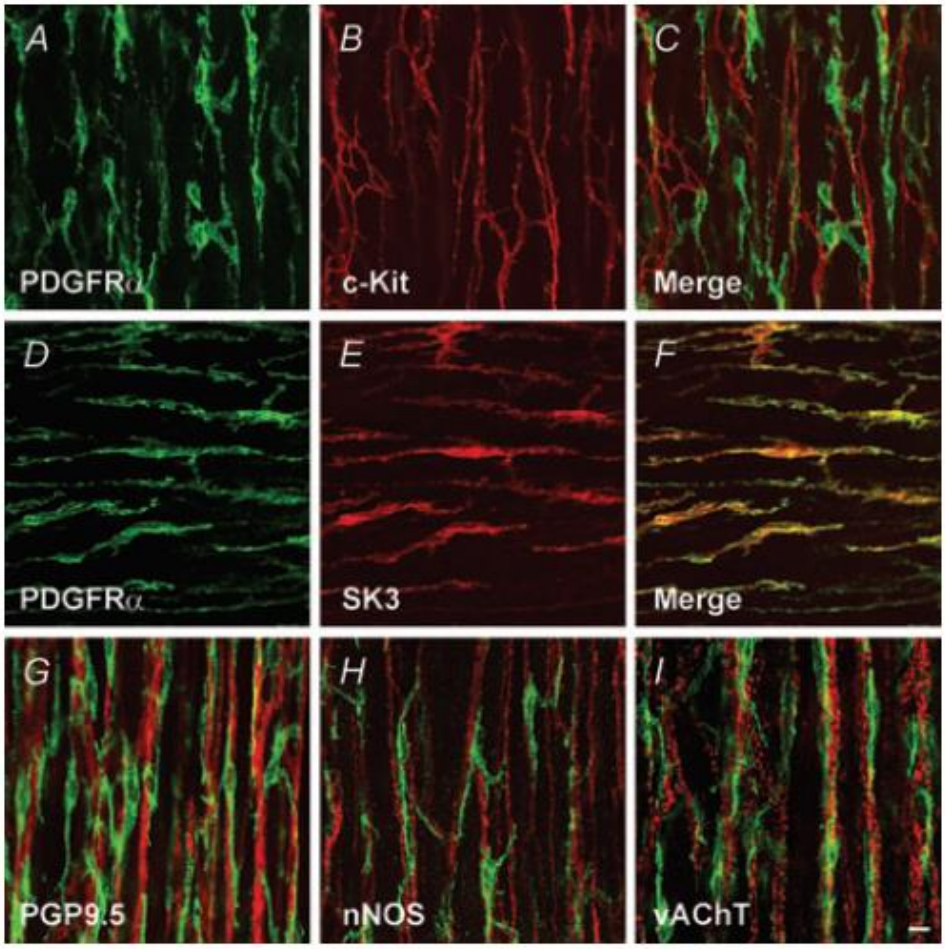


Figure 2.7: (A-C) double immuno-labelling of PDGFR*α* (green) and c-Kit (red), (D-F) double immuno-labelling of PDGFR*α* (green) and SK3 (red), (G-I) immunoreactivity for enteric neurons, inhibitory neurons and excitatory neurons respectively (in GI tract) (Kurahashi et al., 2011)

2.4. ELECTROPHYSIOLOGICAL RECORDINGS

Electrophysiological studies of PDGFRα + cells are studied to determine their role in the hyperpolarization leading to the outward current which are the hall marks of purinergic neurotransmission. PDGFRα + cells express P2ry1, encoding P2Y1 receptors and Kcnn3, encoding SK3. PDGFRα + cells also show transient and large amplitude Ca^{2+} currents in response with purines and these currents coming from PDGFRα + cells are apamine sensitive (a SK3 channel antagonist). These responses are mediated by P2Y1 receptors. Along with SMCs and ICCs, PDGFRα + cells (SIP syncytium) form a post-junctional receptive field for

enteric motor neurotransmitters. The manner in which these cells are electrically coupled in this SIP syncytium, any changes in the conductance of any of the three cell types affect the excitability of the greater syncytium. Thus, enteric motor neurotransmission can only be understood by which cell, receptor, and effectors mediate responses in normal and abnormal muscles. This makes a controversy about the source of outward current due to the purinergic neuro transmissions. Recent studies have showed that the purinergic neurotransmissions are due to the PDGFRα + cells, and SMCs gives out no outward current (Kurahashi et al., 2011,2014).

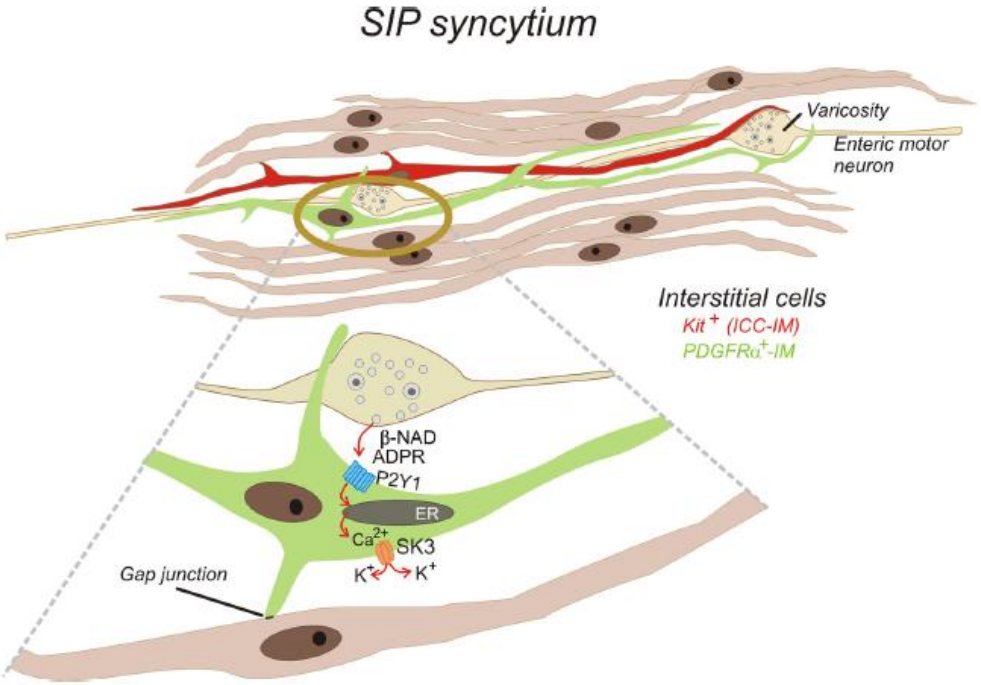


Figure 2.8: A schematic representation of SIP syncytium and the mechanism of enteric inhibitory motor neurotransmissions in PDGFRα + cells (Kurahashi et al., 2014)

Figure 2.10 shows the patch clamp experiment of PDGFRα + cells, and effect of ATP on the membrane currents of these cells. When doses of ATP were given to the cell, the cell responded by giving out an outward current depending on the dose of ATP given. Long exposure to ATP gives sustained current responses. ATP activated a large voltage-independent outward current and caused a negative shift, towards K^+ equilibrium potential in response to ramp depolarization. Under current clamp conditions ATP induced hyperpolarization, and activated an outward current in the same cell after switching to voltage-clamp mode (Lee et al., 2014).

All the purines including neurotransmitters, primary metabolites and selective P2Y1 channel agonists hyperpolarize PDGFRα + cells. hyperpolarization of these cells is blocked when P2Y1 antagonists and SK channel blockers such as apamine are used. When PDGFRα + cells are

hyperpolarized, the membrane potential is taken to the value of E_K, indicating that the responses are due to the activation of K^+ conductance. Also, the magnitude and rate of hyperpolarization indicates the high density of SK3 channels in these cells. When P2Y1 channel antagonists are used in SMCs they show no response to these blockers indicating the absence of purinergic neurotransmissions (Figure 2.10). Therefore, it is possible that the purinergic inhibitory responses of the bladder detrusor muscles are mediated by PDGFRα + cells (Kurahashi et al., 2014, Lee et al., 2014)

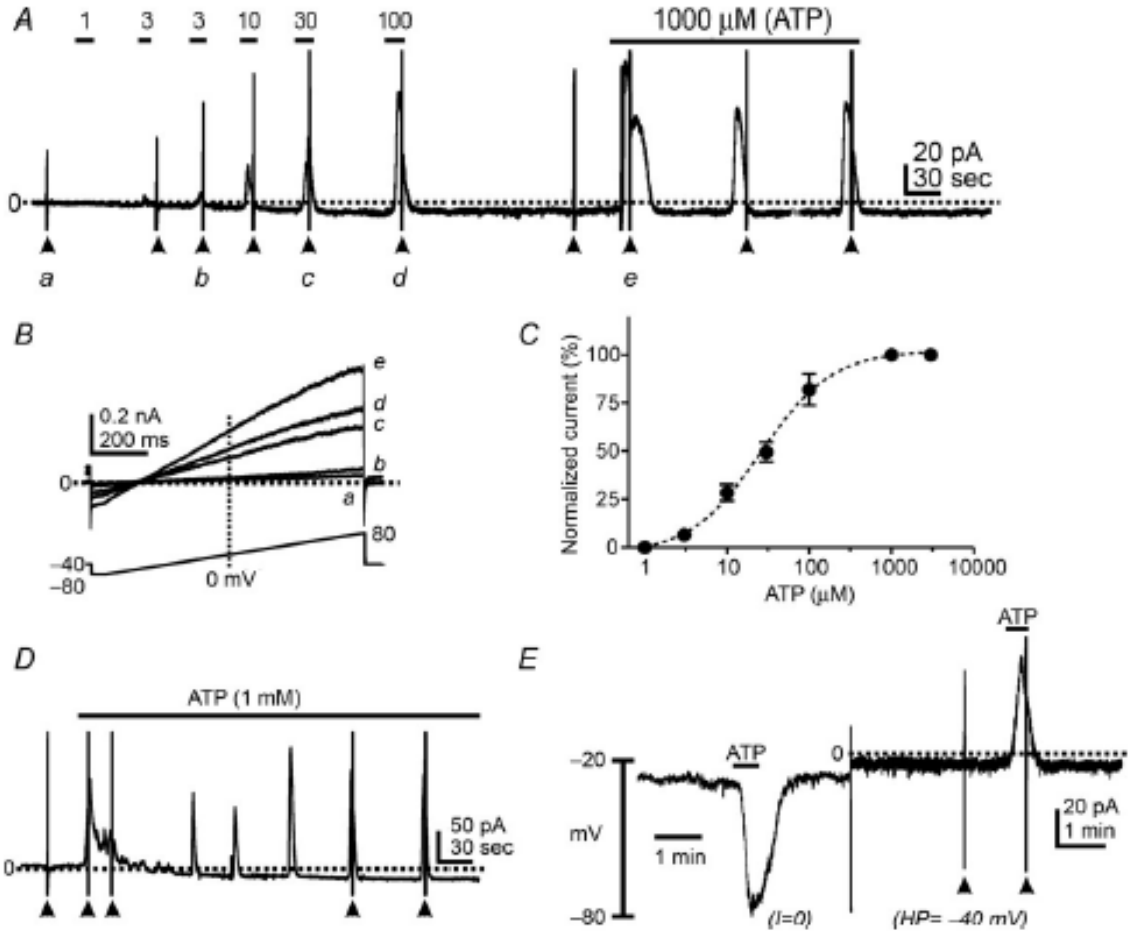


Figure 2.9: Effect of ATP on PDGFRα + cells (Lee et al., 2014)

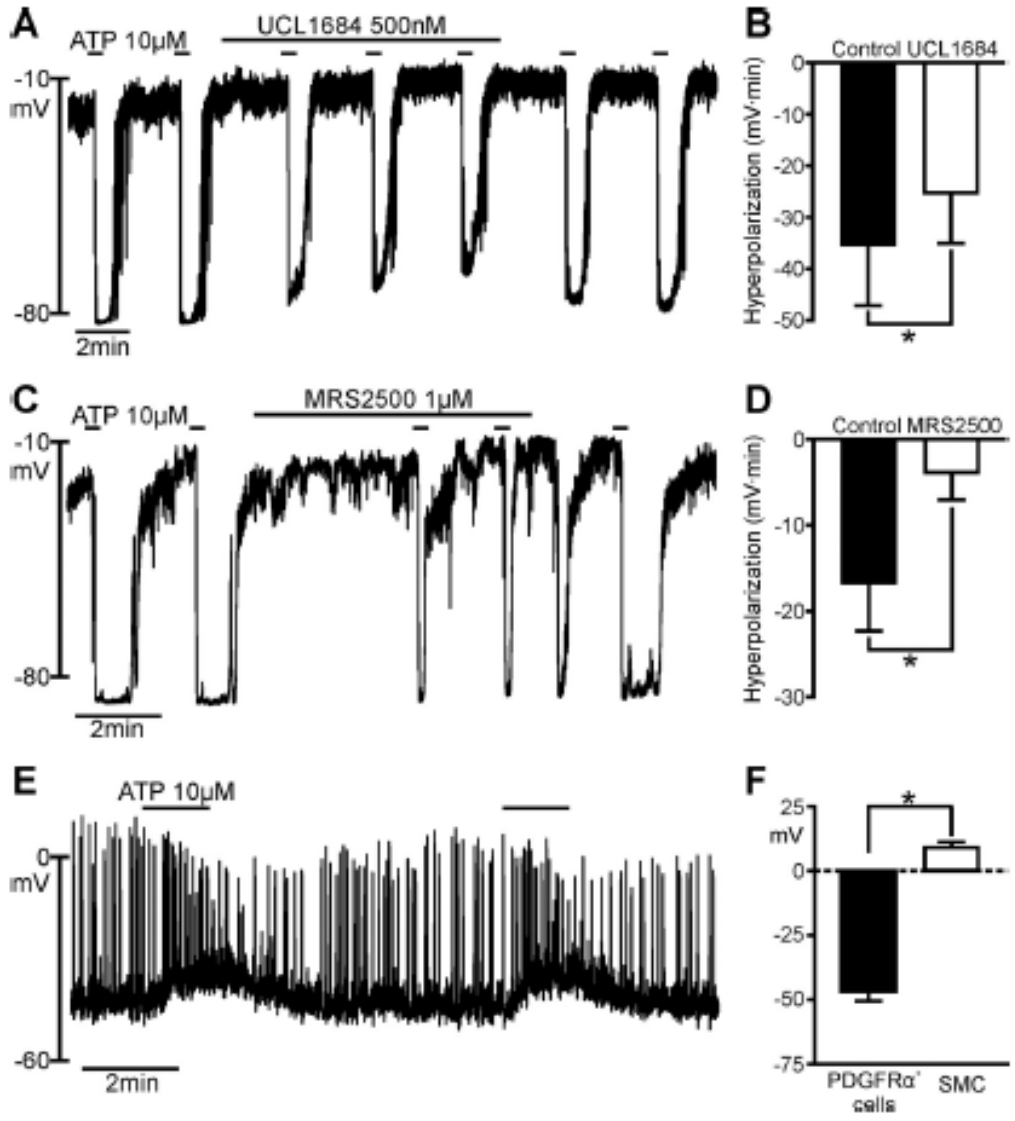


Figure 2.10: Effect of ATP on PDGFRα + cells and SMCs. (A-C) PDGFRα + cells with P2Y1 blockers, (E-F) SMCs (Kurahashi et al., 2014)

2.5. CALCIUM IMAGING STUDIES

PDGFRα + cells were initially identified as the fibroblast cells, because of their close resemblance to fibroblasts morphologically. However, their close association with motor neurons, expression of receptors and effectors for purinergic neurotransmission on their plasma membrane, and formation of post-junctional coupling with ICCs and SMCs suggest a more important functional role of these cells in enteric motor neuron regulation. Further, PDGFRα+ cells generate spontaneous Ca^{2+} transients. As PDGFRα + cells also express SK3 channels, the spontaneous and purine activated Ca^{2+} transients are coupled to the outward current produced by these cells.

Electrical coupling of these cells to SMCs suggests that the purinergic responses produced in the PDGFRα + cells plays a role in the relaxation of the muscles. Evidence that PDGFRα + cells are an integral part of SIP syncytium indicates their role in the regulation of excitability of the SMCs. Purinergic inhibitory neurotransmissions in the post junctional cells are due to the activation of SK3 channels, and it has been reported that PDGFRα + cells express an abundant amount of SK3 channels unlike SMCs and ICCs.

The ICCs play a role in the mediation of excitatory cholinergic and inhibitory nitrergic transmissions, but they play a much-reduced role in the purinergic neurotransmissions. Likewise, the role of SMCs in the purinergic transmissions are also very unlikely as their response to ATP at physiological potentials is limited to activation of non-selective cationic channels and associated membrane depolarization. As well another distinguishing feature is that SMCs responds to ATPs with a net inward current in the bladder (Monaghan et al., 2012; Lee et al.,2013).

Spontaneous Ca^{2+} transient currents in PDGFRα + cells were changed when neuro toxin tetradotoxin (TTX) was added, suggesting the role of PDGFRα + cells in the regulation of basal tone with the release of purines (figure 2.12). Literature shows that TTX and apamine inhibits the spontaneous inhibitory junction potentials (IJPs), these IPJs are due to the activation of P2Y1 receptors (Baker et al., 2013).

P2Y receptors are the main receptor for purinergic neurotransmission and among all the P2Y receptors P2Y1 is the primary receptor responsible for spontaneous IJPs. MRS-2500, an antagonist to P2Y1 receptor when used Ca^{2+} transients elicited by ADP, β-NAD, and MRS-2365 in PDGFRα + cells were blocked, showing the role of P2Y1 receptor in this Ca^{2+} transients (Figure 2.11). However, some PDGFRα + cells retained the Ca^{2+} transients, providing evidence that there are more types of purino-receptors expressed in the PDGFRα + cells (Baker et al., 2013).

Reduction in calcium concentration did not reduce the response to purines by PDGFRα + cells, but blocking Ca^{2+} uptake by SERCA pump blocked the Ca^{2+} transients showing that the Ca^{2+} transients in the PDGFRα + cells is due to the release of Ca^{2+} from intracellular calcium stores such as ER (Figure 2.13) (Baker et al., 2013).

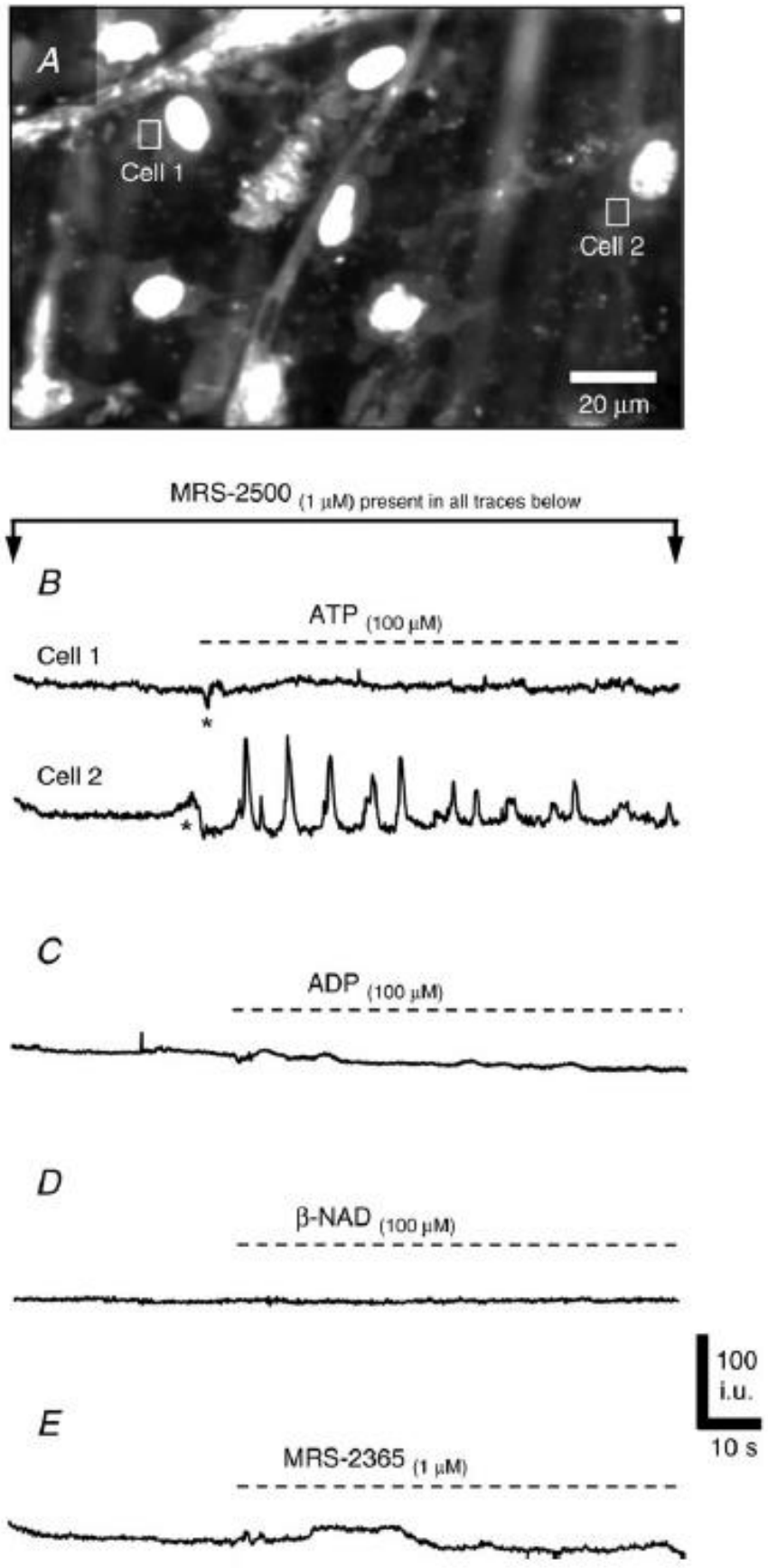


Figure 2.11: Ca^{2+} transients in the presence of MRS-2500 (Baker et al., 2013)

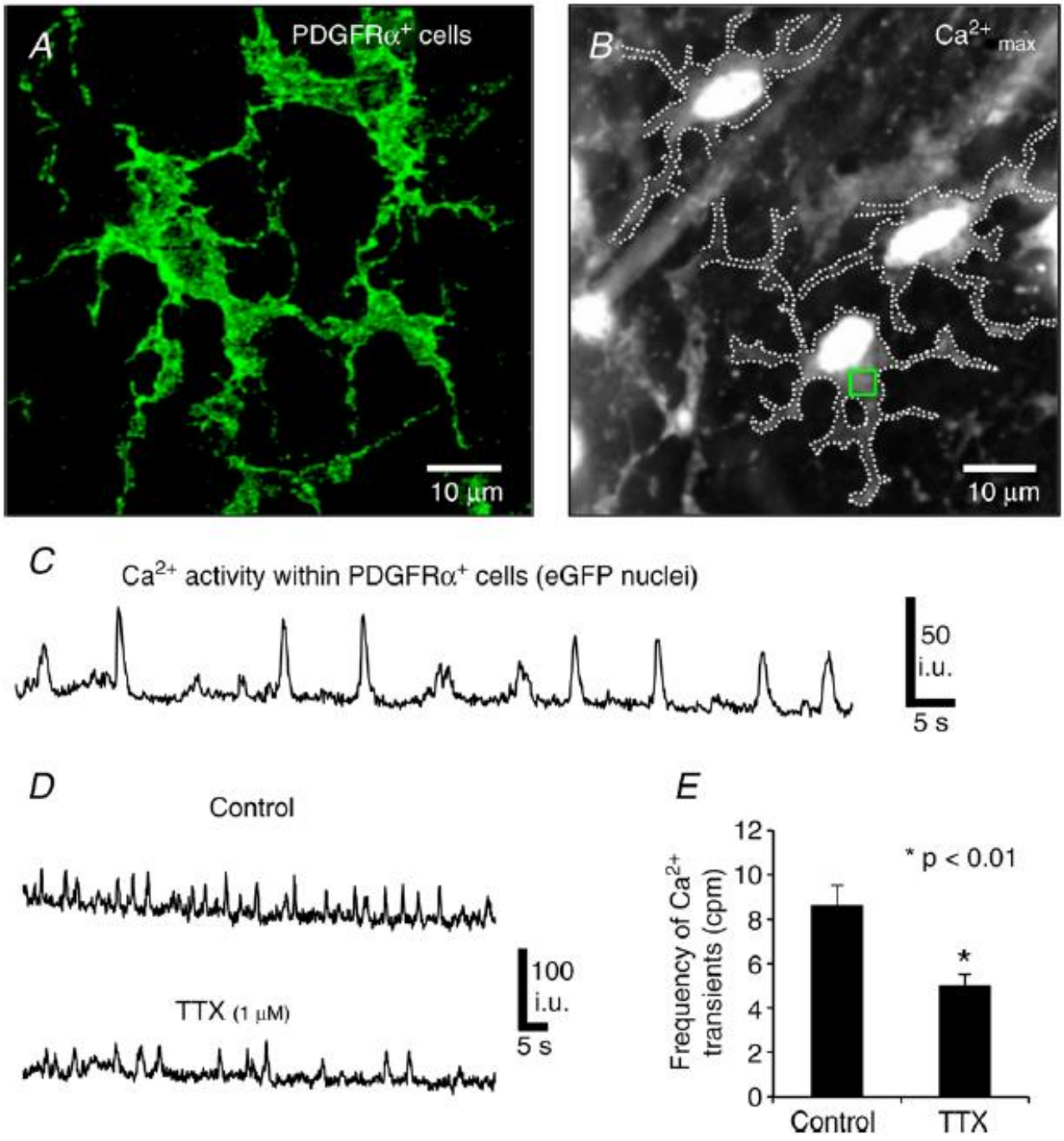


Figure 2.12: (A) PDGFRα + cells in myenteric region (B) Maximum Ca^{2+} fluorescence within eGFP- PDGFRα + cells (Region of interest (ROI)-green square), (C) Ca^{2+} transient in the normal PDGFRα + cells, (D) Ca^{2+} transient with drug, (E) summary of the process (Baker et al., 2013)

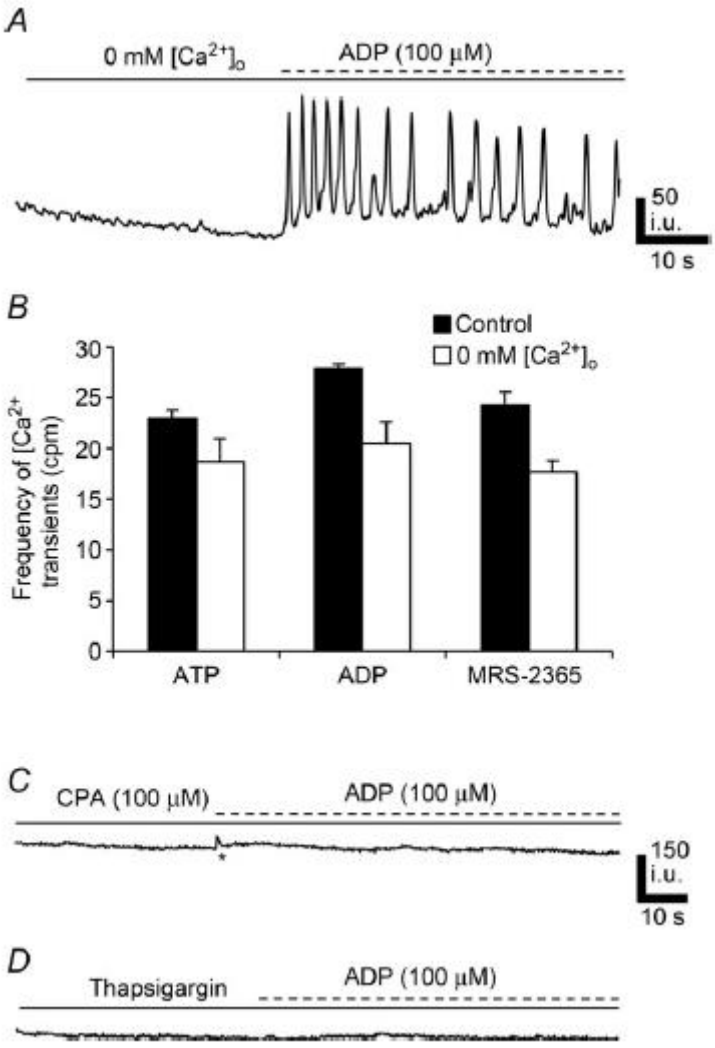


Figure 2.13: Effect of Extracellular and SERCA pump on Ca^{2+} transient of PDGFRα + cells. (CPA and Thapsigargin SERCA pump blockers) (Baker et al., 2013)

2.6. INTERACTION BETWEEN TRPV4 CHANNELS AND SK3 CHANNELS

In urinary bladder the volume increases drastically. However, the change in pressure associated with this volume change is small. This suppression of bladder wall contractions is suggested to be coupled with the activity of SK3 channels, but the underlying mechanism responsible for this process is not well understood. PDGFRα + cells are the cells which express SK3 channels and their activation is associated with the purino receptors such as P2Y receptor as mentioned in the previous sections. The SK3 channels are activated whenever there is a rise in Ca^{2+} ion concentration in the intracellular space. In majority of the cases this increase in concentration is through the activation of voltage dependent Ca^{2+} channels. But functional voltage dependent Ca^{2+} conductance is not reported in PDGFRα + cells. Therefore, this brings up the question of the source of external Ca^{2+} source in these cells. Transient receptor potential (TRP) channels,

which are mechanically activated channels, expressed in these PDGFRα + cells, in which vanilloid TRP (TRPV) channels are permeable to Ca^{2+} ions. Patch clamp experiments performed have confirmed that when an agonist (GSK) to TRPV4 channels was used in PDGFRα + cells they produced an inward current and also GSK activated an outward current in PDGFRα + cells when the cells were held at positive potential to E_K. This outward current is due to the activation of SK3 channel conductance. This suggests that there is a close relation between the stabilization of bladder contractions due to the influx of Ca^{2+} ions via TRPV4 channels and this in turn activates the SK3 channels (Lee et al., 2017).

2.7. REPORTED ROLE IN PATHOPHYSIOLOGY

Distribution of PDGFRα+ cells in the normal urinary bladder and in UPJ obstructed bladder were compared. It is very important to find out the pathophysiological significance of these cells to understand their functional roles. Ultrastructural properties can tell about the similarities shown by these cells to other cells, and the localisation of these cells with respect to other cell such as SMCs, neural cells, etc. during normal and diseased conditions. To find out whether PDGFRα+ cells are structurally associated with SMCs, PDGFRα antibody and phalloidin were double-labelled on UPJ samples. phalloidin labels filamentous actin. It was observed that the PDGFRα+ cells were located close to phalloidin+ SMCs, but they were structurally different from SMCs. PDGFRα+ cells surrounded phalloidin-positive smooth muscle bundles. C-kit is widely used to label ICC-Ls in the upper urinary tract. PDGFRα+ cells were found to be adjacent to these ICC-Ls, but were distinct from c-kit-positive ICC-Ls. ICC-Ls were remarkably reduced in disorders like UPJ obstruction. In order to determine whether PDGFRα+ cells are closely associated with nerve cells or not, a double-labelling immune-histochemical test was performed using a PDGFRα antibody and PGP9.5. PDGFRα+ cells were observed to be in close proximity with the PGP9.5+ nerve cells. In addition to that, in UPJ obstruction disorders, PGP9.5+ nerve cells were markedly reduced compared with normal UPJ cells (Figure 2.15) (Koh et al., 2018).

The expression of SK channels in PDGFRα+ cells and SMCs, a sub-family of Ca^{2+} activated K^+ channels were studied. These channels are found all over the central nervous system (CNS). Double-labelling immune-histochemical studies using SK2, SK3, PDGFRα, and Phalloidin antibodies were performed. Confocal-immunofluorescence-double staining revealed strong SK3 expression within a subset of PDGFRα+ cells (Figure 2.14). Also, SK3 expression in PDGFRα+ cells of the human UPJ was decreased in UPJ-obstructed samples when compared

with normal sample. Confocal-immunofluorescence double staining also showed that SK2 channels were expressed in SMCs, but not in PDGFRα+ cells. There was an increased SK2 immunoreactivity in SMCs of UPJ obstruction sample than the normal sample (Figure 2.16) (Koh et al., 2018).

2.7.1. Western blot

For ensuring the results obtained from immunofluorescence, protein expression levels were investigated using western blot analysis which revealed the expression of PDGFRα, PGP9.5, SK2, and SK3 in human UPJ. PDGFRα protein expression levels in UPJ obstruction samples were not different from the normal sample. However, western blotting confirmed decreased protein expression levels of PGP9.5 and SK3 and increased protein expression levels of SK2 in UPJ obstruction which was also seen in the immunofluorescence studies (Figure 2.17). Along with western blot RT-PCR was also performed to see the mRNA expression in the PDGFRα+ cells, PGP9.5, SK2, and SK3 channels. RT-PCR showed that there was no difference in the mRNA expression during disease and normal sample in case of PDGFRα+ cells. However, in PGP9.5 and SK3 this level was significantly decreased in UPJ obstruction, and in SK2 the gene expression was significantly high (Figure 2.17) (Koh et al., 2018).

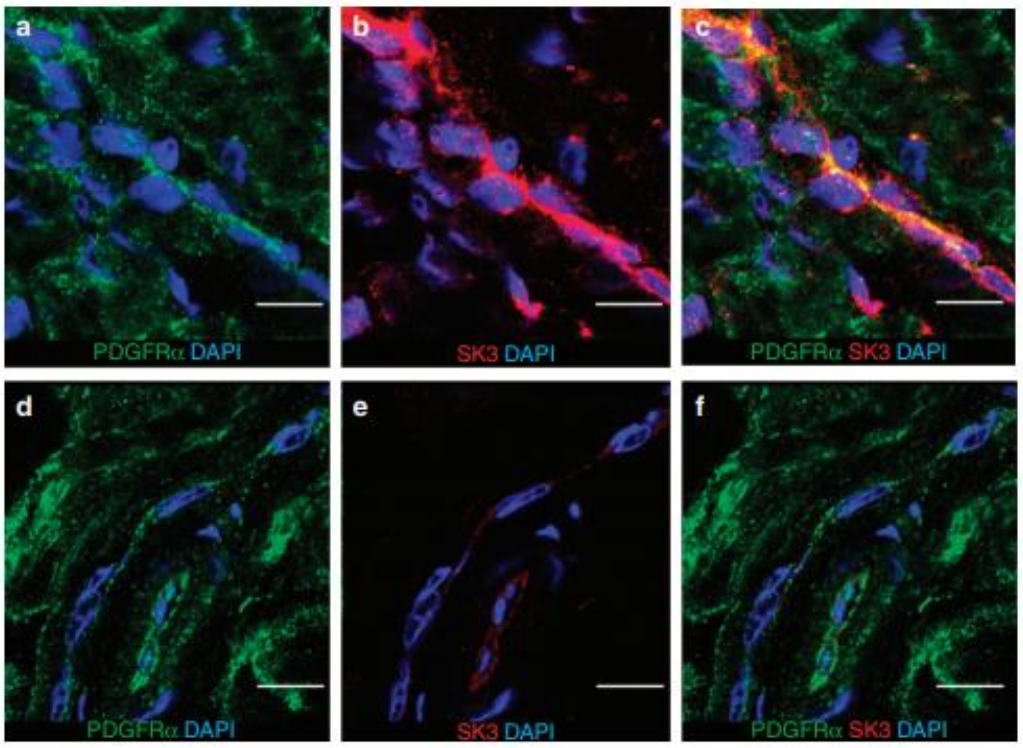


Figure 2.14: Expression of SK3 channels in PDGFRα+ cells and SMCs. (a-c) Normal cells, (d-f) diseased (cells with UPJ obstruction) cells (Koh et al., 2018)

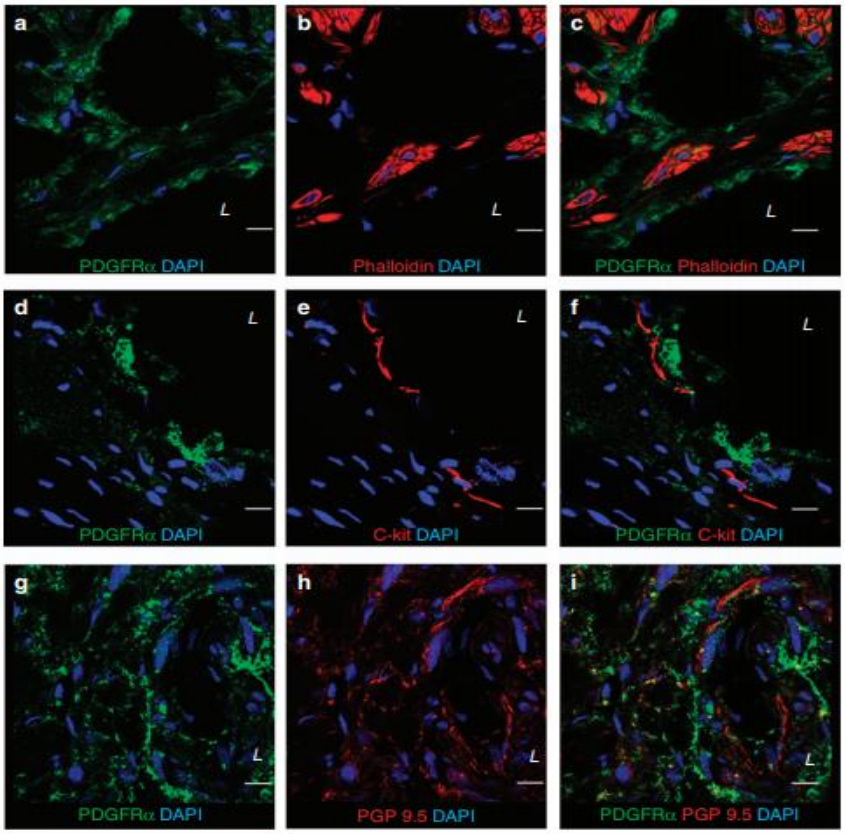


Figure 2.15: (a-c) Expression of PDGFRα+ cells, IC-LCs, SMCs and PGP9.5-positive nerve cells. Double immunolabelled PDGFRα (green) and phalloidin (red) in the muscle layers of the human UPJ. (d-f) Double immunolabelled PDGFRα (green) and c-kit (red) in the muscle layers (g-i) Double immunolabelled PDGFRα (green) and PGP9.5 (red) in the muscle layers (Koh et al., 2018)

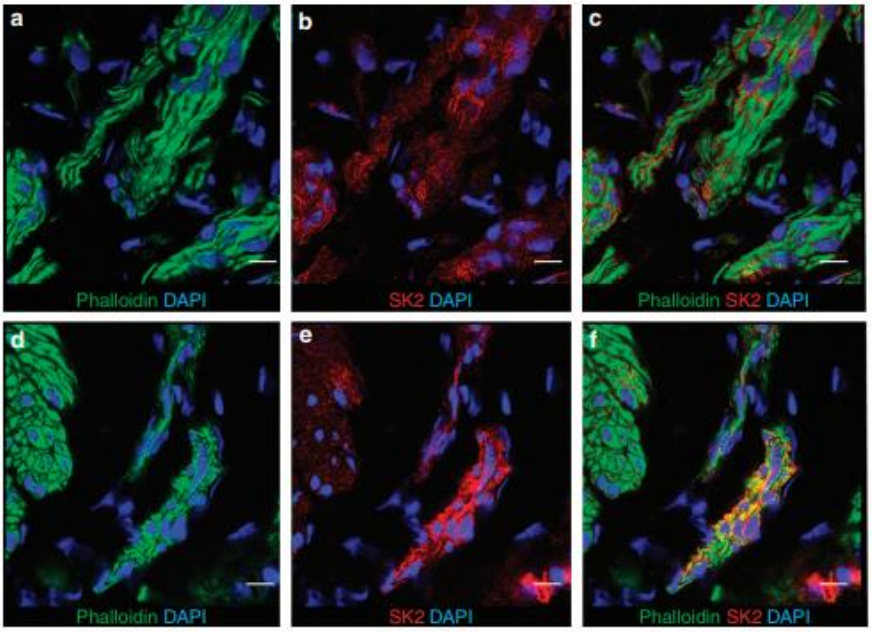


Figure 2.16: Expression of SK2 channels in PDGFRα+ cells and SMCs. (a-c) Normal cells, (d-f) diseased (cells with UPJ obstruction) cells (Koh et al., 2018)

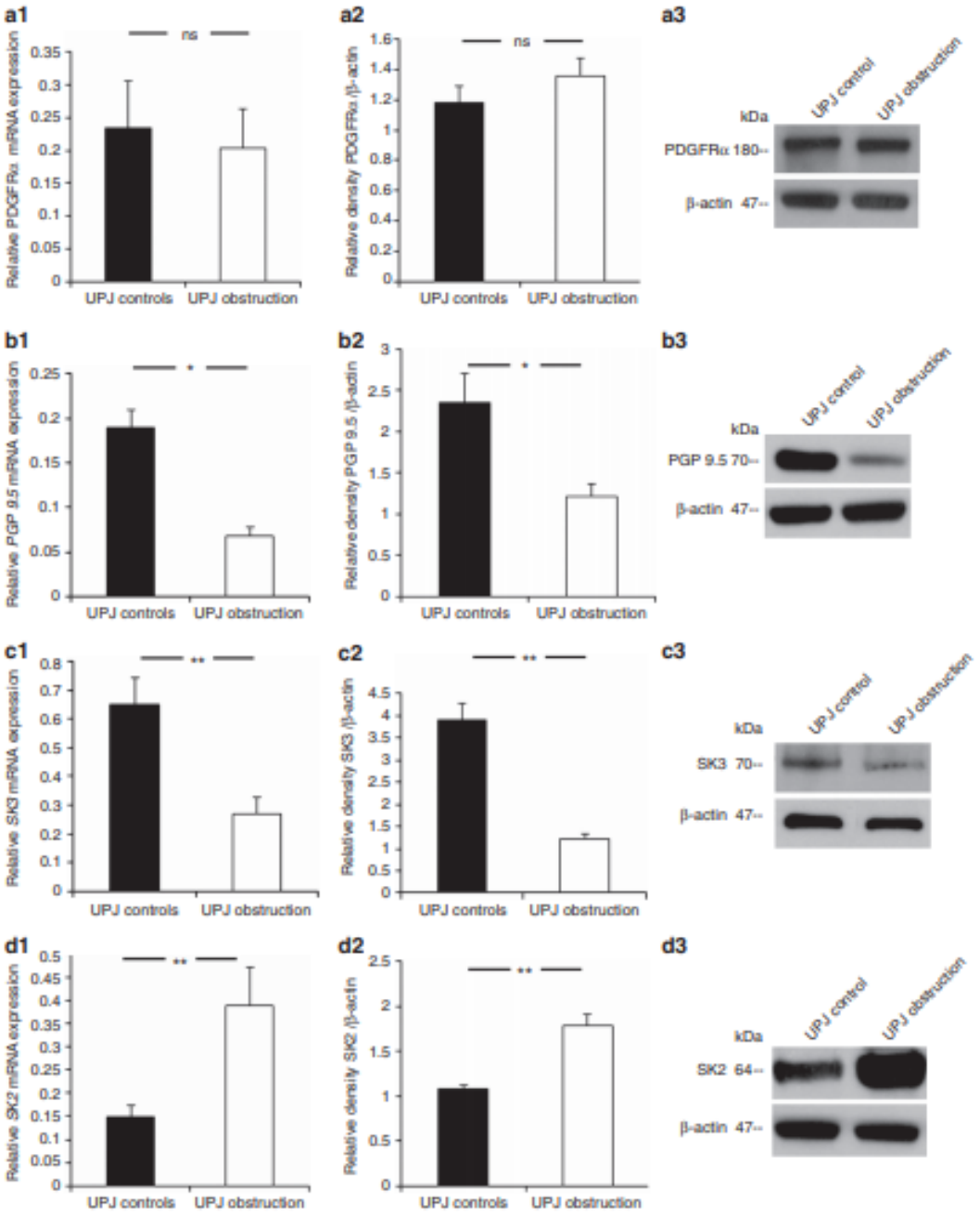


Figure 2.17: comparison of mRNA expression and western blot. Relative mRNA expression levels, western blot, and densitometry of: (a) PDGFRα, (b) PGP9.5, (c) SK2, (d) SK3 (Koh et al., 2018)

2.8. ELECTRICAL SIGNAL CONDUCTANCE AND CALCIUM ION CHANNELS IN THE BLADDER ICs

Koh et al., (2018) have found that most cells that were immune-positive for vimentin were also PDGFRα+. The strain with enhanced green fluorescent protein, driven from the endogenous promoter for PDGFRα was used to identify these cells, PDGFRα+ cells, purified by fluorescence-activated cell sorting, were found to express small-conductance calcium (Ca2+)

activated potassium (K+) (SK3) channels. Patch-clamp experiments showed that these cells expressed SK-channels like conductance. When PDGFRα+ cells were dialysed using 200 nM [Ca2+]i, resulted in spontaneous transient outward currents. When voltage ramp was applied, the cells revealed the voltage-independent outward currents, which were responsible for the reversal of equilibrium potential of K+ ions (Fig. 2.18). Apamin blocked this current as well as the spontaneous transient outward currents. Activation of outward current and hyperpolarization of cells under current-clamp conditions were done by the SK channel agonists CyPPA and SKA-31. When single-channel currents were studied, the amplitude of single-channel currents were found to be 0.14 pA at 0 mV in asymmetrical K+ (5/140 mM) with Ca2+-dependent (EC50 = 98 nM) channel openings. The presence of multiple channels confirmed the high expression of SK channels in PDGFRα+ cells. The currents caused due to SK channels were more difficult to find in detrusor SMCs. But it was found that the current density was low in SMCs and was recorded as 0.5 pA·pF−1 at +10 mV and 12.5 pA·pF−1 at −40 mV in PDGFRα+ cells. No current was found in the normal physiological potentials in SMCs. This concludes that the regulation of excitability of bladder muscle through SK-channels are done by PDGFRα+ cells (Koh et al., 2018).

PDGFRα cells also expresses purine receptors, and ATP produces large-amplitude outward currents with expression of SK3 channels in these cells. These responses were inhibited in cells from P2ry1−/− mice. Purine (P2Y1) receptor antagonists increase the amplitude of the contractions expressed by electrical field stimulation, which shows inhibitory input from purinergic nerves in the bladder (Koh et al., 2018).

P2ry1. P2y2 and P2y4 receptors are expressed in a higher rate in PDGFRα+ cells, along with this P2ry2 and P2ry4 receptors are also seen to be expressed in PDGFRα+ cells. These are activated by pyrimidines, such as uridine triphosphate. This uridine triphosphate in turn activate apamin sensitive outward current in PDGFRα+ cells of murine. This current has the same properties as that of the currents activated by ATP. When current clamp was applied uridine triphosphate lead to hyperpolarization of PDGFRα+ cells and when SMCs were held at negative holding potentials ATP and uridine triphosphate activated inward current (Koh et al., 2018)

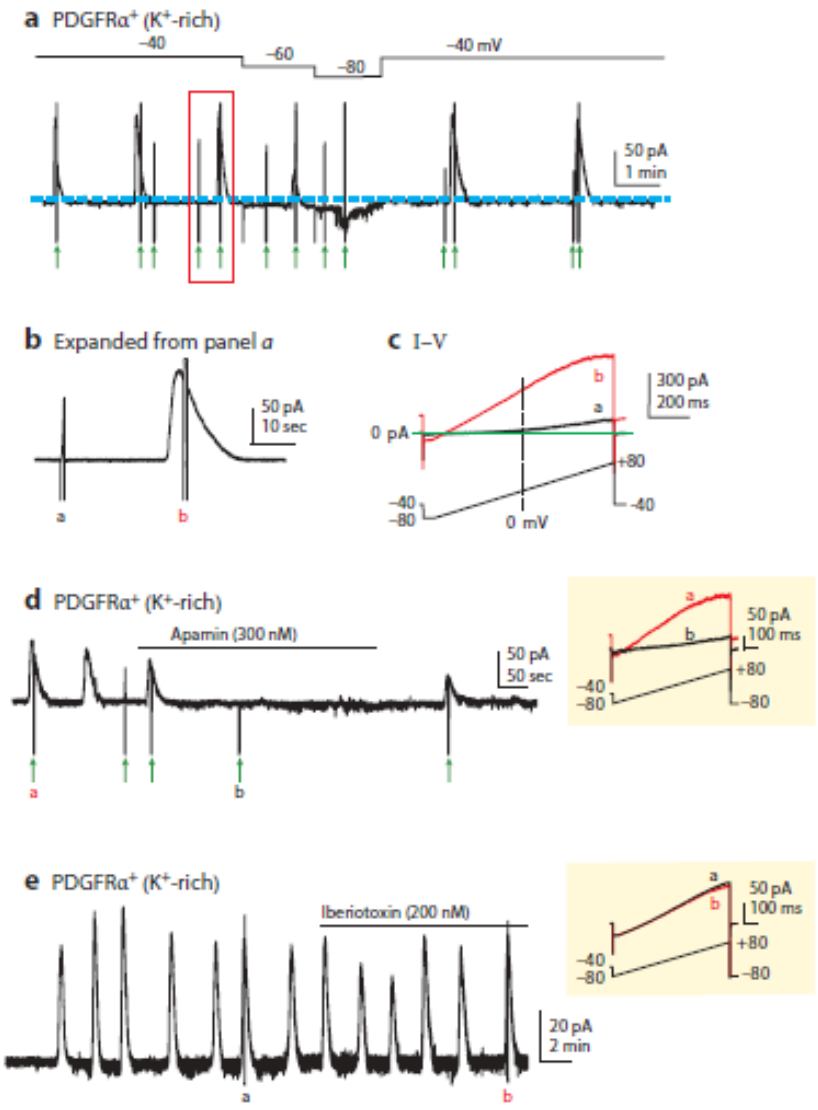


Figure 2.18: The inhibitory conductance expressed by PDGFRα+ in detrusor muscles (a) Shows spontaneous transient outward currents (b) two different ramp depolarizations (c) two different ramp depolarizations (in a and b) (c) current response to ramp depolarizations (d) spontaneous transient outward current inhibited by apamin (Koh et al., 2018).

Which concludes that the hyperpolarization and inhibitory effects of these agonists in detrusor muscles are not mediated by SMCs. Inward current in PDGFRα+ cells is initiated by GSK1016790A, a TRPV4 channel agonist (TRPV4 is mechanosensitive, and these channels mediate Ca2+ influx when activated). This inward current in PDGFRα+ cells in turn activates the SK channels. GSK1016790A hyperpolarizes PDGFRα+ cells under current clamp and also decreased detrusor contractions.

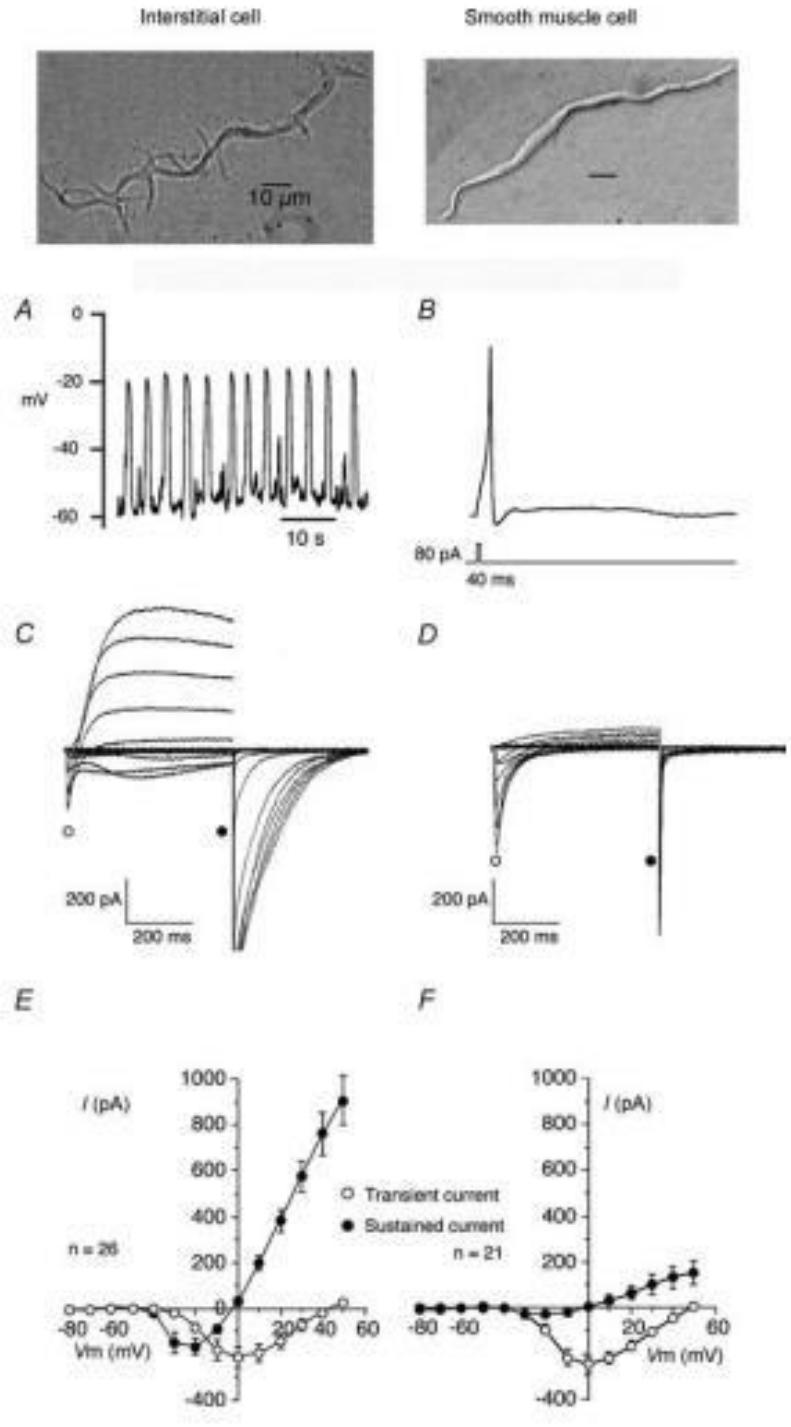


Figure 2.19: Electrical properties of PDGFRα+ cells and SMCs (Koh et al., 2018).

These shows that the stretch-dependent activation of TRPV4 channels may provide calcium2+ ions to activate SK3 channels to stabilize SM excitability (Koh et al., 2018).

Submucosal ICs are considered to be the pacemaker cells of the bladder, as detrusor contractions are regulated by these cells. Also, there are evidence that muscles with intactmucosa shows more powerful contractions than from those muscles from which mucosa were removed. Depolarization starts in these cells by the activating Ca2+-activated Cl− current (CACC). Wu et al suggested that the CACC may be the one responsible for the spontaneous depolarization which are observed in the bladder. It is also noted that the vimentin+ cell population of submucosal ICs had a negative resting membrane potential, −63 ± 14 mV, and this is responsible for the generation of spontaneous action potentials. Also, vimentin+ cells show K+/Na+ hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) immune reactivity (Koh et al., 2018).

Earlier studies have shown the difference in the electrical activities of the SMCs and ICC-Ls (which are now considered as the PDGFRα+ cells). The electrical properties are seen to be contrasting. PDGFRα+ cells were seen to have regular ‘slow-wave’ de-polarizations in current clamp while the smooth muscle cells were quiescent, however, they could produce an action potential in response to depolarizing current. Under voltage clamp conditions, interstitial cells exhibit both L-type calcium currents and calcium-activated chloride currents while the smooth muscle cells have only L-type calcium currents (Figure 2.19). This also put light into the fact that the spontaneous contractions generated has something to do with PDGFRα+ cells (Koh et al., 2018).

Effect small conducting (SK) and large conducting (BK) Ca2+ activated K+ channel blockers and SK channel activators (Figure 2.20) were also studied on ex-vivo bladder preparations. To conduct the studies bladders were treated with apamine, which is an SK channel blocker. The results showed an increase in the transient contractions of the bladder. In the presence of iberiotoxin.

The transient contractions were affected a little, but iberiotoxin along with SKA-31 (a SK channel activator) was added the it totally abolished the transient contractions. Thus the blockage of SK channels in PDGFRα+ cells gave the same effect of that of an over reactive bladder (Koh et al., 2018).

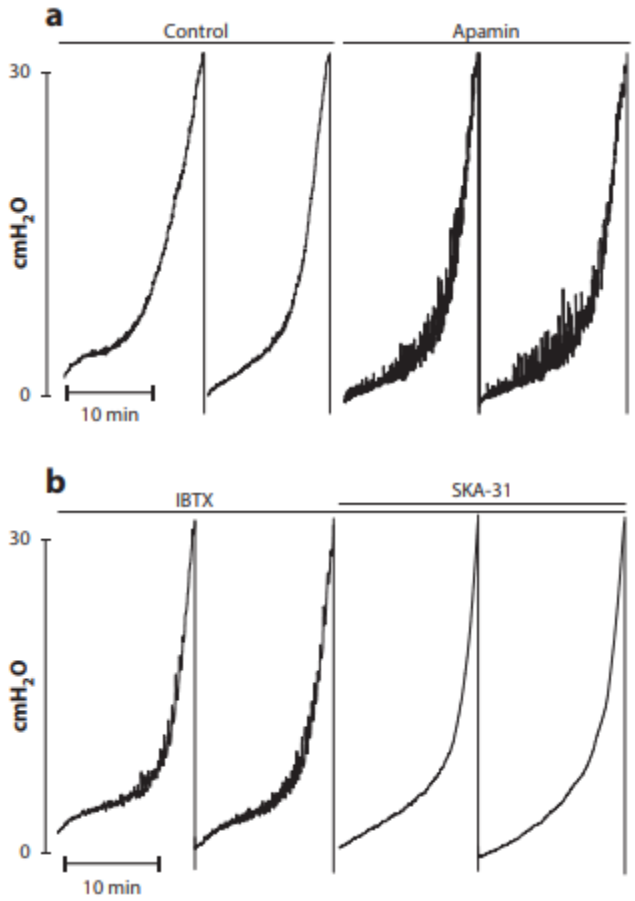


Figure 2.20: Effect of SK and BK channel blockers and SK channel activators on bladder (Koh et al., 2018).

2.9. PHYSIOLOGICAL ROLES AND FUNCTIONS OF ICs

The functioning ICs in the urinary bladder was first known by the help of vimentin+ cell discovery. When exposed to sodium nitroprusside (SNP), these cells produced cGMP. The cell type which produced cGMP was identified by the immunohistochemical studies. These cells which generated cGMP seem to have stellate morphology and were situated near and surrounding the SMCs, which is the location of ICs in the bladder. Also, it was clearly evident that the cells which were involved were ICs and not SMCs. Nitrergic neural transmissions were found in some species and some don't, and this NO transmission can inhibit the detrusor smooth muscle contraction in which NO mechanism is present. This gives an evidence that the ICs are the primary responders to the nitrergic neural transmissions, and they somehow play a major role in the blocking of detrusor SMC contractions (Koh et al., 2018).

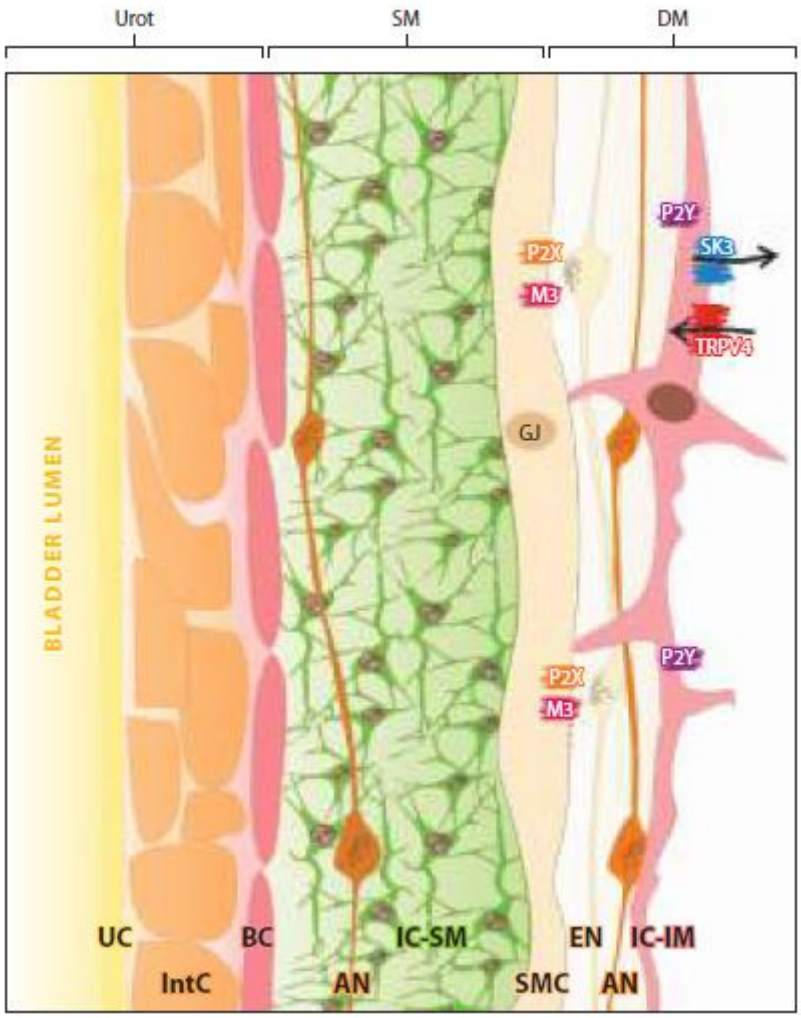


Figure 2.21: A detailed schematic of the ICs in the Bladder (Koh et al., 2018)

To study the origin of the spontaneous contractions from the detrusor, the SMC were filled with fluoro-4. Similar to the above results this study also showed that the ICs were scattered around the smooth muscle bundles. The waves of Ca^{2+} ions were seemed to start from the edge of the SMC bundles and these edges were the places at which the ICs were located. Only 10% of the ICs were responsible for generating the spontaneous Ca^{2+} waves, and the wave generated was of very low frequency compared to that from the SMCs, and also the Ca2+ ion generated waves in SMCs travel independent to that of these waves from ICs. Also, the waves from ICs did not get affected by nifedipine, showing that these waves do not come from the L-type Ca^{2+} channels. However, this study could not find any pace-making role from ICs (Koh et al., 2018).

The development of ICCs in the GI-tract is directly dependent upon the c-Kit. Experiments were conducted to find the contractile properties of the bladder by comparing wild type (WT) and loss-of-function Kit mutants' mice (W/WV). All the electrical responses from them were

blocked using tetrodotoxin. Results of this experiment showed that the post-junctional responses to neural stimuli were similar in both the groups, but purinergic excitor junction responses were stronger in the W/WV mice. However, the number of c-Kit+ and vimentin+ cells were the same in both the types of mice (Koh et al., 2018).

Contraction behaviour of the cells in the bladder were also studied using a tyrosine kinase inhibiting receptor called imatinib mesylate. When imatinib mesylate (in acute concentration) was administered in rat pups, it was seen that the vimentin+ ICs in the submucosal and detrusor muscles were reduced in number. The density of c-Kit+ cells were also reduced and those were seen was also in the intramuscular fibrovascular areas. This resulted in the reduction of spontaneous muscle contractions in the bladder showing that the role of c-Kit+ cells in the detrusor contractions (Koh et al., 2018).

Recent studies show that the majority of the ICs in the detrusor muscles and submucosa are platelet-derived growth factor receptor alpha+ (PDGFRα+) cells. c-Kit+ cells which are present in the bladder are likely to be mast cells. Submucosal (SM) PDGFRα+ cells lie inside the urothelium, which is composed of umbrella cells, intermediate cells, and basal cells (Koh et al., 2018).

The functions of submucosal ICs are not fully understood. However, these cells were seen to convey signals generated from the release of mediators from the urothelium. Within the submucosa there are afferent nerve fibres which can mediate sensory information to the central nervous system. These fibres are seen to have loose associations with ICs, and there has been evidences that ICs modulate sensory input (Koh et al., 2018).

The detrusor muscle contains SMCs, intramuscular ICs (IC-IM), efferent nerve (EN) fibres, and afferent nerve (AN) fibres. The IC-IM are PDGFRα+ cells. As the bladder fills, stretch activates transient receptor potential cation channel subfamily V member 4 (TRPV4) channels in the IC-IM, and the resulting Ca2+ influx activates small-conductance Ca2+-activated K+ (SK3) channels, producing outward current. The outward current stabilizes detrusor SMCs through electrical coupling (GJ) (Koh et al., 2018).

As the bladder fills, stretch also activates nonselective cation channels in SMCs (not shown), and the resulting inward current tends to depolarize SMCs, increase the open probability of L-type Ca2+ channels, and increase the occurrence of transient contractions (TCs) of detrusor SMC bundles. TCs generate significant sensory outflow via the AN fibres within the bladder

wall. The generation of TCs during bladder filling is opposed by the stabilizing influence of PDGFRα+ cells. Finally, when micturition is initiated, ENs release adenosine triphosphate (ATP) and acetylcholine, which activate purinergic (P2X) receptors and muscarinic (M3) receptors on SMCs; the massive inward currents produced by this stimulation overcome the stabilizing influences from PDGFRα+ cells, and voiding contractions occur. Responses to ATP are tempered somewhat, however, by binding of the neurotransmitter to P2Y receptors expressed by PDGFRα+ cells and coupled to the activation of SK3 channels (Figure 2.21) (Koh et al., 2018).

2.10. EXPRESSION & DISTRIBUTION OF PIEZO1 (A MECHANOSENSITIVE CHANNEL) IN THE MOUSE URINARY TRACT

PIEZO channels are stretch activated channels which are commonly found in various organs of the urinary tract like the bladder, kidneys and also present in the prostate, vagina, etc.These channels are activated in response to mechanical stimuli such as increase in the wall tension due to retention of water or flow of blood. It is also activated in response to certain types of shear stress (Dalghi et al, 2019).

PIEZO channels are transmembrane proteins which are homotrimers to form a propeller-like structure. These channels are found to be evolutionary conserved. However their homology with other known protein or channel families is very less. Multiple knockout studies indicate that in the absence of PIEZO channels during the early stage of the development, multiple

congenital diseases. These PIEZO channels can be classified as two types of channels: PIEZO1 and PIEZO2. Of these two subtypes, PIEZO1 is mainly expressed in the interstitial cells underlying the urothelium and in the serosa membrane. These channels release ATP or increase the intracellular Ca2+ ion concentration in response to a stretch stimulus. A study carried out on a mouse model by M.G. Dalghi and team at the University of Pittsburgh indicate that PIEZO channel along with ENAC channel are known to be sensitive to shear stress. Along with the PIEZO channels, there are various mechanoreceptors that are located along the walls of the bladder and oviduct, urothelium, detrusor smooth muscle cell layer which helps to relay information from the bladder wall to the nervous system. PIEZO1 is considered as an important mechanosensor as it meets various criterias to classify it as a channel type mechanosensor. This includes its functional presence on the precise cells during the various stages of the development. It is also observed in studies carried out on mouse models that these channels

show differential expression on various organs depending on the functional relevance of a particular organ during various development stages of the mouse. These channels also respond to changing membrane tensions in the urinary bladder and also to flow like movement by increasing the intracellular calcium ion concentration. Various mutational studies have also indicated that the absence of these channels or in occurrence of a mutational leading to a functional loss of this channel leads to a malfunctioning bladder (Dalghi et al., 2019).

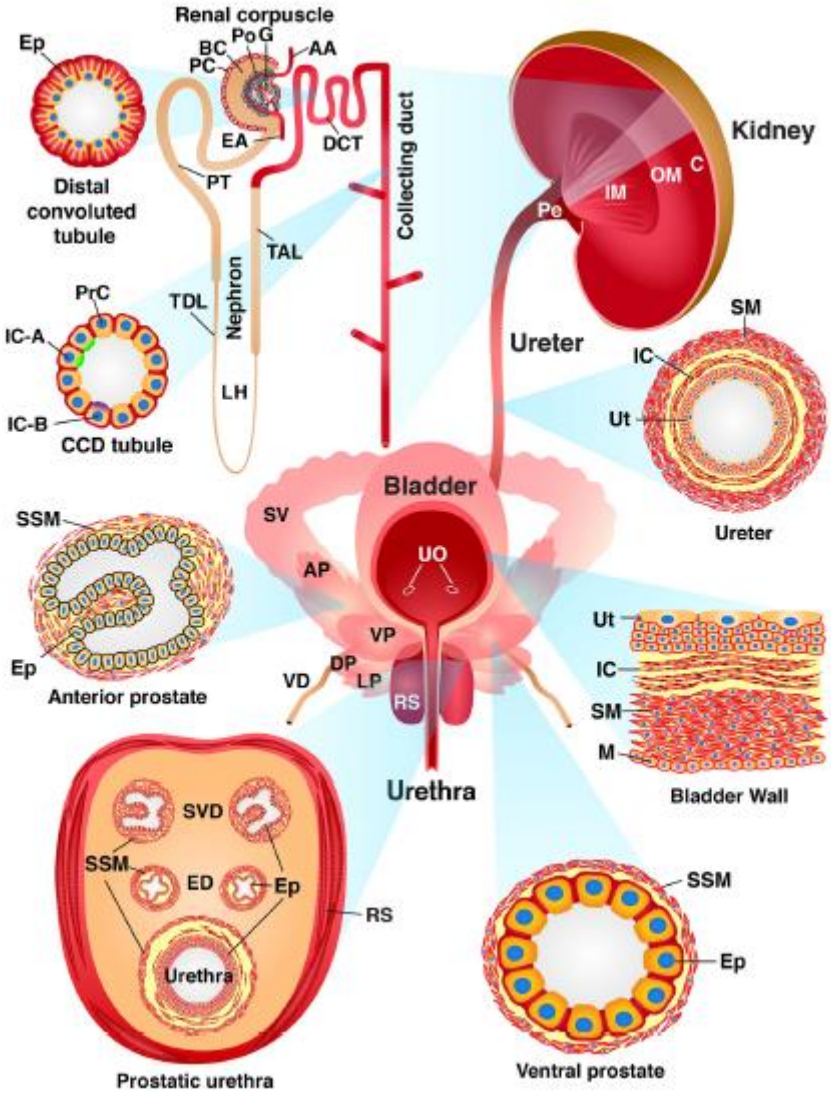


Figure 2.22: Expression of PIEZO1-tandem dimer Tomato (tdT) in urinary tract of mouse (Dalghi et al., 2019)

PIEZO1-tandem dimer Tomato (tdT) is localized in the renal corpuscle and the basolateral surface of epithelia that covers the distal nephron, renal pelvis and collecting ducts in the kidney. PIEZO1- tdT is associated with mesothelium, SMCs, interstitial cells and urothelium of bladder and ureters. PIEZO1- tdT is also distributed in the urethra and closely present organs

of male and female mice like the seminal vesicles, prostate glands and ejaculatory ducts in male mice and vagina in female mice. PIEZO1- tdT was also observed to be present in stromal cells and muscle cells of rhabdosphincter (Figure 2.22) (Dalghi et al., 2019).

2.11. RHYTHMIC EVENTS OF Ca^{2+} IN THE LAMINA PROPRIA OF THE URINARY BLADDER

At the time of prenatal and postnatal development, the micturition reflex goes through characteristic changes. The underlying processes behind these changes are not fully understood. The voiding reflex due to maturation of the central nervous system in the postnatal period is brought in voluntary control requiring involvement of higher brain centres (Ng et. al., 2007). If the nervous system is subjected to injuries, it can lead to loss of voluntary control over the voiding reflex. In new-borns of several species, micturition depends upon a spinal reflex pathway which activates when perineal region of the neonate is licked by the mother. This pathway is required to avoid the retention of urine and is composed of afferent somatic limb present in pudendal nerve and an efferent parasympathetic region present in pelvic nerve. A similar reflex pathway is also present is human neonates. The perineal to bladder reflex diminishes as the new-born develops and is then replaced by the adult form of voiding and an inhibitory perineal to bladder reflex. A spinobulbospinal reflex replaces the spinal micturition reflex as the nervous system matures. This reflex pathway is activated by mechanosensitive afferent nerve activity to trigger micturition (Heppner et al.,2017).

2.11.1. Neurochemistry and morphology of cells in the Lamina Propria

A network of blood vessels, nerves and cells are present just beneath the junction with urothelial cells in the lamina propria. As can be seen in (Figure 2.23), on incubation with Ca^{+2} reporter dyes, the blood vessels, capillaries and cells get loaded with the fluorescent dyes. The cells in the lamina propria are observed to be different in morphology and were found to be highest in number from the tissues prepared from the rat pups aged greater than P21. The size of cells was small and morphology ranged from circular to fusiform with projections. Some cells showed TRPV4 immunoreactivity (Figure 2.23C) whereas PDGFRα immunoreactivity was shown in most cells of lamina propria (Figure 2.23D) in whole mounts cleared of urothelium. Suburothelial nerve plexus was very close to lamina propria cells and the capillary network. This served as an identifiable landmark facilitating the focus on the lamina urothelial junction (Figure 2.23E). Few if any cells were found in the lamina-urothelial junction in pups

aged greater than P25 will were most numerous in pups less than P21 age. Due to this, the observations and analysis of triggered calcium events were restricted to the postnatal stage (Heppner et al.,2017).

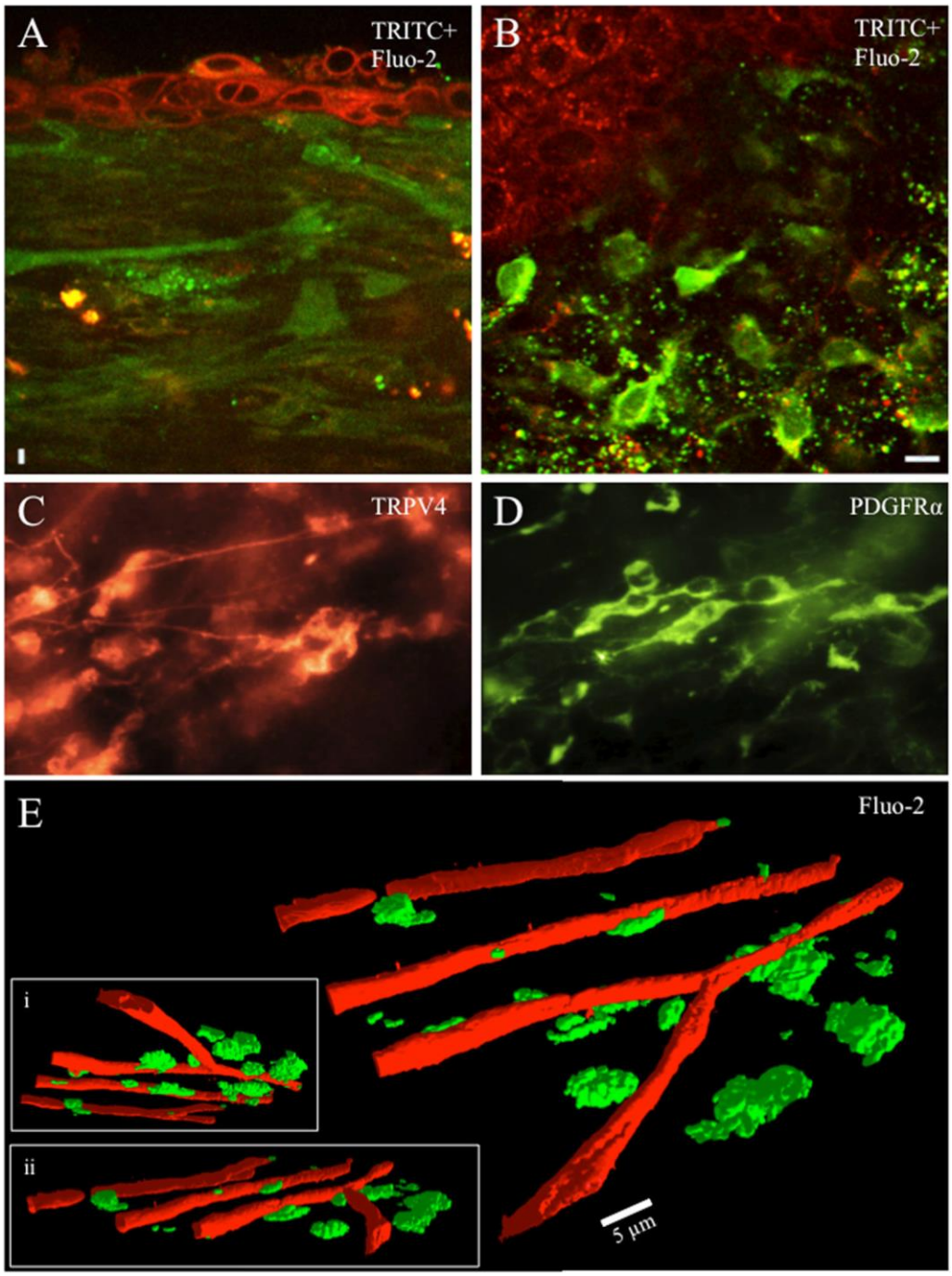


Figure 2.23: Bladder tissue whole mount prepared at urothelial-lamina junction. The mount prepared had a network of blood vessels and cells from rat pups aged less than P21 and picked Fluo-2 green (A, B, E) while the urothelial cells picked TRITC; red (A, B). Many cells in lamina propria showed immunoreactive to PGDFRα while some cells exhibited immunoreactivity to TRPV4 (C, D). At the junction, the network of cells exhibiting immunoreactivity to PGDFRα and TRPV4 were very close to a dense network of capillaries (E) (Heppner et al.,2017).

2.11.2. Analysis of calcium transients and waveform in the lamina propria

At 20x magnification, many cells from lamina propria exhibited calcium transients in postnatal rats (Figure 2.24A-E). The frequency of calcium transients was low with a mean interval of 30 ±8.6 seconds (Figure 2.24F). Calcium events of long duration were seen on analysing waveforms of calcium transients in the cells of lamina propria network (Figure 2.24G). These events had slow downstroke and upstrokes phases (Figure 2.24H). using control recordings from 7 different experiments on 56 cells, the characteristics of calcium transient waveforms were calculated (Heppner et al.,2017).

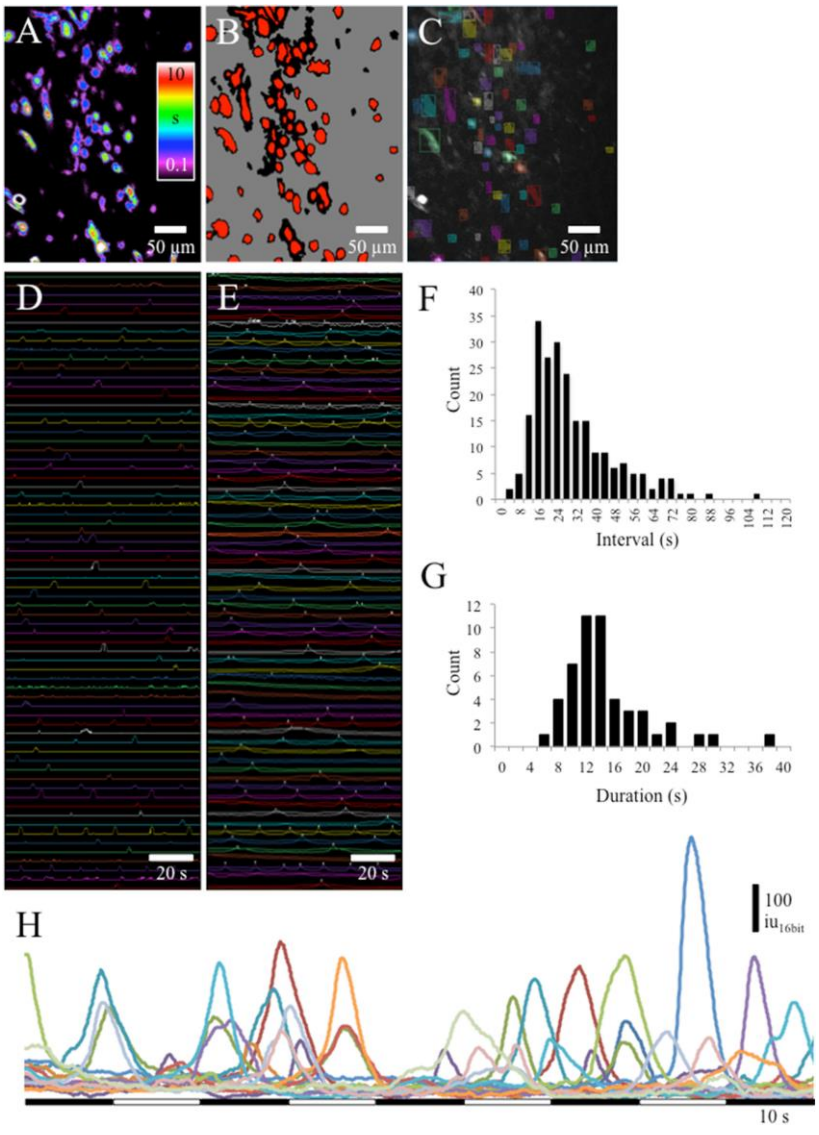


Figure 2.24: (A) Prevalence maps of calcium transients with thresholded PTCLs (B) in order to develop ROI billmasks (C) to find PTCL area (D) and fluorescence induced by calcium (E) from the initial recording. (F) A slow frequency of calcium transients in lamina propria shown in interval histograms. (G) Mean duration of calcium transients was 15.6 ±2.4 seconds. (G) calcium transients examples in 15 lamina propria cells (Heppner et al.,2017).

2.11.3. Calcium activity in the lamina propria cells

Gradually propagating waves of activity were observed at the time of continuous recordings from the lamina propria network in which many cells exhibited prolonged and robust calcium transients (Figure 2.25A-C). The overall activity of propagation can be estimated and any bias in the angle between cells in activation sequence can be determined by spatio-temporal maps of spread of activity in cells of lamina propria network (Figure 2.25A). The velocity of spread of calcium events was often variable in direction but consistent in magnitude at 60-70 μm/second. The bias in angles between cells in activation sequences was calculated to find the overall degree of coupling between cells of lamina propria and the mean value was 10.65 ± 1.63 suggesting that the direction of spread of cell activity is defined and not random independent of whether the wave front was tightly or loosely coupled. Varying degrees of coupling like loose, partial and tight arrangement of lamina propria was demonstrated by spatio-temporal maps (Figure 2.25B and C). These spreading calcium waves suggest that the lamina propria network can act as a functional syncytium (Heppner et al.,2017).

2.11.4. Activation of TRPV4 in Lamina Propria network

In the lamina propria network of postnatal rats, immunoreactivity to TRPV4 was prevalent. On applying activator of TRPV4 (GSK1016790), an increase in the calcium events duration and in the number of cells with calcium events in the lamina propria (Figure 2.26A-C). An increase in integrated calcium activity was observed (Figure 2.26D) although on applying an antagonist (GSK2193874) of TRPV4 channels, number of cells displaying calcium events and the time period of the calcium events were not affected. This indicate that the influx of calcium due to TRPV4 does not affect the calcium signalling at basal level (Heppner et al.,2017).

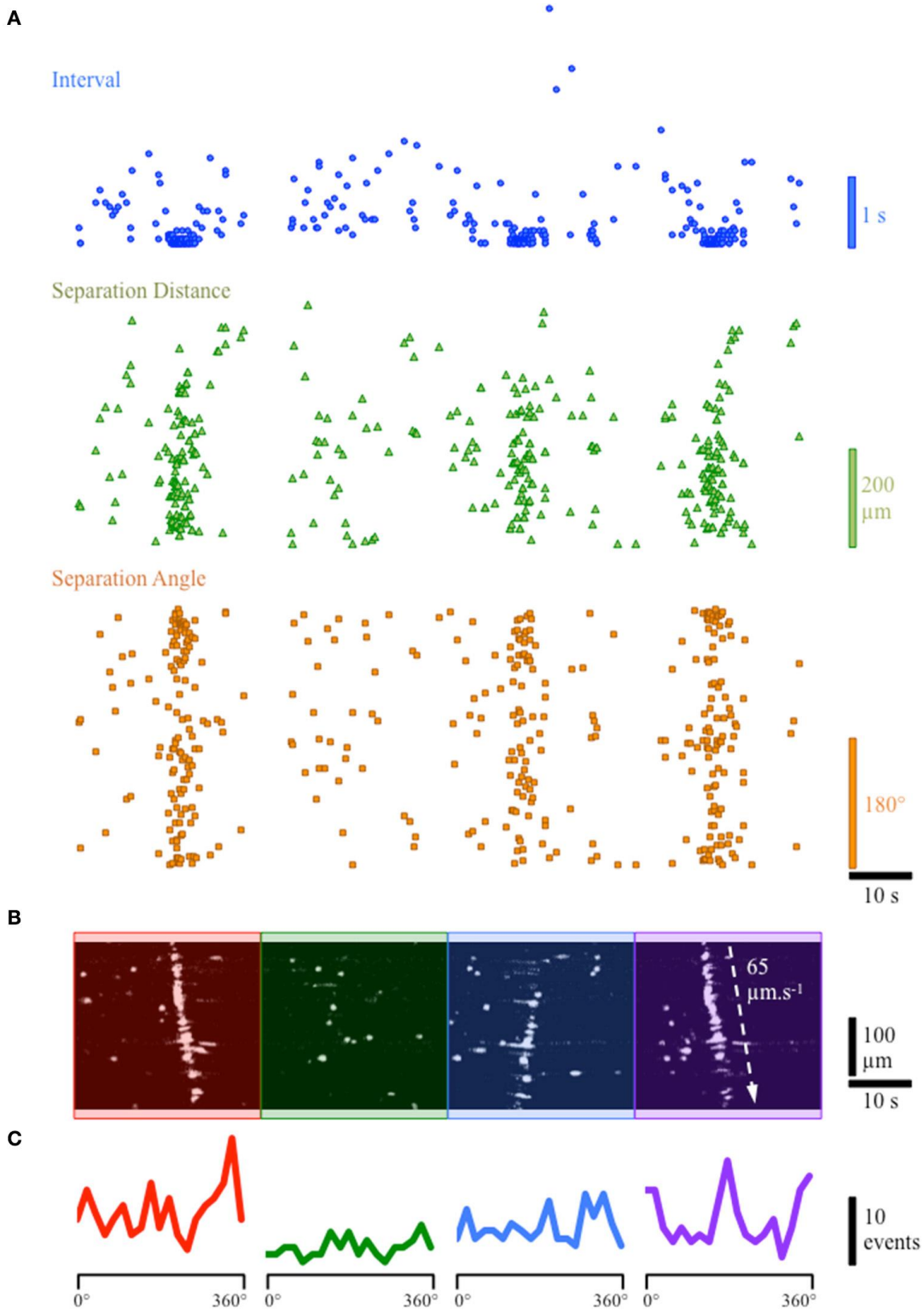


Figure 2.25: Graph of firing characteristics of cell in lamina propria demonstrating distance of separation (green triangles), intervals (blue dots) and angle between cells (orange squares) in forward firing sequence; B: Spatio-temporal map of cells firing in lamina propria showing four network firing events shown in green, red, purple and blue. (C) frequency of angles between next to fire cell demonstrated by histogram (Heppner et al., 2017).

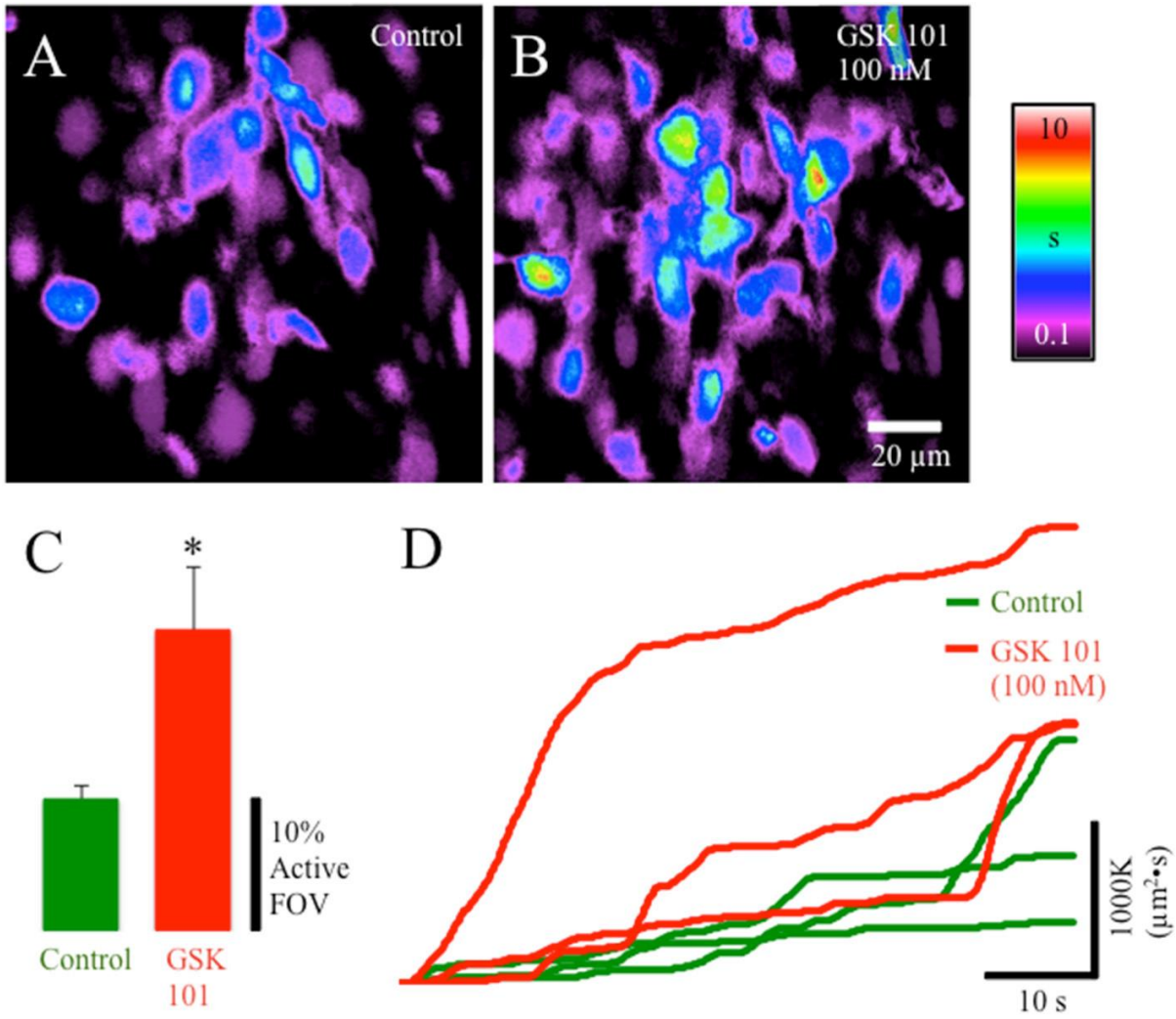


Figure 2.26: Prevalence map of cell control activity in lamina propria; (B) Prevalence map of cell activity after applying agonist of TRPV4; (C) number of cells exhibiting calcium events increases after application with agonist; D: traces demonstrating integrated calcium activity from individual experiments in control conditions and after application of agonist (Heppner et al., 2017).

2.12. SK CHANNELS ACTIVATED BY UTP (A PURINE CHANNEL AGONIST) IN MURINE DETRUSOR PDGFRA+ CELLS

Purines, specifically UTP, are known to be act as activators for the detrusor smooth muscle cells. They induce a transient contraction and long relaxations in these muscles. This occurs when the purines bind to the P2Y (G protein-coupled) receptors that are present on the surface of the detrusor PDGFRα cells. Binding of these purine molecules to these receptors leads to the activation of the small conductance Ca^{2+} activated SK channels. Studies indicate that Kcnn3 a type of SK channel is abundantly expressed by PDGFRα cells (Lee et al., 2015).

P2ry2, P2ry4, and P2ry14 are some of the subtypes of P2Y receptors that are expressed on the surface on the detrusor muscle. Binding of UTP to these receptors results in the generation of an outward current however, these currents are blocked by the SK channel blockers which

reduces the detrusor activity when the bladder experiences stretch indicating the functional importance of UTP during a mechanical stretch (Lee et al., 2015).

It was also noted that the current generated is directly corelated to the level of expression of SK channels on the detrusor PDGFRα cells. A parallel pathway for activation was also found when the SK channel was blocked. The parallel pathway included the activation of the $G_{q/11}$-coupled receptors which lead to an increase in the intracellular Ca^{2+} levels via the synthesis of IP_3 and thereby the activation of the IP_3 receptor-operated intracellular Ca^{2+} stores.

The detrusor SMCs also express P2X receptors which get activated when ATP molecules bind to then leading to a transient depolarisation causing the contraction and relaxation of the bladder. The hyperpolarisation of the SK channels along with the coupling with SMCs leads to the activation of L type channel leading to the passage of Ca^{2+} ions into the SMCs (Lee et al., 2015).

Knockout studies have shown that in the absence of SK channels the bladder loses its contractibility when it receives a mechanical stimulus rendering it useless. This indicates the importance of SK channels in the bladder and understanding its mechanism in detail may help to treat various overactive or underactive bladders (Lee et al., 2015).

CHAPTER 3

METHODS FOR MODELLING

3.1 OVERVIEW

As part of the first stage MTP, we have implemented a mechanistic model for PDGFRα+ cells as described by Yeoh et al., 2016. The model consists of 5 principal mechanisms, namely, (i) ATP activated G Protein Coupled Receptor (GPCR-P2Y); (ii) IP3 metabolism (production and degradation); (iii) Endoplasmic Reticulum induced calcium dynamics via the J_{IP3}, J_{SERCA_PUMP} and J_{Leak} fluxes; (iv) Calcium activated potassium conductance SK3; and (v) Non-specific cationic conductance (NSCC). Each of these mechanisms are represented by component equations which will be described in this chapter. In addition to individual component equations, there are six differential equations that compute the rate of change of model parameters that reflect different facets of PDGFRα+ cell physiology. Figure 3.1 below shows a schematic of the PDGFRα+ cell model implemented as part of the MTP 1st Stage work.

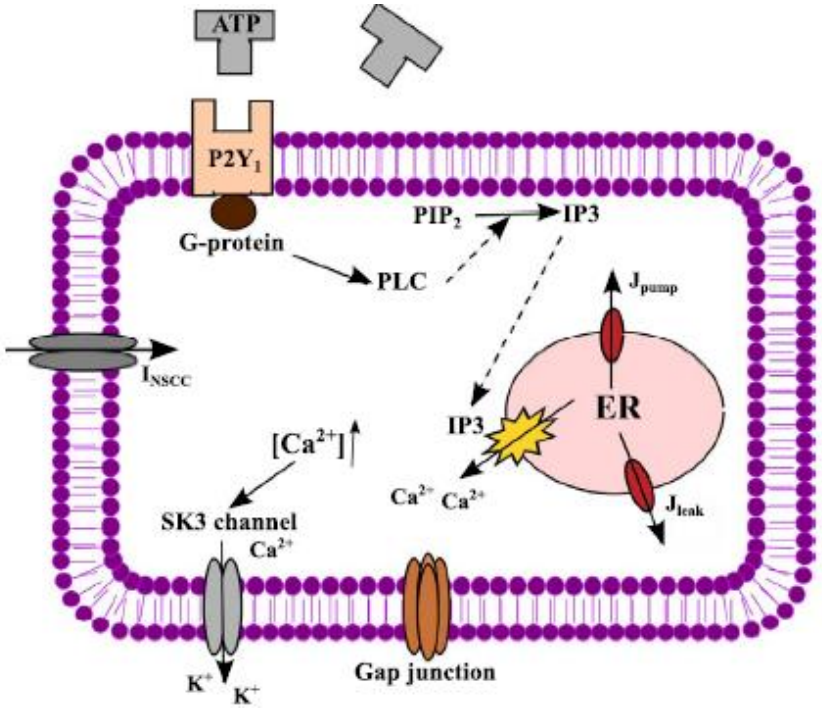
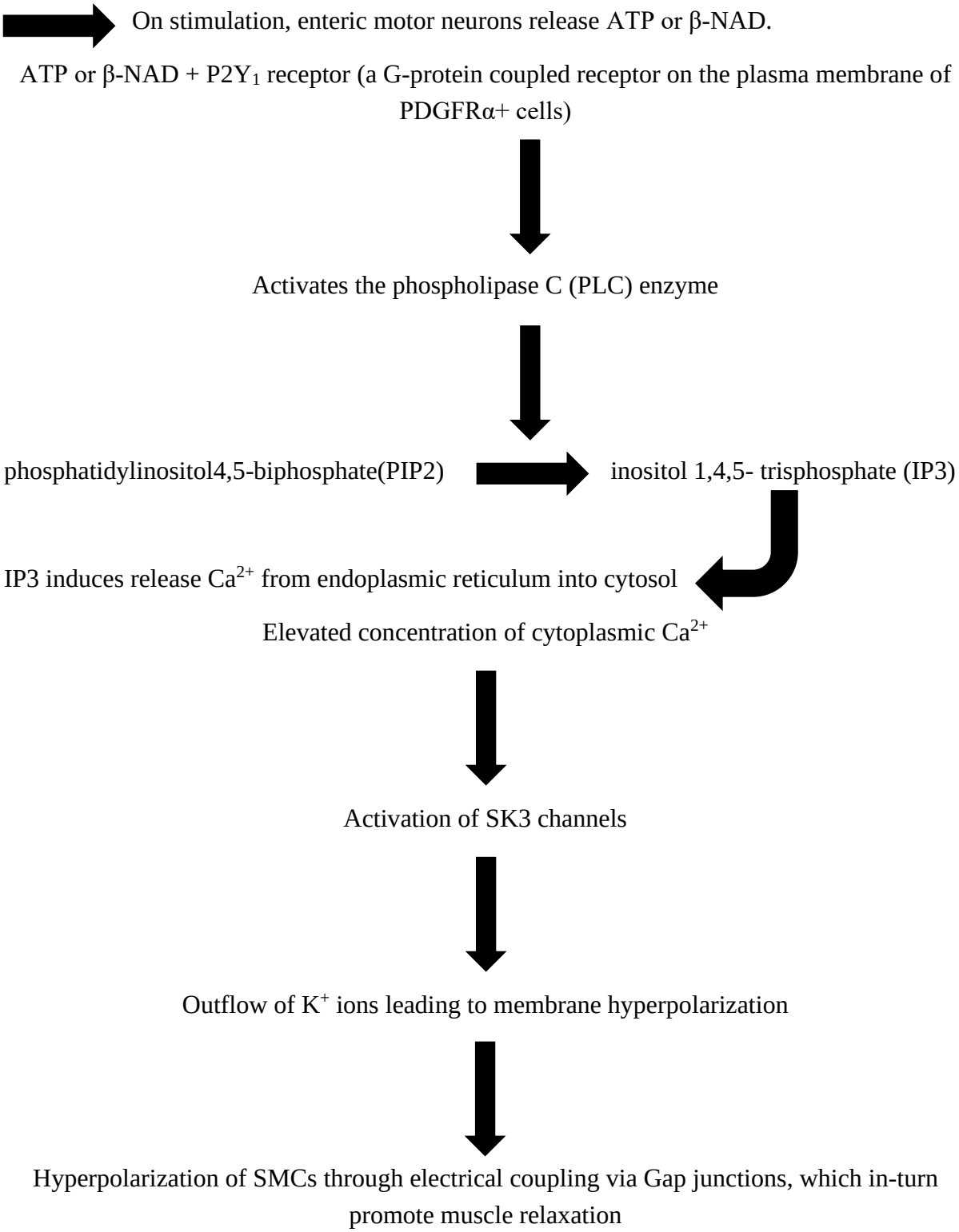


Figure 3.1 A schematic representation of the PDGFRα+ cell model (Source: Yeoh et al., 2016)

3.2 MODEL DESCRIPTION

The model by Yeoh et al., 2016 describes one pathway via triggered P2Y receptors that mediates purinergic inhibitory neurotransmission to smooth muscle cells via PDGFRα+ cells. The following flowchart describes the functional interaction happening at cellular level in PDGFRα+ cells.



3.3 GPCR RECEPTORS

The activation of purino-receptor $P2Y_1$ is mediated by the ATP released from the enteric motor neuron terminals. The following equation govern this model:

$$L + R \underset{k_1^-}{\overset{k_1^+}{\longleftrightarrow}} LR \qquad \text{eq. 3.1}$$

where L is the ATP ligand, R is the unbound $P2Y_1$ receptors. The bound receptors can be given by the following equation:

$$\rho = [LR] / [R_T] \qquad \text{eq. 3.2}$$

where $[R_T] = [R] + [LR]$, and R_T is the total number of $P2Y_1$ receptors. A fast binding kinetics was assumed with an instantaneous equilibrium, which is

$$k_1^+ [LR] = k_1^- [LR] \qquad \text{eq. 3.3}$$

From the above equation, the binding fraction can be given as:

$$\rho = [ATP] / K_R + [ATP] \qquad \text{eq. 3.4}$$

where, K_R = k1-/ k1+ is the dissociation constant associated with the binding of ATP to the P2Y1 receptor. P2Y1 receptors are GPCR's that set into motion a cascade of cellular events involving numerous secondary messengers. Therefore, the binding fraction can indirectly tell us the number of activated G-protein and this in-turn gives a measure of activated P2Y1 receptors and the coupling strength of these receptors to the G-protein activation (Yeoh et al., 2016).

3.3.1 G-protein Cascade

A simplified model is proposed here to describe the G-protein activation (Yeoh et al., 2006). The rate of G-protein activation is directly proportional to the number of active P2Y1 receptors and the number of inactive G-protein. Whereas the G-protein deactivation rate is directly proportional to the amount of active G-protein. This reaction can be given as follows:

$$G_{in} \underset{k_d}{\overset{k_a(\rho+\delta)}{\longleftrightarrow}} G \qquad \text{eq. 3.5}$$

where, Gin is the inactive G-protein, G is the activated G-protein, k_a and k_d represents the activation and deactivation rate constants respectively. ρ and δ represent the binding fraction and the activity ratio of the unbound and bound receptors respectively. Further, δ is due to the background activity present in the cell. This activity results in the activation of some fraction of G-protein by the unbound P2Y1 receptors even in the absence of ATP ligand. The total G-protein is given by the following equation:

$$G_T = G + G_{in} \qquad \text{eq. 3.6}$$

From the above conditions given, we can write the resulting equation for activated G-protein as:

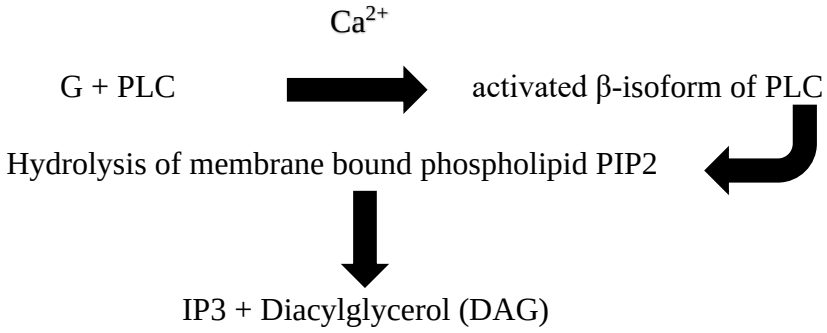
$$\frac{d[G]}{dt} = k_a (\delta + \rho) ([G_T] - [G] - k_d[G]) \qquad \text{eq. 3.7}$$

By assuming fast kinetics, the rate of change will be zero, (d[G])/dx = 0, and the activated G-protein ratio is given as:

$$G^* = (\delta + \rho) / (K_G + \delta + \rho) \qquad \text{eq. 3.8}$$

where, $K_G = k_d/k_a$

3.4 IP3 PRODUCTION



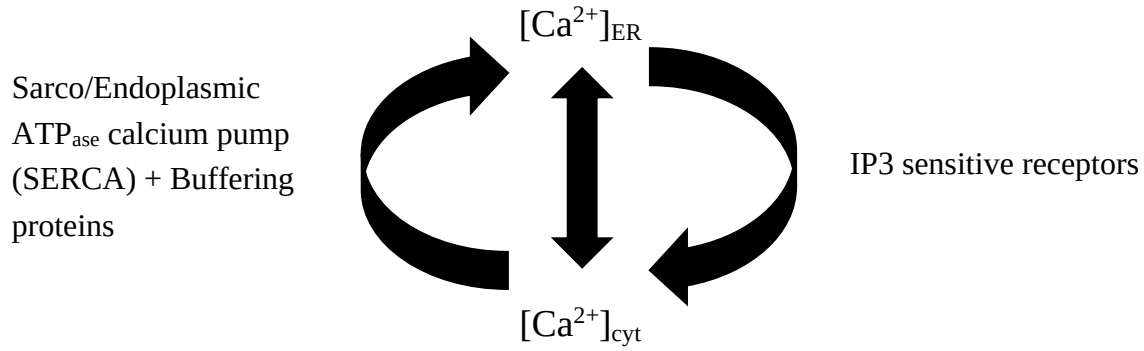
The above flowchart summarises the production of IP3 upon activation of the P2Y GPCR. The IP3 molecules produced are secondary messengers that diffuse into the cytosol to mediate signal transduction. They get degraded by intracellular kinases. The IP3 production and degradation is explained as represented by the following equation:

$$\frac{d[IP3]}{dt} = r_h{*}G^* - k_{deg*}[IP3] \qquad \text{eq. 3.9}$$

where, r_h* is the IP3 production rate independent of Ca2+ concentration, k_{deg} is the degradation rate of IP3. The model produces IP3 in the physiological range i.e. 350-800 nM. The activity ratio is given from the above equations by considering binding fraction as zero. This equation is formulated by applying a steady state assumption to the above equation:

$$\delta = K_G * k_{deg} * [IP3]_0 / (r_h* - k_{deg} * [IP3]_0) \quad \text{eq. 3.10}$$

3.5 INTRACELLULAR CA^{2+} DYNAMICS



The equations which governs the above pathways are as follows:

$$d[Ca^{2+}]_{cyt} / dt = \beta (J_{IP3} + J_{leak} - J_{SERCA\_Pump}) \quad \text{eq. 3.11}$$

where, J_{IP3}, J_{leak} and J_{pump} are the Ca^{2+} fluxes from the IP3 sensitive channels, leakage from ER, and SERCA pump respectively. The free calcium in the cytosol is regulated by the association and dissociation equilibrium maintained by the buffering proteins. For this reason, the factor β is included in the above equation. From the literature the value for β is given as:

$$\beta = \{1 + ([B]_{end} / K_{end})\}^{-1} \quad \text{eq. 3.12}$$

where, $[B]_{end}$ is the concentration of the endogenous buffer and K_{end} is the dissociation constant of that buffer. IP3 sensitive channels open and trigger the release of Ca^{2+} to cytosol, when IP3 and Ca^{2+} are bound to the IP3 activation site, calcium activation site and calcium inactivation site. The governing equation is as follows:

$$J_{IP3} = J_{max} \left\{ \frac{[IP3]}{[IP3]+K1} * \frac{[Ca^{2+}]_{cyt}}{[Ca^{2+}]_{cyt} + Kact)} * h \right\}^3 \left\{ 1 - \frac{[Ca^{2+}]_{cyt}}{[Ca^{2+}]_{ER}} \right\} \quad \text{eq. 3.13}$$

where, J_{max} is the maximum flux, K_1 and K_{act} dissociation constants for the IP3 molecules binding to their receptors and Ca^{2+} binding to the calcium activation site respectively, and h

represents the probability of Ca^{2+} occupying an inhibitory site. The rate of change of h is given by the following equation:

$$\frac{dh}{dt} = k_{on} \{k_{inh} - ([Ca^{2+}]_{cyt} + k_{inh})\, h\} \qquad \text{eq. 3.14}$$

where, k_{on} and k_{inh} are the binding rate of Ca^{2+} to the inhibitory site and the corresponding dissociation constant respectively. Initial value of h is given by:

$$h_0 = k_{inh} / ([Ca^{2+}]_0 + k_{inh}) \qquad \text{eq. 3.15}$$

The equation for SERCA pump is given by the following Hill equation:

$$J_{pump} = V_{max} \{[Ca^{2+}]^2_{cyt}/([Ca^{2+}]^2_{cyt} + K_p^2)\} \qquad \text{eq. 3.16}$$

where, V_{max} and K_p represents the maximum pump flux rate and the corresponding dissociation constant respectively. The leak flux is given by:

$$J_{leak} = P_L \{1 - ([Ca^{2+}]_{cyt}/ [Ca^{2+}]_{ER})\} \qquad \text{eq. 3.17}$$

where, P_L is the leak constant. In this model we consider the cell as a closed system, from this assumption the total free Ca^{2+} per cytosolic volume is give as:

$$[Ca^{2+}]_{tot} = c_1[Ca^{2+}]_{ER} + [Ca^{2+}]_{cyt} \qquad \text{eq. 3.18}$$

where c_1 is the volume ratio between the ER and cytosol. This value is taken from the literature, where they calculated the volume ratio of fibroblast cells, which is morphologically very similar to PDGFRα+ cells. From this the Ca^{2+} in the ER is calculated as follows,

$$d[Ca^{2+}]_{ER}/dt = \frac{\beta}{c_1} (-J_{IP3} - J_{leak} + J_{pump}) \qquad \text{eq. 3.19}$$

3.6 SMALL CONDUCTANCE CA^{2+} ACTIVATED K^+ CHANNELS (SK3)

IP3 channels → Activation of calcium sensitive SK3 channels

↓

K^+ ions flow outward

→ Large transient outward current

The SK3 channel current is modelled based on the HH formalism and the SK3 current is given by the following equation:

$$I_{SK3} = g_{max} * P_{o} * (V_m - E_K) \quad \text{eq. 3.20}$$

where g_{max} is the maximum channel conductance reported from voltage-clamp experiments conducted by (Kurahashi et al., 2011), P_o the channel open probability, V_m is the membrane potential and E_K Nernst potential of K^+ ion. The channel kinetics was described using a Hodgkin-Huxley type gating variable P_0, who rate of change w.r.t is given by:

$$\frac{dP_o}{dt} = (P_{inf} - P_o)/\text{Tau\_SK3} \quad \text{eq. 3.21}$$

where, Tau_SK3 is time constant for activation, P_{inf} is the channel open probability (steady-state value). A steady state equation is used to describe P_{inf} (Silva et al., 2007), given as,

$$P_{inf} = 1/\{1 + (EC50/[Ca^{2+}]_{cyt})^n\} \quad \text{eq. 3.22}$$

Here EC50 is the half maximal effective concentration and n represents the Hill coefficient

3.7. MEMBRANE POTENTIAL

The membrane potential of the PDGFRα+ cell is determined using the HH approach where the cell membrane is represented as a equivalent electrical circuit consisting of capacitances, representing the lipid bilayer, which are connected in parallel with the membrane conductances that represent the ionic pathways. The rate of change of membrane potential is given as follows:

$$\frac{dV_m}{dt} = -\frac{1}{C_m}(I_{SK3} + I_{NSCC}) \quad \text{eq. 3.23}$$

where, C_m is the membrane capacitance and I_{NSCC} is a non-selective ionic current. The I_{NSCC} current is represented by the following equation:

$$I_{NSCC} = g\,(V_m - E_{NSCC}) \quad \text{eq. 3.24}$$

CHAPTER 4

RESULTS AND DISCUSSION

The model by Yeoh et al., (2016) described in Chapter 3 was implemented on MATLAB (2019A). We will describe the results in the following sections. In section 4.1, the ATP given to the system is for 100 seconds. From section 4.2 onwards, please note that the duration of ATP exposure was set at 0.5s. We used the forward Euler method to compute the rate change of the state variables described in the model. The time-step dt was set at 0.0001s.

4.1 ATP INDUCED Ca^{2+} ELEVATION

In this experiment (simulation), ATP was provided for a period of 100s. For each iteration, the ATP concentration was changed as indicated in the legend. The level of ATP was changed from 0.01 µM to 1mM as shown in figure 4.1.

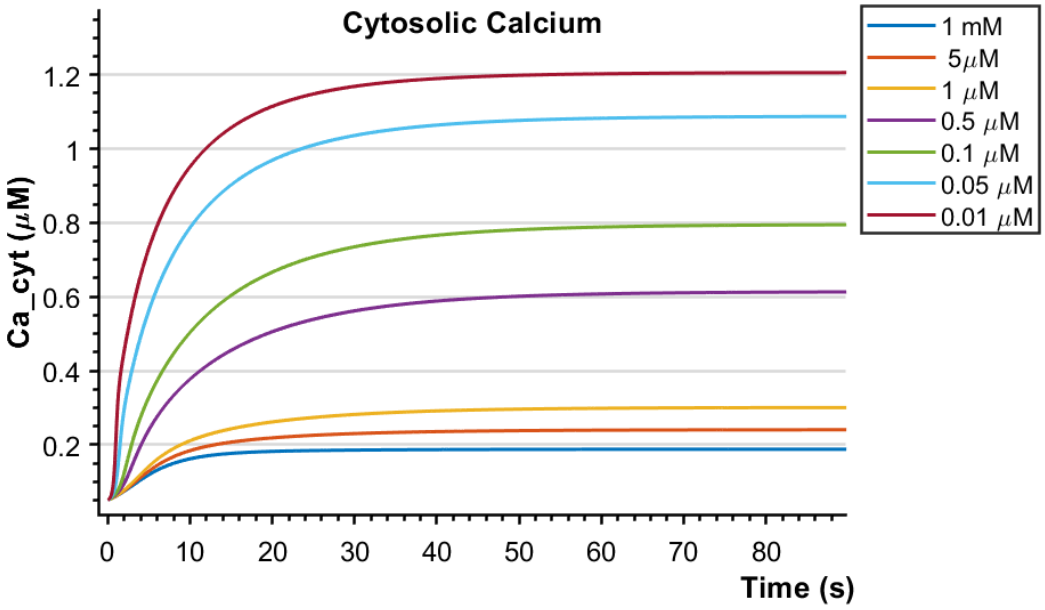


Figure 4.1: Change in cytosolic Ca^{2+} concentration in response to variation in ATP input

As seen in Figure 4.1, as the ATP input increases, the GPCR pathway gets triggered inducing release of calcium from the ER store via activation of IP3 channels by the secondary messenger IP3. This results in increase in levels of cytosolic calcium. This is a simulated experiment that

correlates to the simulation carried out in the work presented by Yeoh et al., (2016). However, this does not necessarily represent a physiological scenario. To be noted: While the trend in the rise of cytosolic Ca^{2+} is replicated in our simulation, the quantitative value of the cytosolic Ca^{2+} does not match with the results shown by Yeoh et al., (2016). The results in the following section 4.2 will explain the intervening steps in the signal transduction pathway that gets triggered upon purinergic input to the PDGFRα+ cells.

4.2 ATP INDUCED MEMBRANE HYPERPOLARIZATION

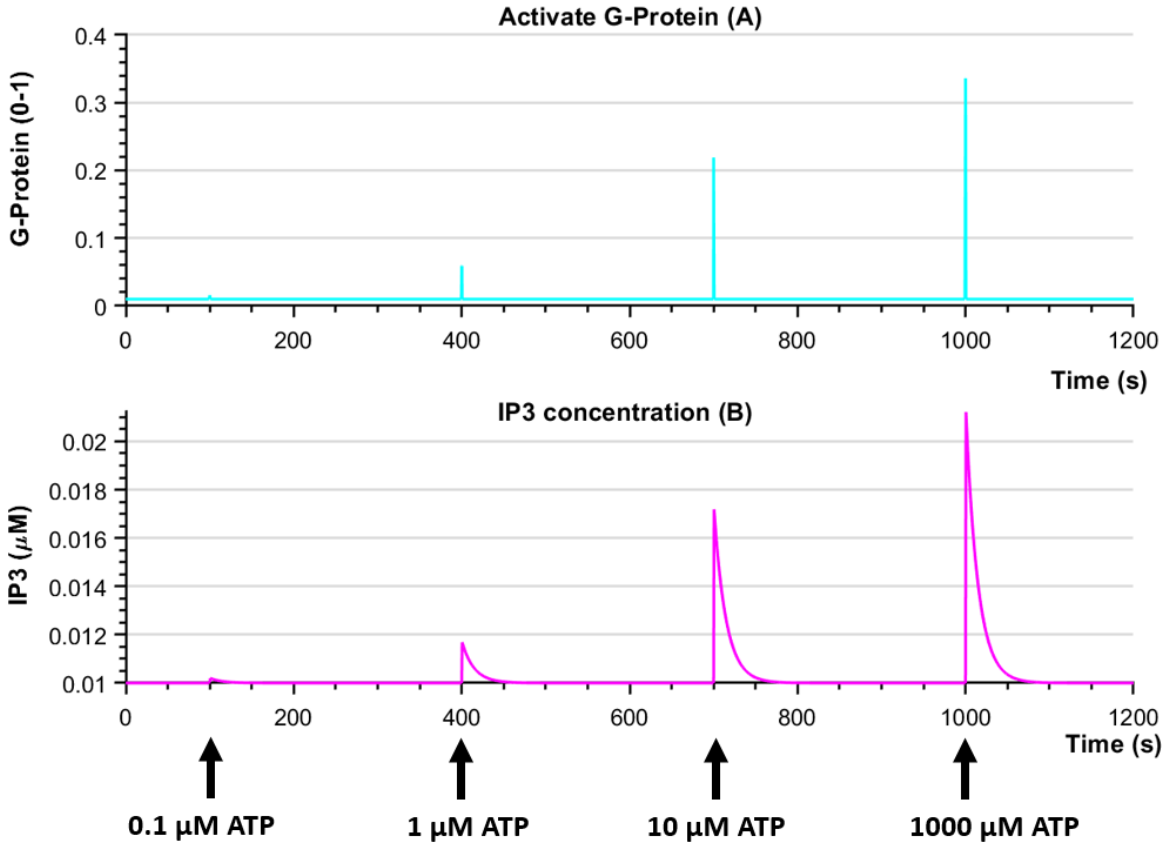


Figure 4.2: (A) Change in the level of G-Protein (ratio of Activated G-Protein) and (B) the production of IP3 in response to purinergic input at (100s; 400s; 700s; 1000s). Please note: the time duration of ATP stimulus is 0.5s

As shown in figure 4.2, in response to ATP stimulus at different time instants, the GPCR pathway (via P2Y receptor) is triggered. This results in the metabolism of IP3 molecule as explained in Chapter 3. Please note, the different levels of production of IP3 in response to different levels of ATP.

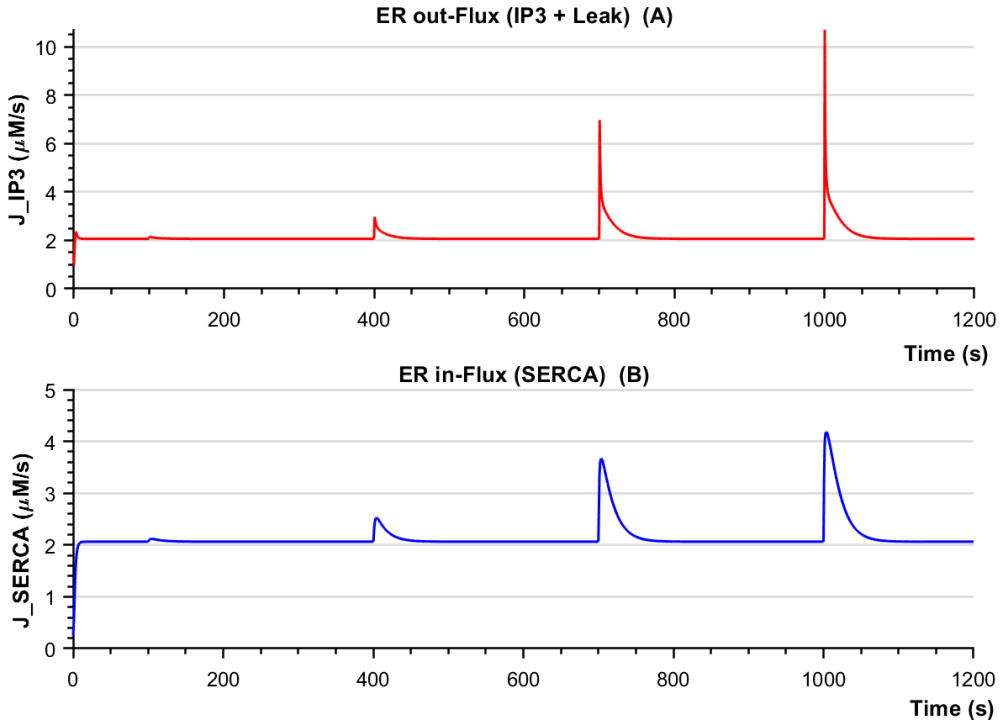


Figure 4.3: ER calcium fluxes in response to purinergic input at (100s; 400s; 700s; 1000s). Please note: the time duration of ATP stimulus is 0.5s

As shown in figure 4.1, there is production of IP3 in response to ATP stimulation. The IP3 produced triggers release of calcium from the ER store, as seen in Figure 4.2 (A). This results in activation of the SERCA pump flux as seen Figure 4.2 (B). SERCA retrieves the released calcium back into the ER.

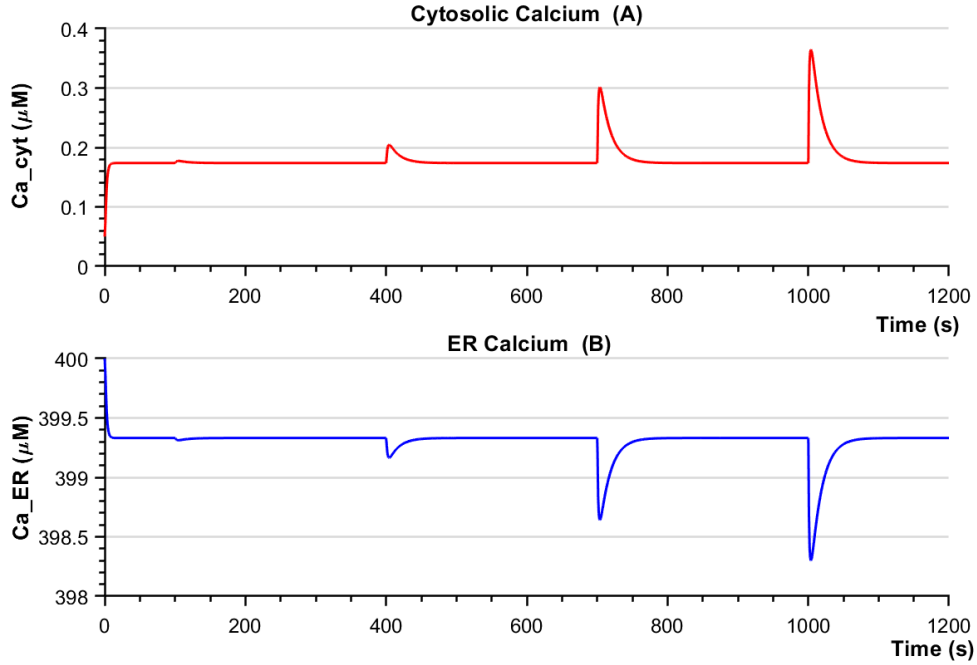


Figure 4.4: Calcium concentration in cytosol (A) and ER (B) in response to purinergic input at (100s; 400s; 700s; 1000s). Please note: the time duration of ATP stimulus is 0.5s

As seen in Figure 4.3, the responses i.e. rate of efflux and influx from the ER has different time courses. The IP3 flux activates faster and then inactivates. The SERCA flux activates slower and its activity returns to baseline once the ER calcium levels are restored. Please note, there is a baseline activity of the SERCA pump as a result of the leak flux (efflux) from the ER.

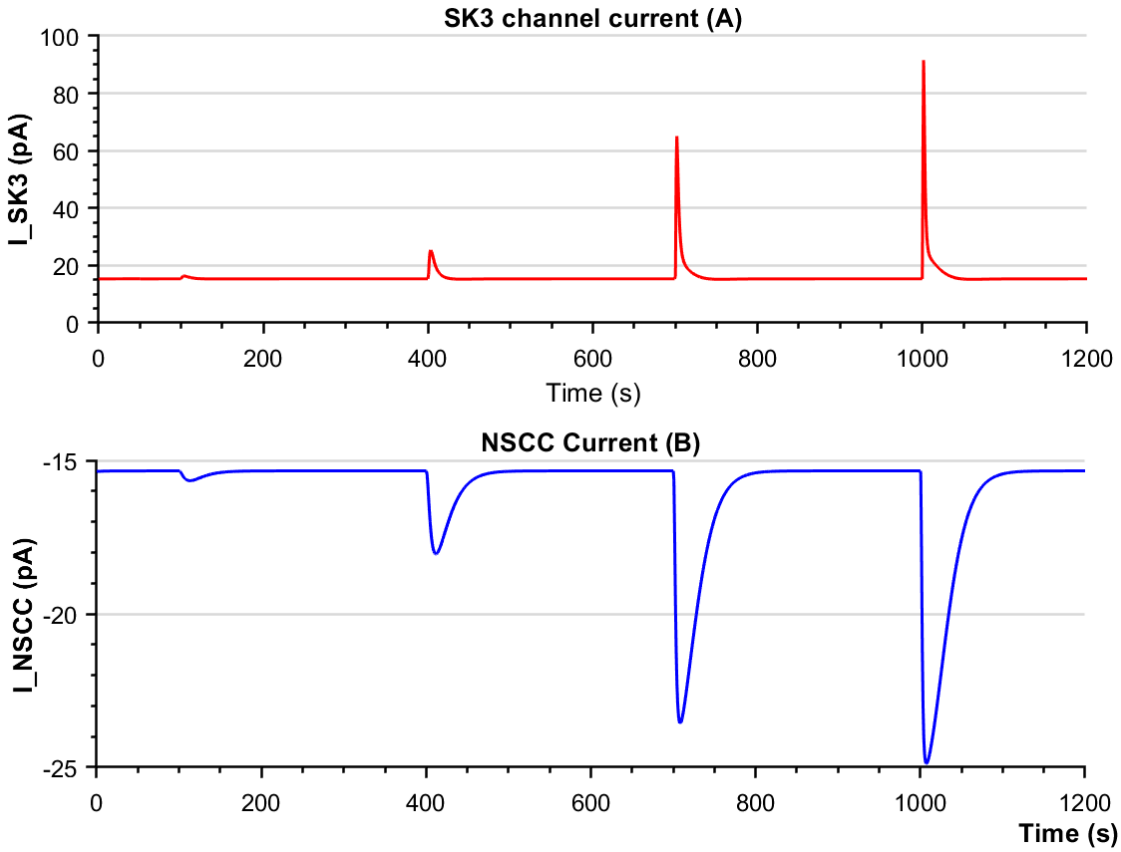


Figure 4.5: Membrane currents (A) outward K^+ via SK3 and (B) inward re-polarizing NSCC current; in response to purinergic input at (100s; 400s; 700s; 1000s). Please note: the time duration of ATP stimulus is 0.5s

The increase in the levels of cytosolic calcium activate the SK3 channels (calcium activated potassium channels). The SK3 channels mediate an outward potassium current that hyper-polarizes the membrane as seen in figure 4.5(A). This results in the hyperpolarization of the membrane (see figure 4.6), and the level of hyperpolarization is determined by the SK3 current amplitude which in turn is dependent on the level of ATP stimulation.

As the calcium levels in the cytosol return back to baseline (0.05 µM), the SK3 current returns to its baseline level. Do note that the SK3 current assumes a non-zero value at the baseline level. An inward non-specific cationic current (NSCC), as seen in figure 4.5 (B) brings the membrane voltage back to RMP (~ -50mV) as seen in figure 4.6.

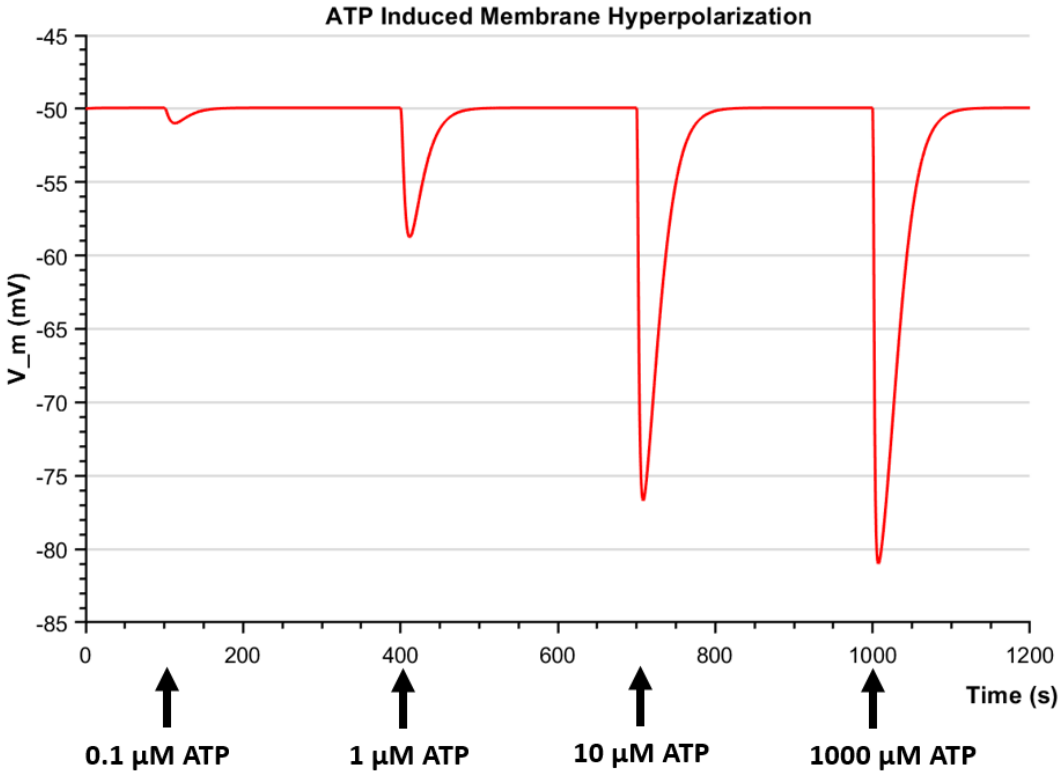


Figure 4.6: ATP induced membrane hyperpolarization in a PDGFRα+ cell in response to purinergic input at (100s; 400s; 700s; 1000s). Please note: the time duration of ATP stimulus is 0.5s

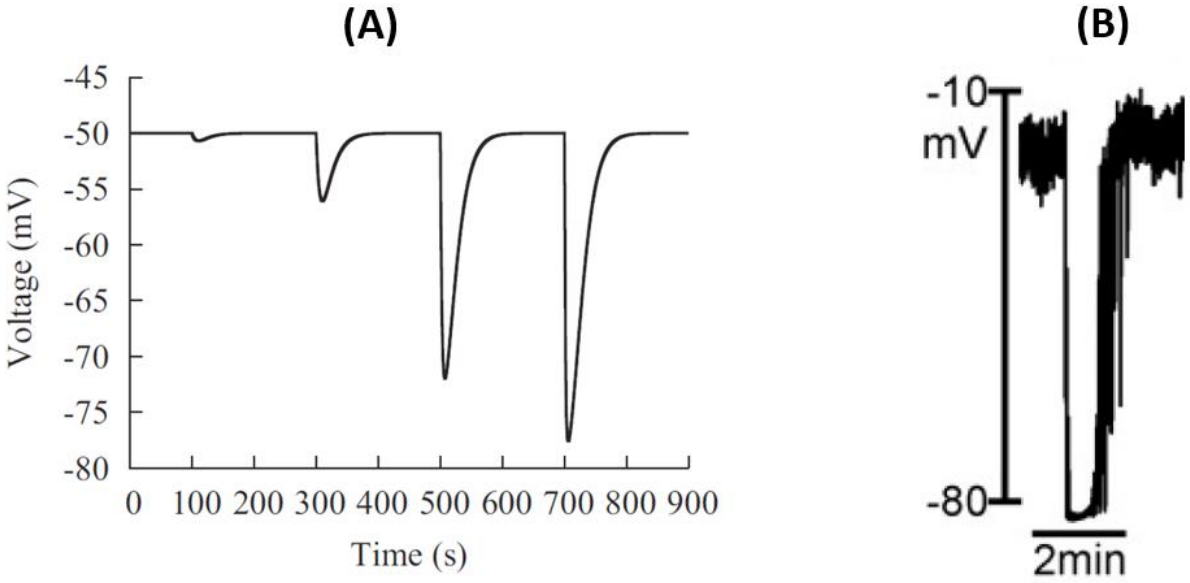


Figure 4.7: (A) ATP induced membrane hyperpolarization in a PDGFRα+ cell reported in the simulations by Yeoh et al., (2016); (B) Experimental recording of membrane voltage from a PDGFRα+ cell, membrane hyperpolarisation induced by ATP input (10 µM for 2 min) (Kurahashi et al., 2014).

4.3 DISCUSSION

As described in the literature survey, the principal role of PDGFRα+ cells in both the bladder as well as the GI tract is to mediate inhibitory neurotransmission via the purinergic pathway. The purinergic pathway is triggered when ATP binds to the P2Y receptors thereby leading to production of IP3. This feature of PDGFRα+ physiology is captured by this model. As seen in figure 4.2, different levels of ATP stimulation results in different levels of IP3 production. As well, this results in increase in cytosolic calcium, which in turn activates SK3 channels to produce the membrane hyperpolarisation. As seen in Figure 4.6, our implementation of the model produces a good match with the results reported in Yeoh et al., (2016). However, as seen in the experimental recording of the membrane voltage in response to 10 µM ATP, results in a net hyperpolarization of -70 mV in the PDGFRα+ . As well, the RMP seems to fluctuate around -10 mV in the absence of ATP input. These features of the PDGFRα+ cell physiology is not captured by the Yeoh et al., (2016) model which reports an RMP of -50 mV and does not capture the oscillation of RMP in the absence of ATP input.

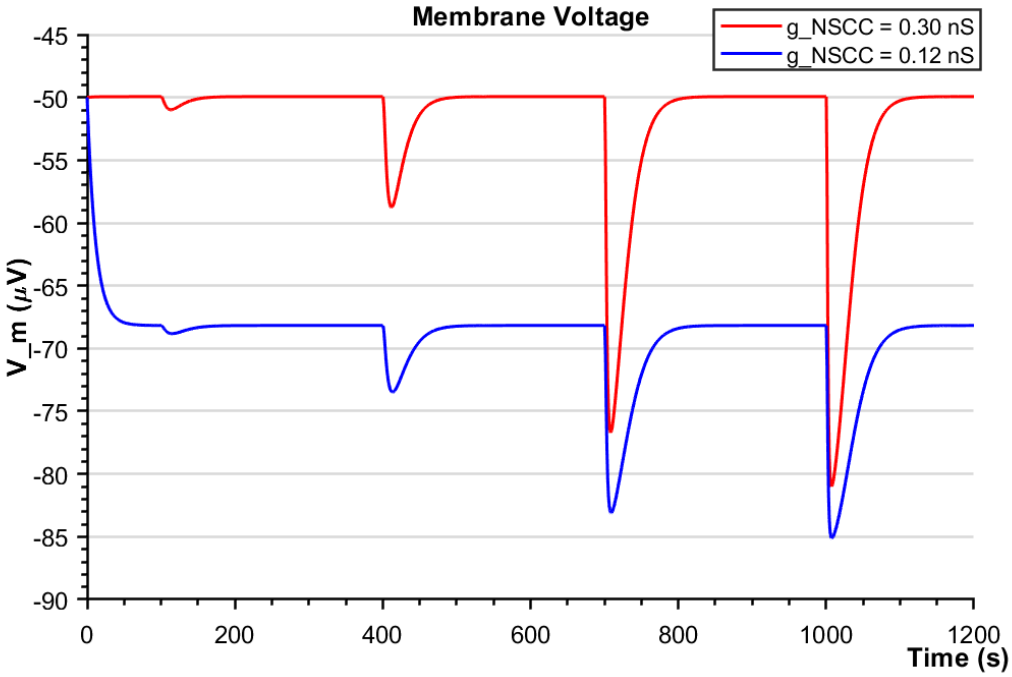


Figure 4.8: Difference in traces of membrane voltage in response to purinergic input with different values of NSCC conductance i.e. Yeoh et al., 2016 value (blue trace); and tuned value of NSCC conductance (red trace)

There were a few anomalies in the results when the model was initialized with the values reported by Yeoh et al., (2016). One such instance was the reported value of the NSCC

conductance. Here, when we simulated the activity of the PDGFRα+ cell, we found that for the reported value of NSCC conductance, the RMP was hyperpolarised to −68 mV. This was not in accordance to the results shown by Yeoh et al., (2016) which showed a stable RMP of −50 mV as seen in figure 4.7 (A). Once again this value does not match with experimental values, where the cell is depolarized to around −10 mV for isolated PDGFRα+ cells. To overcome this, we tuned the value to the NSCC conductance to obtain the reported RMP as seen in Figure 4.8.

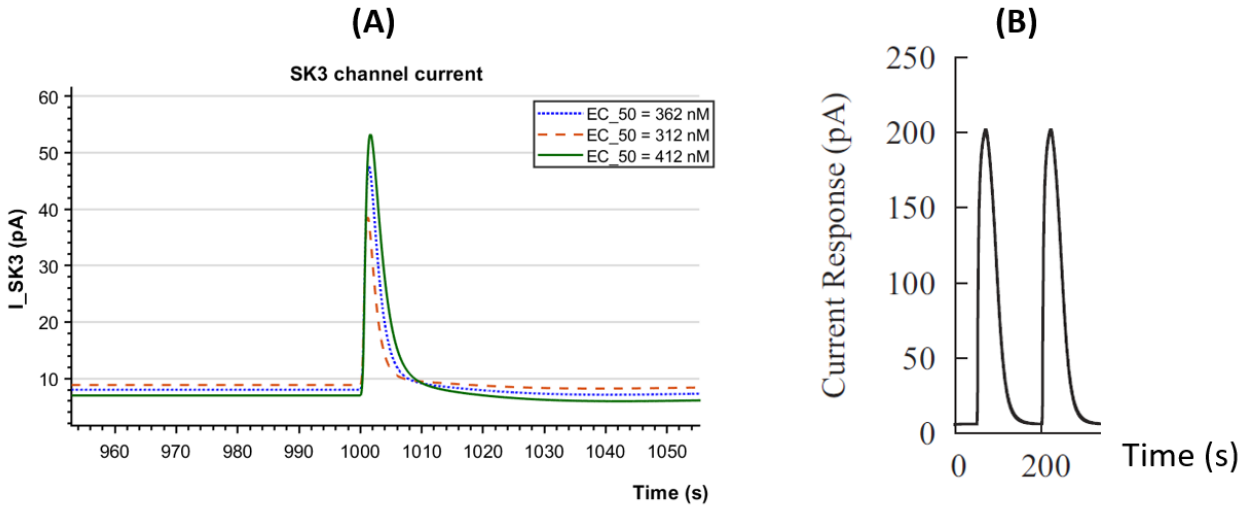


Figure 4.9: (A) Difference in traces of I_{SK3} current in response to purinergic input (ATP = 10 µM) for different values of EC_50 as reported by Yeoh et al., 2016. (B) I_{SK3} current trace as reported by Yeoh et al., (2016).

We also observed certain irregularities with the SK3 conductance. The first of these irregularities is the difference in the amplitude of the SK3 current. Our simulation is producing an amplitude of 48 pA whereas their simulation is producing an amplitude of 200 pA. Secondly, the time of activation of the current is for around 10s whereas their simulation shows the time of activation of the current to be around 100 s. The difference in the temporal profiles of the two currents is a matter that remains to be resolved. Lastly, the model takes a few seconds to stabilize. With the reported values of the initial condition by Yeoh et al., (2016), we are getting an initial bump in the SK3 current in the absence of the ATP input. We had to offset this erroneous component of the current by adjusting the open channel probability of the SK3 channel in our code during the initial 50s of the simulation. There are other aspects of PDGFRα+ physiology that is not captured by this model. We will present these aspects in the next chapter as part as the future work to be carried out during the second stage of the MTP.

CHAPTER 5

FUTURE DIRECTIONS

As indicated in the previous section, there are important aspects of the PDGFRα+ physiology that are not captured by the model described by Yeoh et al., (2016) especially from the point of view of developing a bladder specific PDGFRα+ mathematical model. The following are some of the future work directions to be included into the model framework in the context of both improving the existing model as well as adding relevant bladder specific cellular mechanisms for PDGFRα+ cells:

(i)	SK3	To verify and validate the model for the SK3 current for PDGFRα+ in tune with the experimental data for the same as reported in literature.
(ii)	TRPV4 + Piezo1	As mentioned in the literature survey (Chapter 2), the TRPV4 mediated stretch induced Ca^{2+} current and possibly the Piezo induced Ca^{2+} are pathways via which elevation in cytosolic calcium is achieved to trigger the SK3 induced hyperpolarization, and therefore, these need to be modelled in order to develop an accurate biophysically detailed model of PDGFRα+ cells in the bladder.
(iii)	NSCC	The authors use a dummy leakage conductance to model the repolarizing component of the membrane current. We need to look into the different ion channels which can possibly contribute to maintenance of a significantly depolarized RMP (~10 mV).
(iv)	P2 receptors	In addition to the role of P2Y(1), the possible roles of other P2Y and P2X family receptors reported to be present in PDGFRα+ cells need to be further investigated.
(v)	T type	T type calcium channels are present in abundance in PDGFRα+ cells. Future studies need to investigate any potential functional role for these channels in PDGFRα+ cell electrophysiology.
(vi)	Calcium Dynamics	Transient spontaneous calcium oscillations have been observed in PDGFRα+ cells. These might be the underlying cause for an unstable

RMP in PDGFRα+ cells. Therefore, the framework needs to be updated to understand the genesis of these signals as part of the modeling work.

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