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Single-Channel Analysis of TRPC Channels in the Podocytes of Freshly Isolated Glomeruli

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Abstract

One of the most important functions of the kidney is the filtration of the blood that takes place in the glomeruli. Glomerular epithelial cells (podocytes) have several functions, including regulation of the filtration process and glomerular basement membrane turnover. Dysfunction of podocytes is a major cause of glomerular kidney diseases. Gain-of-function mutations in the TRPC6 channel underlie a subset of familial forms of focal segmental glomerulosclerosis (FSGS). While growing evidence supports an important role of TRPC channels in podocytes, the regulation of these channels has yet to be investigated in freshly isolated glomeruli. Native settings in glomeruli provide, by all means, the most appropriate as well as one of the most challenging environments to study ion channel regulation. Thus, it is important to develop new methods that would better reflect the native settings of the podocytes. To address this question, we have established an experimental approach that allows studying podocytes in the freshly isolated decapsulated intact glomeruli. Here we describe the preparation of the rat glomeruli for patch-clamping, focusing on special conditions required for single-channel analysis of TRPC channels. Several tricks useful for cell-attached patch-clamping of the glomerular podocytes and solutions appropriate for registration of the TRPC channels are also provided.

Keywords

TRPC channels; TPC6; Glomeruli; Podocytes; Patch clamp; FSGS

1 Introduction

One of the main functions of the kidney is blood filtration that is tightly controlled by renal glomeruli. Glomerular podocytes are the central components of the renal filtration barrier. Podocytes are very dynamic cells which, at least in part, play a role in the pathogenesis of most proteinuric glomerulopathies (1, 2).

Transient receptor potential canonical (TRPC) channels belong to the larger superfamily of the TRP channels. TRPC channels play an important role in the pathogenesis of renal and cardiovascular diseases (3–6). Several members of the TRPC family, including TRPC6, are essential components of the podocyte slit diaphragm, where they are integrated into a

signaling complex that interacts with a number of podocyte structural proteins, including nephrin, podocin, alpha-actinin-4, and calcineurin (7–10). It was demonstrated that TRPC6 protein is the genetic impetus for an autosomal dominant form of focal segmental glomerulosclerosis (FSGS) (11–13). The known TRPC6 gain-of-function mutations cause an increase in calcium current in podocytes.

A number of studies provided new insights into the role of TRPC channels, and particularly TRPC6, in the functionality of the glomeruli and highlighted their role in mediating concomitant diseases (11, 12, 14). Most of these studies were conducted on the cultured podocytes (15–17) or in the recombinant systems (18,19) which, although being quite sustainable, cannot provide the comprehensive setting for understanding the regulation of the channels under physiological conditions. The lack of such studies has not been a result of their unimportance, but rather resulted from the fact that electrophysiological recording of endogenous ion channels in their native surrounding is a complicated procedure requiring a combination of pure isolation of glomeruli, separation of endogenous currents, electrophysiological skills, etc. Gloy et al., who have developed a technique allowing to measure membrane voltages and ion conductances of podocytes in isolated glomeruli (20), stated later describing the approach they developed that “it is important to develop new methods that better reflect the in vivo situation of podocytes, but studying podocytes function in situ is methodologically very difficult” (2).

Taking into account the emerging need to comprehend the functionality of ion channels of the podocytes, we have attempted to perform single-channel analysis of endogenous TRPC-like ion channels in the podocytes of isolated decapsulated glomeruli on the basis of the technique suggested by Gloy and colleagues (2, 20). The method described here consists of several steps including removal and perfusion of the rat kidney, isolation of the glomeruli fraction by sequentially pushing the kidney cortex through the steel sieves of different mesh, preparation of the glomeruli for patch-clamping, and electrophysiological cell-attached recordings themselves.

In contrast to Gloy et al., who removed the Bowman's capsule mechanically with a small broken glass pipette, we used sieving of the glomeruli as it was described previously (21–23). This approach allowed to avoid a time-consuming and potentially podocytes-damaging step of manual removal of the glomerular basement membrane. Fluorescence and electron microscopy techniques confirmed the integrity of podocytes in the isolated glomeruli after this isolation. Thus, the technical approach developed by us and described here could be used to search for physiologically relevant mechanisms of various ion channels regulation in both physiological and pathophysiological conditions and opens new directions of research into glomerular diseases.

2 Materials

2.1 Rat Kidney Isolation Surgery and Perfusion

1. Eight-week-old Sprague–Dawley male rat (we used internal MCW colony; however this rat strain is broadly commercially available (for instance, Charles River, USA, strain code 400)).

2. 25 mL of the cold Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, USA).
3. 100 μ L of pentobarbital (Sigma-Aldrich, USA).
4. 10 mL of the physiological salt solution (saline) (Sigma-Aldrich, USA).
5. Polyethylene tubing (PE50, Sigma-Aldrich, USA).
6. Syringe pump-based perfusion system (Harvard Apparatus, USA).
7. 1 $\times$ 2 teeth tip 12 cm Adson forceps (Fine Science Tools, USA).
8. Straight sharp 13 cm surgical scissors (Fine Science Tools, USA).
9. Straight 10 cm Graefe forceps (Fine Science Tools, USA).
10. Straight 12.5 cm Halsted-Mosquito hemostats (Fine Science Tools, USA).
11. Schwartz 26 mm sharp-bend micro serre fi nes (Fine Science Tools, USA).
12. Straight 11 cm Dumont #4 forceps (Fine Science Tools, USA).
13. Surgical suture, braided silk (Surgical Specialties Corporation).
14. Temperature controlled surgical table for rodents (Harvard Apparatus, USA).
15. Nitrile gloves (Kimberly-Clark, USA).
16. Binocular microscope (Nikon Eclipse TS100, Nikon, USA).

2.2 Isolation of the Rat Glomeruli

1. One freshly removed perfused kidney of an 8-week-old Sprague–Dawley male rat, kept on ice in HBSS.
2. Single-edge steel razor blade (Fisher Sci, USA).
3. Steel sieves (#100 (150 μ m), 140 (106 μ m) (Fisher Sci, USA)) and mesh 200 (screen for CD-1, Sigma-Aldrich, USA).
4. 5 mL of 30% albumin solution from bovine serum (BSA) (Sigma-Aldrich, USA).
5. 25 mL of culture media solution RPMI 1640 without antibiotics or FBS (Sigma-Aldrich, USA).
6. Steel “spoonulet” lab spoon (Fisher Sci, USA).
7. 6.75 cm straight Mayo dissecting scissors (Fisher Sci, USA).
8. Two 10-cm diameter plastic Petri dishes (TPP, Switzerland).
9. Five Fisherbrand Urisystem disposable plastic transfer pipettes (Fisher Sci, USA).
10. One 15 mL plastic conical Falcon tube (Santa Cruz, USA).

2.3 Single-Channel Analysis of TRPC Channel Activity Using Patch-Clamp Method

1. 25 $\times$ 25 Cover glass #2 (Corning, USA) cut into 5 $\times$ 5 mm pieces.
2. 0.01% solution of MW 70,000–150,000 poly-D-lysine (Sigma-Aldrich, USA).

3. Multiclamp 700B patch-clamp amplifier (Molecular Devices, USA).
4. Digidata 1440A analog-to-digital board (Molecular Devices, USA) interfaced with a personal computer running the *pClamp 10.2* software suite (Molecular Devices, USA).
5. MP-225 motorized micromanipulator (Sutter Instrument Co., Novato, USA).
6. Microvibration isolation table equipped with Faraday cage (TMC, USA).
7. Inverted microscope (Nikon Eclipse Ti , USA).
8. Model P-87 Flaming/Brown micropipette puller (Sutter Instrument Co., USA).
9. MF-830 microforge (Narishige, Japan).
10. Borosilicate glass capillaries (World Precision Instruments, USA) pulled and forged to 7–10 M Ω for cell-attached patch-clamp recordings.
11. Recording/perfusion chamber RC-26 (Warner Instruments, USA).
12. Multichannel valve perfusion system (Valve Bank II, AutoMake Scientific, USA).
13. Intracellular pipette solution: 126 mM NaCl, 1.5 mM CaCl₂, 10 mM HEPES, 10 glucose; pH 7.4 (all salts and chemicals purchased from Sigma-Aldrich, USA, unless noted otherwise).
14. Extracellular bathing solution: 135 mM NaAsp, 1 mM CaCl₂, 10 mM HEPES, 2 mM MgCl₂, 10 mM glucose; pH 7.4.
15. Chloride and potassium channels inhibitors in their final concentrations: 100 μ M niflumic acid (Sigma-Aldrich, USA) or DIDS (Sigma-Aldrich, USA), 10 mM TEA chloride (Tocris, USA), 10 nM iberiotoxin (Sigma-Aldrich, USA), 10 μ M nicardipine (Sigma-Aldrich, USA).
16. Adjustable volume pipette (10–100 μ L) with appropriate tips (Eppendorf Research plus 100 μ L, Eppendorf, USA).

3 Methods

For the successful isolation of rat kidneys, male rats of approximately 8 weeks old are used. The kidneys are flushed with PBS to clean out blood and urine. In addition to the electrophysiological measurements described below, this procedure may be useful for several other applications, e.g., immunohistochemical, biochemical, or molecular analysis of the tissue, etc. Figures 1 and 2 demonstrate steps required to isolate the glomeruli. Figure 3 shows the electron microscopy of the freshly isolated glomerulus fixed with 2% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.4). As seen on these images acquired by the transmission electron microscopy, a normal configuration of podocytes with coordinated pattern of foot processes was observed (Fig. 3). With the help of the patch clamp technique, we have examined the biophysical properties of native TRPC channels in the podocytes of the freshly isolated decapsulated glomeruli. Our results demonstrate that it is possible to study functional channels in these intact glomeruli. The use of specific inhibitors within the patch pipette together with the composition of the pipette and bath solutions and the

parameters of the current–voltage dependencies allowed distinguishing two channel types with distinct TRPC properties. However the development of this approach and identification of the precise composition of TRPC channels in podocytes require additional studies.

3.1 Rat Kidney Isolation Surgery and Perfusion

1. Appropriate ethical approvals need to be obtained before the work can be carried out; i.e., in our experiments, animal use and welfare adhered to the *NIH Guide for the Care and Use of Laboratory Animals* following a protocol reviewed and approved by the IACUC at the Medical College of Wisconsin.
2. 8-week-old male rats are anesthetized with pentobarbital *i.p.* injections (50 mg/kg) (see Notes 1 and ²). The rat is restrained manually and a 25 gauge or smaller needle attached to a syringe is inserted into the lower right quadrant of the abdomen. Before injecting, the syringe plunger is withdrawn to ensure that the needle has not entered a blood vessel or possibly the bowel. While anesthetized, the monitoring of anesthetic depth via assessments of toe-pinch withdrawal, respiratory rate, and related observational methods are utilized.
3. The anesthetized animal is brought to the animal preparation room where it is weighed and inspected.
4. After anesthesia the animal is placed on a temperature-controlled surgical table before a midline incision of the abdomen is made (see Fig. 1 for the schematic illustration of the following procedure).
5. The abdominal aorta is then catheterized with a polyethylene tubing (PE50), the vessel clamp is removed, and chilled physiological salt solution (PBS) infused for 2–3 min at a rate of 3 mL/min.
6. A clamp is placed just below the renal arteries around the abdominal aorta. The abdominal aorta is then ligated below the clamp and the celiac and superior mesenteric arteries also ligated (see Note 3 for a reference to other methods of the kidney perfusion).
7. The vena cava near the renal veins is incised to prevent pressure buildup from fluid.
8. After 2–3 min of infusion, the kidneys are excised and the diaphragm is cut to euthanize the animal.
9. The kidneys are put on ice into a 50 mL tube with 25 mL of the HBSS solution.

¹Non-survival surgeries are performed in septic but clean conditions. Instruments used should be clean, but not necessary sterile. All solutions used in this preparation should be sterile and kept on ice.

²Anesthesia can also be performed using isoflurane gas. In the case of inhalant anesthesia, the rat is placed in a transparent induction chamber. Isoflurane is delivered to the chamber via a precision vaporizer and compressed O₂. For induction, the percentage of isoflurane may be as high as 5%. Once the animal is unconscious, it is removed from the chamber and placed on a heated surgical table and a nose cone applied to continue delivery of anesthetic. The nose cone is attached to the vaporizer and oxygen source. At this point the concentration is reduced to that level which maintains the proper plane of anesthesia; typically this is between 1 and 3%.

³There are other slightly different methods of kidney perfusion than we described in this procedure. Here we provide a brief description of two additional methods.

3.2 Isolation of the Rat Glomeruli

1. Before the experiment, fresh solution of the RPMI 1640 with BSA is prepared adding 5 mL of 30% BSA to 25 mL of the media (see Note 4) (from here on referred to as *solution A*).
2. The kidney is taken from the HBSS solution and decapsulated with the help of the surgical scissors. See Fig. 2 that illustrates all the steps of the procedure for reference.
3. The decapsulated kidney is coronary cut in two halves with a razor blade (see Note 5) and then cortex is isolated and placed into approximately 4 mL of the ice-cold solution A.
4. The kidney is minced with a razor blade and mixed with a fine transfer pipette to obtain a homogenous substance (see Note 6).
5. The homogenate from step 4 is transferred onto the top of the 100 mesh sieve (see Note 7 that comments on the mesh size) and is pushed through the sieve with a spoon-shaped end of the spatula and is collected into a Petri dish (see Note 8).
6. The solution from a Petri dish from step 5 is transferred onto the top of the presoaked 140 mesh steel sieve, is let to flow through the sieve by gravity force, and is collected into a new Petri dish.
7. The solution collected at step 6 is filtered through the pre-soaked 200 mesh sieve and the filtrate is discarded (see Note 9). The glomeruli fraction sediments on the top of the sieve.
8. The top of the 200 mesh sieve is washed with approximately 15 mL of the solution A, and then glomeruli fractions are collected into a 15 mL tube and stored on ice (see Notes 10 and ¹¹).

⁴We have tested different solutions for isolation of the glomeruli to lower the cost of this procedure (i.e., RPMI media with

⁵We suppose that it might be more convenient to cut the kidney coronary in the ratio 1:3; this will allow removing the medulla from one piece, only. The second part will consist of the cortex only.

⁶It is supposed that 2–3 mL of the solution A is added to the fraction isolated after every step and then it is mixed with a fine transfer pipette until homogeneity is reached.

⁷The mesh size of the sieves listed in this chapter is adjusted for the 8-week-old male Sprague–Dawley rats; if you are going to isolate the glomeruli from mice or rats of other age, we would recommend adjusting the mesh size accordingly. The probable starting point for adjusting the mesh size for mice can be found in the works of Akis et al. and Rops et al. (25, 26).

⁸Steel sieves need to be soaked in the solution A. For large sieves, a drop of the solution is to be spread on the top of the sieve in the area you are going to use (up to 6–7 cm in diameter), and the small sieve is to be fully soaked in the solution A. The flow of the homogenate can be facilitated with the spoon-shaped end of the spatula. The homogenate that stayed on the reverse side of the sieve and did not get into the Petri dish can be collected from the reverse side of the sieve with a razor blade. Homogenate pushed through a new sieve always requires being collected into a new Petri dish.

⁹We would recommend being very careful with the last sieve as the pure fraction of the glomeruli rests of the top of it and it is likely to lose them at this step, so it would be reasonable to seep the solution onto it slowly, drop by drop.

¹⁰After the glomeruli fraction settles down to the bottom of the tube, we recommend removing most of the supernatant and leaving about 800 µL of the solution in the tube.

¹¹Takemoto et al. (27) suggested that the glomeruli of the mice can be efficiently isolated with the Dynabeads. In brief, the kidney is perfused with the solution containing magnetic Dynabeads, then minced, digested with collagenase, pushed through the sieve, and then the Dynabeads-containing glomeruli are separated with a magnetic particle concentrator. However, this approach seems to be more time-consuming and disturbs the native setting of the glomerulus with the Dynabeads.

3.3 Single-Channel Analysis of the TRPC Channel Activity Using Patch-Clamp Method

1. Immediately after isolation, the 15 mL tube with the fraction of the glomeruli (see Note 12) is left on ice for up to 20 min to let the glomeruli settle down and concentrate at the bottom of the tube (see Note 13).
2. After sedimentation the supernatant is removed and the rest of the solution containing the concentrated fraction of the glomeruli is kept on ice.
3. Before the patch-clamp experiment, the solution containing glomeruli is mixed and 50 μ L of obtained solution are removed by a pipettor and placed on the poly-D-lysine pre-coated 5 $\times$ 5 mm cover glasses (see Note 14); the chip is left at room temperature for 5–10 min to let the glomeruli settle down and attach to the surface. As demonstrated in Fig. 3, the podocytes of isolated glomeruli are intact.
4. The glass chip placed into the recording chamber of the patch-clamp setup is filled with the extracellular bathing solution and the chamber is perfused with the solution for 2–3 min to remove the unattached glomeruli and ensure the replacement of the BSA-containing solution with the bath solution.
5. Directly before the patch-clamp experiments the pipette solution used for the single-channel recordings is complimented with various inhibitors to prevent the registration of the unrelated chloride, calcium, and potassium currents: 100 μ M niflumic acid or DIDS, 10 mM TEA, 10 nM iberiotoxin, 10 nM nicardipine (see Note 15).
6. The patch pipette is slowly lowered to the surface of the glomerulus (see Note 16) and a high-resistance seal is formed with application of gentle suction. The formation of a gigaohm seal (>1 G Ω) is monitored by the pipette resistance that is increasing from 7 to 10 M Ω to more than 1 G Ω after the suction is applied. Figure 4 illustrates the decapsulated rat glomerulus in the patch-clamp setup with a patch pipette attached to a podocyte on the edge of the glomerulus.
7. Upon the formation of a high-resistance seal and compensation of the offsets, the cell membrane patch isolated by the pipette is voltage clamped with the potential set in the software. The currents evoked by the potentials are recorded and stored in the PC hard drive. For the cell-attached measurements, the currents are low-passed at 300 Hz by an eight-pole Bessel filter and digitized at 1 kHz.

¹²The fraction that is isolated with this preparation is highly pure and consists mostly of the decapsulated glomeruli; however, up to 10% of the glomeruli in the solution might still have a capsule. The final mixture contains very low quantity of other nephron segments. We (24) and others (21) successfully used this preparation for other applications such as Western blotting.

¹³The glomeruli fraction can be spun (5 min at 500 $\times$ g) to facilitate the sedimentation if the preparation is used for biochemical assays. However, we do not recommend this step if preparation is to be used for patch-clamp experiments as spinning makes the glomeruli more difficult to patch.

¹⁴Glass coverslips cut into pieces of approximately 5 $\times$ 5 mm are covered with poly-D-lysine before the experiment. We do not recommend using coverslips with lysine coating that are more than 3 days old.

¹⁵100 μ M niflumic acid or DIDS are added to block Ca²⁺-activated Cl⁻ channels; 10 mM TEA is used to inhibit the activity of the large-conductance Ca²⁺-dependent K⁺ channel; 10 nM iberiotoxin blocks the Ca²⁺-activated K⁺ channels; and 10 μ M nicardipine inhibits the N-type Ca²⁺ channels (24, 28). We recommend keeping the pipette solution on ice to maintain the inhibitors active.

¹⁶The podocytes are found on the surface of the glomerulus; the most amenable for patching podocytes are usually found on the edges of the glomerulus and have an oval prominent form on its surface (see Fig. 4 for reference).

8. The membrane is voltage clamped at -60 mV test potential using Clampex in the gap-free mode. If the channels activity is present then a current–voltage dependency is recorded. Figure 5a, b illustrates the activity of the two distinct types of ion channels registered in the cell-attached conditions at different potentials (from -120 to $+70$ mV) in the membrane of the podocytes of the freshly isolated glomeruli. The data revealed that the channels recorded were active throughout the range of holding potentials tested (see Notes 17 and ¹⁸). The current–voltage dependency shown at Fig. 5d allowed distinguishing two channel types. The identified TRPC-like channels were characterized by different conductances and displayed similar gating properties. Data analysis revealed two major channel populations with the conductance values of 10.3 ± 0.8 pS ($n = 11$) and 20.1 ± 0.7 pS ($n = 10$) (Fig. 5d). Both channels had a reversal potential (E_{rev}) of 0 mV. We termed these channels as “big” and “small” TRPC-like channels, respectively. Different conductances were sometimes found to coexist in the same patch as illustrated in Fig. 5c (24).
9. The cell-attached configuration allows applying the drugs and observing the changes in the channels activity in the native preparation of the intact glomerulus. Figure 6 shows the modulation of the TRPC-like channels in the podocytes as represented by inhibition by 500 μ M of the nonsteroid anti-inflammatory drug diclofenac (see Notes 19 – ²¹).
 - a. Ligate the distal abdominal aorta and distal inferior cava vein and clip the abdominal aorta and inferior cava vein by vessel clamp below the renal arteries and veins. Then catheterize the abdominal aorta and ligate the mesenteric and celiac arteries and abdominal aorta above the renal artery. After that remove the clamp and cut the inferior cava vein to ensure the flow of the perfusion solution and start per-fusing the kidney.
 - b. Another method of kidney perfusion suggests that the distal abdominal aorta and distal inferior cava vein, as well as the superior mesenteric and celiac arteries, should be ligated. Then the thoracic aorta is to be catheterized with venous retention needles (24 G) and a hole should be cut in the inferior cava vein to ensure venous drainage. FBS, DMEM media with FBS) and got a lower yield of the glomeruli, and the preparation was

¹⁷In our experiments the next day after isolation of the glomerular fraction, we succeeded to obtain stable patches with channels with approximately the same activity as on the same day of isolation. We have not tried to keep the glomeruli any longer, but it is possible that they might be vital for up to 1 week if they are kept at 4 °C. If this works, the same approach can be successfully used for over- or down-regulation of proteins of interest.

¹⁸Active channels were observed in up to 30% of the cell-attached patches obtained, whereas in approximately 50% of the patches that initially showed no channel activity, channels were activated by respective drugs (data not shown). Thus, there is a possibility that majority of channels are silent in this preparation.

¹⁹As reported by Gloy and colleagues, whole-cell current measurements are also possible on the freshly isolated preparation of the glomeruli (20).

²¹Biophysical properties, inhibition by SKF-096365 (blocker of TRP cation channels (29), insensitivity to the inhibitors within the patch pipette, and the parameters of the current–voltage dependence (Fig. 5) strongly suggest that the channels recorded in the membrane of the freshly isolated rat glomeruli are the members of the TRPC superfamily. However, several types of TRPC channels are identified in the podocytes, and formation of homo- and heteromeric complexes of these subunits are proposed (10, 30–33). Thus, precise identification of TRPC composition remains to be identified.

very difficult to patch; the glomeruli seemed to be too soft compared to original preparation.

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Fig. 1.
Schematic illustration of the rat kidney perfusion protocol

Fig. 2. Scheme of the glomeruli isolation protocol. Kidney cortex was isolated from the Sprague–Dawley rat kidney and then homogenized with a blade. The homogeneous cortex fraction was pushed through the consecutive steel sieves of the different mesh size and then collected into a Petri dish

Fig. 3.

Electron microscopy of freshly isolated decapsulated glomerulus. Fragments of the decapsulated glomerulus were visualized with electron microscopy at 1,500 $\times$, 5,000 $\times$, and 20,000 $\times$ (*a*, *b*, and *c*, respectively; scale bars are shown). *PB* podocyte body, *PN* podocyte nucleus, *EF* endothelial fenestrations, *FP* foot processes (reproduced from ref. 24 with permission from Elsevier)

Fig 4.

A representative image of a decapsulated rat glomerulus in the patch-clamp setup with a patch pipette attached to a podocyte on the edge of the glomerulus (40× and a close-up image) (reproduced from ref. 24 with permission from Elsevier)

Fig. 5.

Single-channel biophysical properties of TRPC-like channels identified in podocytes of freshly isolated rat glomeruli. All recordings were performed in cell-attached configuration in voltage-clamp mode. Representative current traces of “big” (a) and “small” (b) channels identified in the podocytes. The holding membrane potentials are indicated near the traces. c and o denote closed and open current levels, respectively. (c) Typical current traces held at -60 mV demonstrating coexistence of the two types of the channels in the same patch. Y_1 denotes the open current levels of channels with different conductances. (d) Single-channel current–voltage relationship for two types of channels identified. Values are means $\pm$ SEM of at least four experiments (reproduced from ref. 24 with permission from Elsevier)

Fig. 6.

Diclofenac modulate the activity of TRPC channels in podocytes of isolated glomeruli.

Representative experiment from a cell-attached patch containing TRPC channels.

Continuous current trace as well as the fragments of the traces at the expanded time scale are shown. *Arrows* demonstrate addition of diclofenac to the external bath solution. The patch was held at a -60 mV test potential during the course of the experiment. The c and o_i denote closed and open current levels, respectively (figure reproduced from ref. 24 with permission from Elsevier)