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RESEARCH PAPER

Real-time Imaging of Ca^{2+}-handling in Intact Renal Glomeruli Using Confocal Microscopy

Muhammad Nabeel Ghayur* and Luke Jeffrey Janssen

Department of Medicine, McMaster University, St. Joseph's Hospital, Hamilton, Ontario, Canada

Abstract. Glomeruli are filtering units in the kidneys. Being multicellular and complex in structure, many aspects of glomerular function are yet to be elucidated. Most studies use glomerular cells in culture, which may exhibit altered physiology compared to native cells. Confocal microscopy has opened new avenues in exploring in situ glomerular function and physiology. In this report, we propose experimenting with glomerular cells in renal cortical slices and isolated intact glomeruli for Ca^{2+}-handling studies. Cortical slices (100 μm thick) were obtained from mice while intact glomeruli were isolated from rats using the sieving method. These were loaded with fluo-4 and then placed in a confocal microscope. Fluo-4 was excited using a 488 nm photodiode laser and images were collected at 1 frame/sec. Changes in average fluorescence intensity (AFI) were interpreted as changes in $[Ca^{2+}]_i$. AFI increased to 37.1 ± 6.7% and 84.3 ± 20.9% with Ang II (0.01 and 0.1 μM respectively). Norepinephrine (10 μM), arginine vasopressin (0.1 μM) and K^+ (30 mM) also elevated AFI by 26.5 ± 6.8%, 22.3 ± 1.0% and 39.8 ± 10.3% respectively in the glomerular cells. Likewise in isolated glomeruli, Ang II (0.1-10 μM), K^+ (30-90 mM) and endothelin-1 (0.01-1 μM), all showed elevation in $[Ca^{2+}]_i$. These results give an impetus for future studies examining Ca^{2+}-handling by confocal microscopy in glomerular cells using renal cortical slices and isolated intact glomeruli. The results support the utility of this system for study of glomerular physiology and pharmacology.

Correspondence: Dr. Muhammad Nabeel Ghayur, Department of Medicine, McMaster University, St. Joseph's Hospital, Room L-314, 50 Charlton Avenue East, Hamilton L8N4A6, Ontario, Canada. Tel: 1-905-5221155 x 34327. Fax: 1-905-5406510. E-mail: nghayur@mcmaster.ca

Abbreviations used: MC, mesangial cells; Ang II, angiotensin II; AVP, arginine vasopressin; ET-1, endothelin-1

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1. Introduction

There are some estimated 500 million people worldwide, about one in ten, with varying degrees of chronic renal disease [1]. As a consequence, more than a million people die every year around the world from end-stage renal disease [2]. Renal vascular disease and glomerulonephritis collectively account for approximately 32% of kidney failure in new patients [1]. The renal glomerulus consists of a network of capillaries engulfed in two epithelial layers (visceral epithelial cells also called podocytes), while the entire glomerular tuft is supported by mesangial cells (MC) which are contractile pericytes resembling vascular smooth muscle cells in respect to their signalling and cytoskeletal responsiveness to hormones [3]. MCs are secretory and produce growth factors for normal cell turnover, are targets of numerous inflammatory mediators, produce matrix proteins for structural support of capillaries and sometimes take the role of macrophages [3]. Contraction of MCs leads to decreased renal ultrafiltration coefficient, decreased capillary surface area, reduced permeability of capillaries and a resultant attenuation of GFR which can pathologically lead to compromised renal blood flow, increase in renal vascular resistance and thus renal hypertension [4]. This glomerular hypertension is mainly considered to contribute to glomerulosclerosis observed in a number of nephropathies [5]. Thus, it is imperative to study the effects and signalling of the different mediators which are known to be physiologically active in the renal glomerulus, such as angiotensin II (Ang II), arginine vasopressin (AVP), endothelin-1 (ET-1) and adenosine [3].

Most of the studies performed on glomerular cells (e.g., MCs, podocytes and endothelial) from mouse, rat or human kidneys have been done using cultured cells [6,7]. The physiological status of receptors and ion channels may be doubtful in such preparations, given the well-recognized phenomenon of phenotypic change during cell culture and passaging [7]. Thus many researchers doubt the contractile behaviour of MCs based on studies done on cultured cells, since the latter show the presence of smooth muscle α-actin while native cells do not [8]. Also, monocultures lack the complex cell-cell interactions which are present in the multicellular environment of the intact glomerulus.

We have been successfully using a slicing technique to study contractility and Ca^{2+}-handling in airways of different lung tissues [9]. In this study, we have adapted that approach, with modifications, to examine Ca^{2+}-handling in fresh mouse renal cortical slices (100 μm thick) and isolated intact renal glomeruli from rats using confocal microscopy.

2. Materials and Methods

2.1. Animals

Adult Balb-C mice and Sprague-Dawley rats were purchased from Charles River Laboratories (Saint-Constant, Quebec, Canada). Animals were housed at the animal quarters of St. Joseph's Hospital in environmentally-controlled, specific pathogen-free conditions, were given a standard mouse chow ad libitum and allowed free access to tap water. All studies complied with the guidelines of the Animal Research Ethics Board of McMaster University, St. Joseph's Healthcare Research Ethics Board, Canadian Council on Animal Care and the National Institutes of Health's (NIH) 'Principles of laboratory animal care' guidelines.

2.2. Chemicals and Solutions

Angiotensin II (Ang II), arginine vasopressin (AVP), endothelin-1 (ET-1), norepinephrine and potassium chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hanks' balanced salt solution (HBSS; without sodium bicarbonate and phenol red; Invitrogen Inc., Burlington, ON1, Canada) was supplemented with 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer (HEPES, 0.02 M) and was titrated to pH 7.4 with NaOH at 37ºC.

2.3. Preparation of Mouse Renal Cortical Slices

A modified methodology, originally employed for mouse lung slicing [9-11], was used.

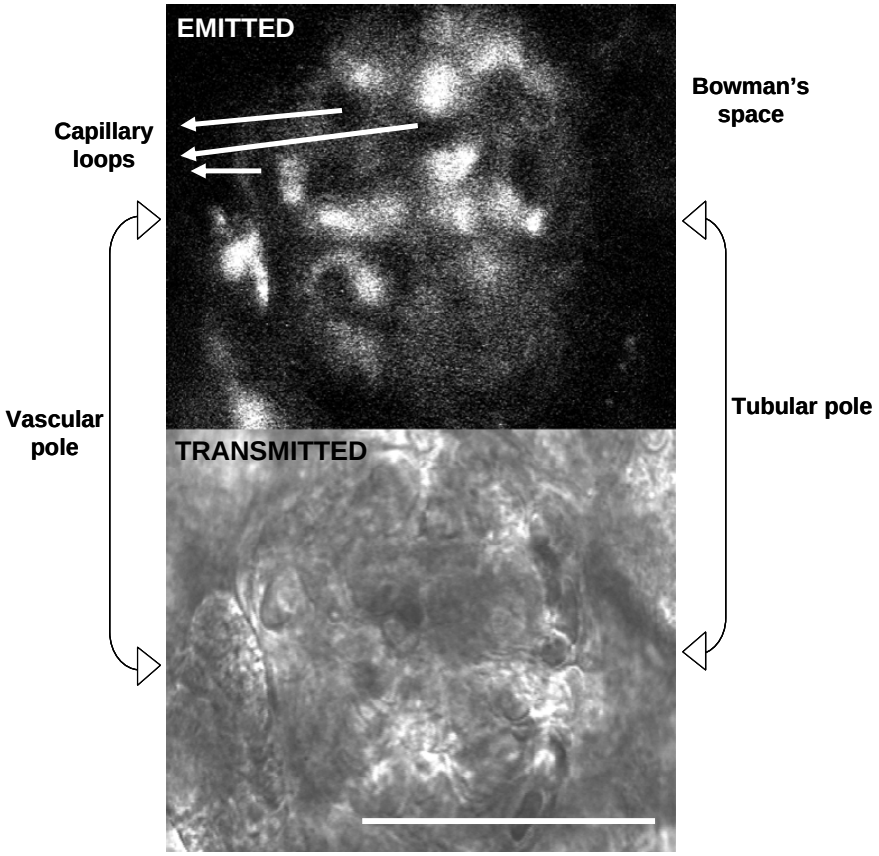

Figure 1. **Emitted and transmitted images showing different areas in a glomerulus. The glomerulus is captured in a mouse renal cortical slice (100 μm thick) surrounded by renal tubules (see the transmitted image). The slice was loaded with a Ca^{2+} indicator dye (fluo-4, 7 μM), then visualized under a 40x oil-immersion objective of a confocal laser scanning microscope (white scale bar = 50 μm).**

After euthanizing the mice with carbon-dioxide suffocation, the kidneys were removed through a midline abdominal incision. These were then transferred in cold HBSS. The kidneys were cleaned of fat and connective tissues, decapsulated and sliced longitudinally into half. The portion with the renal pelvis was discarded while the other half was used to create cortical slices using an EMS-4000 Tissue Slicer (Electron Microscopy Sciences, Fort Washington, PA). For better loading with Ca^{2+}-indicator dye, slices of 100 μm thickness were obtained. These cortical slices were kept refrigerated at 4ºC in HBSS solution and used within 2-3 hours; each slice was used for one experiment only. For each group of experiments, slices from 4 to 5 different mice were used.

For use in a confocal microscope, the slices were loaded for 1 h at room temperature with 7 μM of a Ca^{2+}-sensitive fluorescent probe, fluo-4 AM (Molecular Probes, Eugene, Oregon) dissolved in dimethyl sulphoxide with 0.01% pluronic F-127 added to enhance solubility [10]. The slices were then mounted between two glass cover slips, held in position by a piece of a nylon mesh, and placed on the stage of a custom-built confocal microscope equipped with a x40 oil-immersion objective. The bathing solution for all experiments was carbogen-aerated HBSS, maintained at 37°C, which was exchanged constantly via superfusion throughout the experiment. Tissues were washed for 30 min prior to the start of

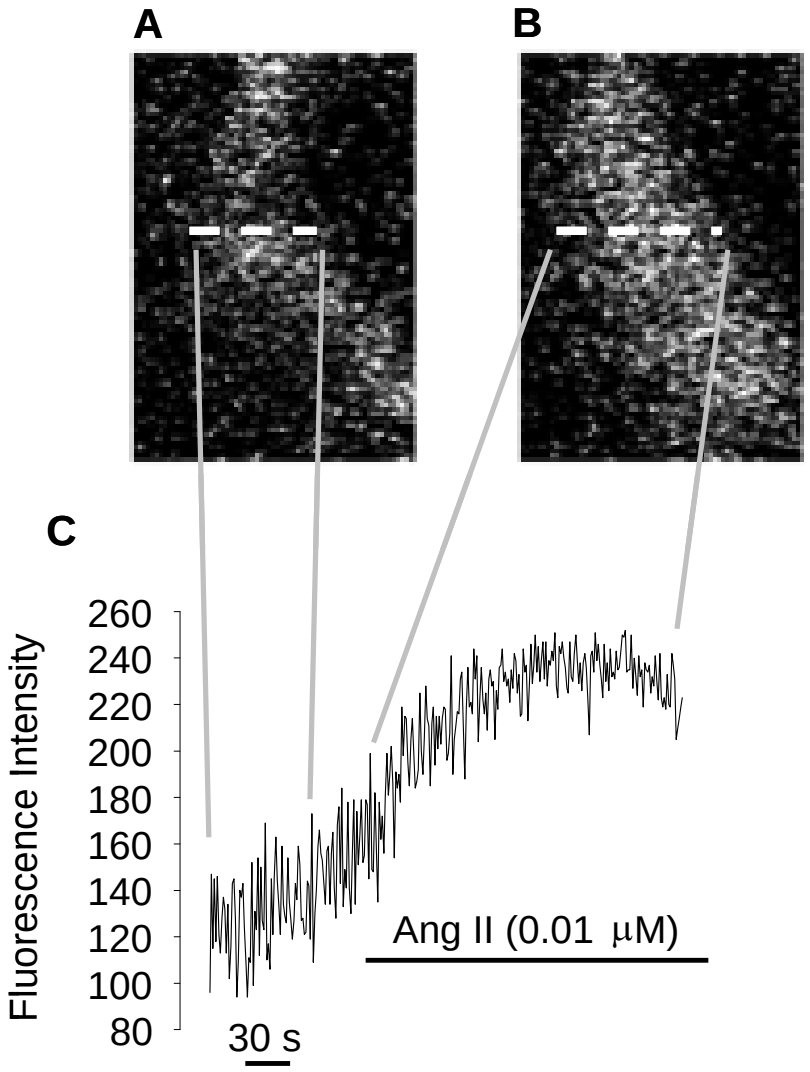

Figure 2. **Angiotensin II (Ang II) elevated $[Ca^{2+}]i$ in renal glomerular cells. A representative tracing showing a glomerular cell, A without and B with Ang II (0.01 μM) treatment that results in an increase in average fluorescence intensity (AFI) or $[Ca^{2+}]_i$ in response to the agonist. C shows a line graph of the increased AFI in response to Ang II (0.01 μM).**

experiment to remove extracellular fluo-4 AM.

2.4. Isolation of Rat Intact Renal Glomeruli

Glomeruli were isolated from renal cortex by a graded sieving technique [12]. After sacrificing the mice, kidneys were immediately removed and placed in cold HBSS solution at pH 7.4. These were then decapsulated, cut longitudinally into two halves and the isolated outer cortex was minced into small pieces with scissors. This cortical homogenate was passed gently (facilitated by use of ice cold HBSS and a spatula) through calibrated stainless steel sieves, kept on ice, of decreasing mesh sizes. Specifically, the chopped tissue was passed through a 250 μm sieve. The filtrate was collected and was drawn in and out of a 20 G needle three times before be-

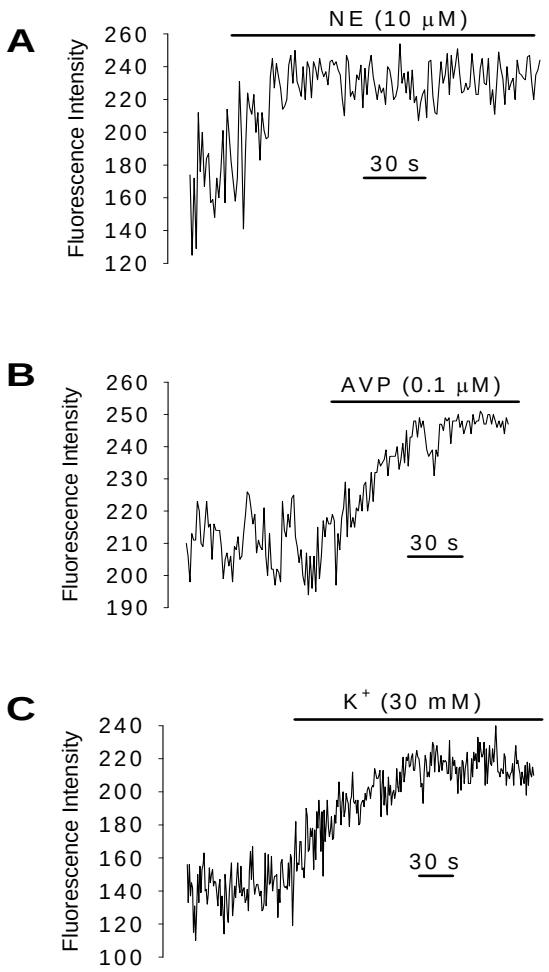

Figure 3. Increase in fluorescence intensity by different agonists. Tracings showing change in average fluorescence intensity evoked by norepinephrine (NE), arginine vasopressin (AVP) and K^+ [concentrations indicated] in fluo-4 loaded murine whole kidney slices via confocal microscopy.

ing placed on a 106-μm sieve. This was again gently sieved and finally the pulp was collected on a 53 μm sieve. The collected material which consisted mainly of glomeruli (~95%; mostly without Bowman's capsule) with minimum tubular contamination (as determined under a light microscope) was washed off the sieve, resuspended in HBSS and centrifuged at 4ºC for 5 min at 1610 rcf. Around 10% of the glomeruli were found with attached pre- and post-glomerular arterioles. Using this technique, the outer glomerular epithelium is usually damaged, but the remaining peripheral epithelium, endothelium and mesangial cells are intact. These isolated glomeruli were stored at 4ºC and used until about 2-3 h of their initial isolation.

For use in the confocal laser-scanning microscope, the glomeruli were loaded for 1 h at 37ºC with fluo-4 AM with 0.01% pluronic F-127 added to enhance solubility. The loaded glomeruli were transferred to a bath chamber with poly-L-lysin coated cover slides in the bottom and were allowed to attach for 15 min. The chamber was later mounted on the stage of confocal microscope. Tissues were washed for 30 min prior to the start of experiment. The bath solution for all experiments was carbogen-aerated HBSS at 37°C, introduced in the bath chamber as con-

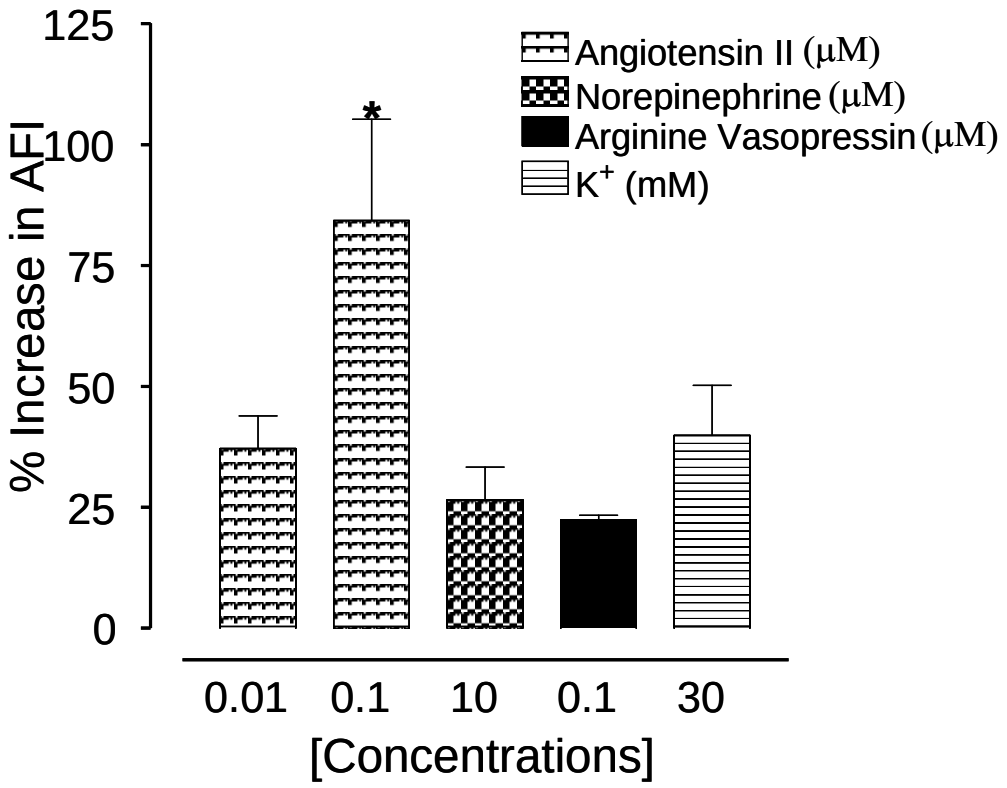

Figure 4. **Pooled data for effect of different agonists on $[Ca^{2+}]_i$. Mean changes in average fluorescence intensity (AFI) evoked by angiotensin II (n = 6), norepinephrine (n = 5), arginine vasopressin (n = 4) and K^+ (n = 5) in fluo-4 loaded murine whole kidney cortical slices recorded via confocal microscopy (values shown are mean ± SEM; * $P < 0.05$, Student's t-test).**

tinuous superfusion.

2.5. Image acquisition and measurement of $[Ca^{2+}]_i$

The tissues were illuminated using 488 nm light from a 20mW photodiode laser (Coherent Technologies; CA) and two distinct images (480 x 640 pixels) were collected simultaneously at 1 Hz: one comprised the light emitted by the dye (top image in Fig. 1, only wavelengths greater than 500 nm, using a long-pass filter) to indicate the changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) within individual cells, as well as the 488 nm laser light passing through the tissue to provide structural details of the whole tissue (transmitted or laser image shown in bottom part of Fig. 1). Images were formed and visualized on a computer screen using the recording software 'Video Savant' (IO Industries; London, ON). Exposure of the tissue to the laser light during the experiment was limited to the image collection period to minimize photobleaching. The digitized images for each time point following stimulation were analysed by selecting regions of interest of 10 × 10 pixels for measurement of $[Ca^{2+}]_i$ while cell pixel intensities were analyzed frame by frame using custom-written macros in the image analysis software 'Scion' (Scion Corporation, Frederick, Maryland). Fluorescence intensities of the regions of interest were saved and plotted against time. An increase in average fluorescence intensity (AFI) was interpreted as an increase in $[Ca^{2+}]_i$. Autofluorescence was evaluated in non-loaded preparations and was found to be less than 5% of the fluorescence of

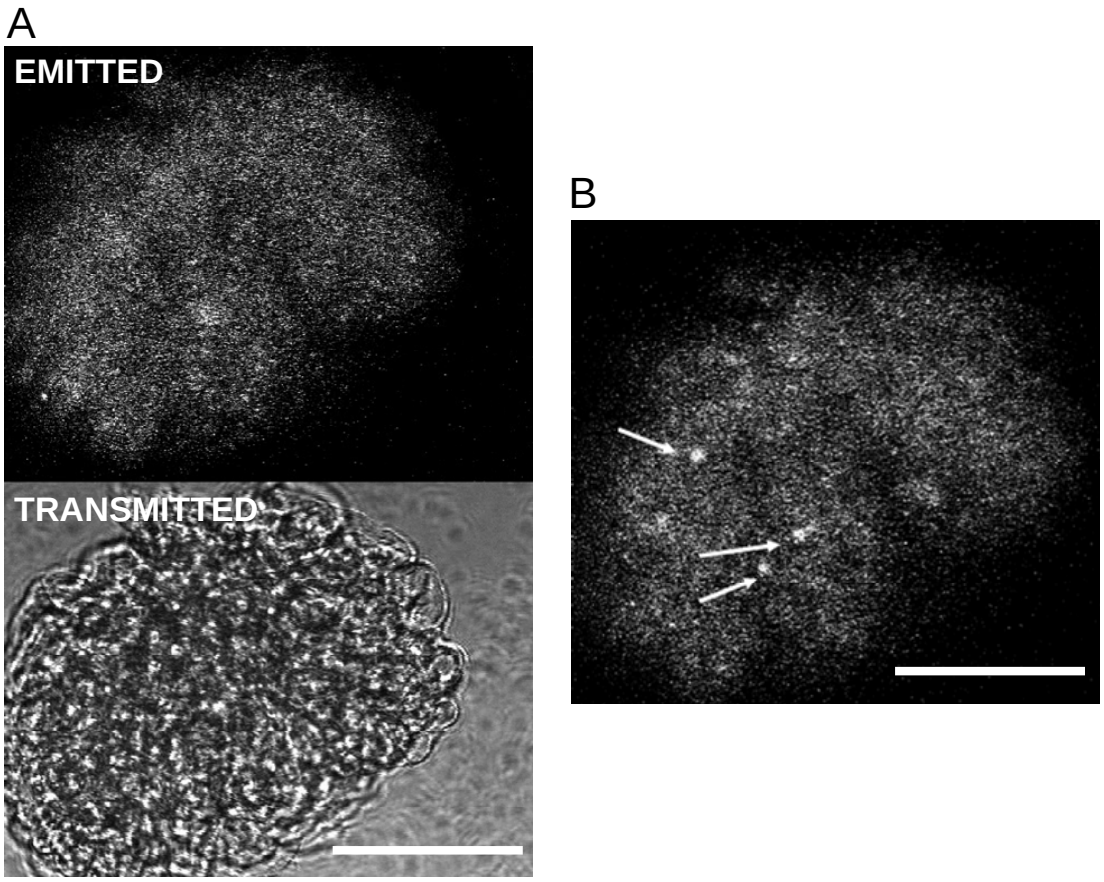

Figure 5. **Images showing an isolated rat glomerulus. A shows a comparison of the emitted (fluorescent) and transmitted (laser) images of an isolated decapsulated rat glomerulus at rest. The glomerulus was loaded with a Ca^{2+} indicator dye (fluo-4, 7 μM), then visualized under a 40x oil-immersion objective of a confocal laser scanning microscope.**

fluo-4 loaded tissues.

2.6. Data Analysis

All the data expressed are mean ± standard error of mean (SEM, n = number of experiments). The statistical tests used were Student's *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's test with $P < 0.05$ noted as being significantly different (GraphPAD program, GraphPAD, San Diego, CA, USA).

3. Results and Discussion

Kidneys play a major role in the regulation of blood pressure (BP). Proper functioning of kidneys ensures optimum control of BP. Studies have shown that if normotensive rats are transplanted with kidneys taken from normotensive rats that are genetically hypertensive-prone, the former also develop hypertension [13], a phenomenon also shown in humans [14,15]. If the kidneys are unable to regulate BP, then hypertension can ensue which leads to renal hypoperfusion and elevated glomerular pressure and glomerular filtration rate (GFR).

In addition to the mechanisms of tubuloglomerular feedback and myogenic responsiveness or autoregulation [6,16] that regulate GFR, contractility of glomerular cells, like MCs and podocytes, can also lead to overall contractility of the glomerulus, and thus regulation of

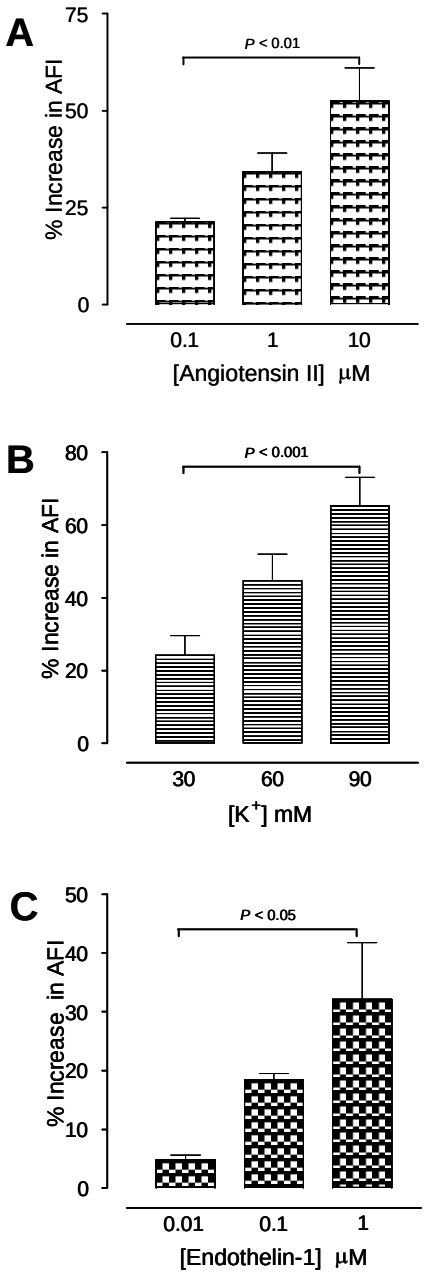

Figure 6. Pooled data for stimulatory effect of different agonists on $[Ca^{2+}]_i$. Mean ± SEM (one-way ANOVA) increases in AFI in response to angiotensin II (n = 3-9) (panel A), K^+ (n = 5-10) (panel B) and endothelin-1 (n = 3-4) (panel C) in isolated fluo-4 loaded rat renal glomeruli recorded via confocal microscopy.

GFR [6,12,17]. Glomerular cells contract to decrease the renal ultrafiltration coefficient, which reduces capillary surface area and permeability of the capillaries resulting in reduced GFR [4]. But this pathway is still controversial [18], mainly because of the multicellular nature of glomeruli and the difficulty in specifically targeting a particular cell type within a glomerulus [7,19]. Much of the data suggest glomerular cells such as MCs can contract [20-22]. However, because these cells can't be visualized directly in situ (given that they are buried within the multicellular glomerulus), most of the studies have been done with cultured MCs. It is well known that the physiological status of receptors and ion channels may be doubtful in cultured cells [23,24]; the same is true with glomerular cells [21,25]. Ouardani et al. [19] showed that cultured MCs undergo phenotypic modulations when compared with normal MCs. Different contractile proteins have also been identified in cultured MCs including myosin, tropomyosin, actin, α-actinin [26] and dystropin [19] which are not known to be present in these cells in vivo [8,27].

Keeping the controversies in mind, we have tried to come up with a technique where the glomerular cells can be imaged in real-time for Ca^{2+} signalling. This involved confocal microscopy of native renal cortical slices and isolated glomeruli loaded with fluo-4 in order to look directly into the live glomerulus and its resident cells. Although coming up to a stage to observe responses in result to administered stimuli took a lot of effort and time, here in this communication we have only given and discussed specifications that worked and yielded the results [28]. Using this approach, we were able to obtain emitted (fluorescent, $[Ca^{2+}]_i$) and transmitted (laser, morphological) images of a glomerulus under study at a frame rate of 1 Hz, showing distinct cellular areas within the filtering unit (Fig 1). This attempt has purely been to provide the proof-of-principle that it is possible to study Ca^{2+}-handling in intact glomeruli and to provide the application of this technique. It should be noted that it is not simply a paper describing the responses to a variety of autacoids. Thus we have refrained from dissecting the signalling pathways and mechanisms of action for the responses that were observed.

In the preliminary experiments performed on cortical slices, we focused on individual

glomeruli and found that Ang II increased fluorescence in discrete cells (Fig. 2) representative of increased $[Ca^{2+}]_i$ in those particular cells. Ang II was the most efficacious among all the agonists tested, showing a concentration-dependent increase in fluorescence of 37.1 ± 6.7% and 84.3 ± 20.9% (n=6; $P < 0.05$) at 0.01 and 0.1 μM respectively (Fig. 2 and Fig. 4). Among the other excitatory agonists tested, noradrenaline (10 μM), AVP (0.1 μM) and K^+ (30 mM) also showed increases of fluorescence of 26.5 ± 6.8% (n = 5), 22.3 ± 1.0% (n = 4) and 39.8 ± 10.3% (n = 5) respectively (Fig. 3 and Fig. 4). All these agonists are known to be excitatory to glomerular cells when tested by other techniques [3,29]. Ang II levels within the kidney are known to be approximately 1000 times greater than they are in the circulating blood, further implicating the significance that this peptide has in the kidneys [30].

Similar results were obtained in the experiments with isolated glomeruli. Figure 5A shows the different emitted and transmitted images of an isolated decapsulated glomerulus. When different agonists like Ang II, K^+ and ET-1 were tested on isolated glomeruli in increasing concentrations, all of the agonists showed an increase in AFI indicating increased $[Ca^{2+}]_i$. Ang II, with concentrations of 0.1, 1 and 10 μM, showed an increase in AFI of 21.3 ± 0.88%, 34.2 ± 4.9% and 52.4 ± 8.5% (n = 3-9) respectively (Fig. 6A). K^+ (30, 60 and 90 mM) increased the AFI by 24.2 ± 5.4%, 44.7 ± 3.7% and 65.2 ± 7.9% (n = 5-10) respectively (Fig. 6B) and ET-1 (0.01, 0.1 and 1 μM) exhibited increases of 4.8 ± 0.8%, 18.5 ± 1.0% and 32.1 ± 9.6% (n = 3-4) respectively (Fig. 5B and Fig. 6C).

Although these results are preliminary, they clearly demonstrate the potential usefulness of this technique. In comparison to studies on cultured cells, this technique retains endogenous interactions between the different cell types thus allowing for the monitoring of detailed cellular/molecular events in cells still in their native environment, with intercellular connections and interactions intact. This technique would not only allow Ca^{2+} imaging but can also be used with other fluorimetric dyes to image other ions, membrane potential, pH, etc. The slices under study can be fixed and stained for structural and immunohistochemical correlations after their use. By increasing the acquisition rate (up to 60 frames per second), one can extend to look at Ca^{2+} sparks and oscillations. Apart from all these potential advantages, there still is more work to be done to further develop it in terms of identifying the resident glomerular cell type(s) that show the changes in $[Ca^{2+}]_i$.

This study reiterates the importance of imaging microscopy in unravelling the different glomerular mechanisms taking place in a real-time fashion. We propose this technique to be employed to examine different physiological and pharmacological questions in this area of Ca^{2+}-handling in renal glomeruli.

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