Acetylcholine Increases the Free Intracellular Calcium Concentration in Podocytes in Intact Rat Glomeruli via Muscarinic M₅ Receptors

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Abstract. The effects of acetylcholine (ACh) on the free intracellular calcium concentration ([Ca²⁺]) of microdissected glomeruli were investigated using fura-2 fluorescence digital imaging and two-photon confocal microscopy. ACh caused a concentration-dependent [Ca²⁺] increase with an initial peak followed by a sustained plateau, which was suppressed by reduced extracellular Ca²⁺ concentrations. The [Ca²⁺] plateau was not affected by the L-type Ca²⁺ channel blocker nicardipine, whereas gadolinium and lanthanum (both at 1 μM) blocked the plateau. Diphenylacetoxy-N-methylpiperidine methiodide (100 nM), an M₃/M₅ receptor antagonist, and pirenzepine (1 μM), an M₁ receptor antagonist, completely inhibited the effect of ACh. [Ca²⁺] measurements using two-photon excitation of fluo-3 and staining of the cells with calcine/acetoxyethyl ester, for observation of the capillary network together with the glomerular cells, showed that [Ca²⁺] was increased in single podocytes. Immunohistochemical studies did not demonstrate M₃ receptor expression in glomerular cells. M₁ receptors could be detected only in the parietal sheet of Bowman’s capsule, whereas M₅ receptors were found only in podocytes. The data show that ACh increases [Ca²⁺] in podocytes of intact glomeruli, most likely via muscarinic M₅ receptors.

Acetylcholine (ACh) modulates renal medullary circulation, induces vasodilation in isolated perfused kidneys, increases renal plasma flow, and produces natriuresis and diuresis (1). The effects of ACh can be modulated by locally produced hormones; for example, inhibition of cyclooxygenases by indomethacin reversed the effects of ACh on renal plasma flow, natriuresis, and diuresis (2,3). ACh is probably released from cholinergic renal nerves, as has been directly demonstrated in kidney cortex slices (4,5). Knowledge regarding the role of ACh in glomeruli is limited. ACh increases glomerular intracellular cGMP accumulation and produces glomerular contractions, probably via a Ca²⁺-dependent mechanism (6,7). Lebrun et al. (8) demonstrated that the parietal sheet of Bowman’s capsule and single intact glomeruli exhibit intracellular calcium concentration ([Ca²⁺]) increases in response to ACh. However, it was unclear, because of the limitations of conventional fluorescence assays, which of the three resident cell types of the glomeruli, i.e., podocytes, endothelial cells, or mesangial cells, responded to ACh (8). Each of the three glomerular cell types can be propagated in cell culture. It has been demonstrated that cultured glomerular endothelial cells do not respond to ACh with [Ca²⁺] increases (9) and, to our knowledge, ACh-mediated [Ca²⁺] increases in mesangial cells or podocytes have not been reported. However, the interpretation of possible effects of ACh in cultured cells is difficult, because many cell types that possess muscarinic receptors in vivo lose their muscarinic receptors under in vitro conditions. In addition, changes in the expression of muscarinic receptor subtypes may occur under cell culture conditions (10). To overcome these problems, we recently developed a technique that allows measurement of [Ca²⁺] in intact glomeruli (11). The aim of this study was to investigate whether ACh affects [Ca²⁺], in intact glomeruli and whether podocytes are involved in the [Ca²⁺] response to ACh.

Materials and Methods

Isolation and Preparation of Glomeruli

The method for the preparation of isolated glomeruli was previously described in detail (12). In brief, rat glomeruli were obtained using the sieving technique (the sieve sizes used were, in descending order, 150, 100, and 50 mesh size). A single glomerulus (with intact capsule) was transferred into a bath chamber mounted on the stage of an inverted microscope. The glomerulus was maintained at room temperature and immobilized at the vascular pole with a holding glass pipette. After incubation with 1 g/liter collagenase IV (collagenase A, catalog number C 5138; activity, 369 U/mg solid; Sigma Chemical Co., Deisenhofen, Germany) for 1 to 2 min and subsequent wash-off of the collagenase, the capsule was mechanically stripped off with a
second glass pipette. The integrity of podocyte morphologic features and the intactness of the glomerular basement membrane after such an experimental procedure have been proved in electron microscopic and immunofluorescence studies (12).

**Measurement of \([\text{Ca}^{2+}]_i\)**

The decapsulated glomerulus was held in the perfusion chamber with two micromanipulator-controlled glass pipettes and was loaded with fura-2/acetoxymethyl ester (AM) (5 μM) at room temperature for 20 to 25 min, as described previously (11). Experiments were performed using two different experimental configurations. One configuration, for fluorescence digital video microscopy (FDIM), consisted of an inverted fluorescence microscope (Axiovert 100 TV; Zeiss, Jena, Germany) equipped with a ×100 objective (Fluar 40/1.3 oil; Zeiss), a fast-switching monochromator (TILL; Photonics, Planegg, Germany) to change the excitation wavelength, and a generation 3, intensified, charge-coupled device camera (ICCD 350; Videoscope International, Sterling, VA) for fluorescence imaging (13). Fluorescence images (all emission signals above 505 nm) were acquired with an average of 16 frames, to increase the signal/noise ratio. Control of the experiment, image acquisition, and data analysis were performed with the software package MetaFluor/MetaMorph 3.5 (Universal Imaging, West Chester, PA). The number of the recorded ratiometric images was varied during the experiment. Shortly before and during hormone application, the ratiometric image acquisition rate was near 0.7 Hz; during control periods, the rate was decreased to 0.2 to 0.05 Hz. Optical filters and dichroic mirrors were obtained from Delta Light & Optics (Lyngby, Denmark).

**Confocal and Two-Photon Microscopy**

The one-photon excitation measurements with fluo-3 were performed with 488-nm excitation and collection of the fluorescence emission above 505 nm with a conventional confocal microscope (LSM 510; Zeiss). The confocal pinhole was set to achieve an optical slice thickness of 1 μm. Time series of fluorescence images (40/1.2 water objective, C-Apochromat, 60 to 150 images of 512 × 512 pixels, 2- to 4-μs pixel time) were recorded before and during ACh stimulation. A prototype two-photon microscope system (LSM 560; Zeiss) with a turnkey fiber laser (780 nm; pulse width, <180 fs; maximal power, 7.4 mW) was used to image fluo-3-loaded glomeruli (loading for 20 min with 5 μM fluo-3/AM at room temperature) with a ×40 objective (Plan-Neofluar 40×/1.3 oil; Zeiss). The mean laser power at the objective plane was reduced to 1.2 mW using gray filters. The two-photon excitation wavelength of 780 nm allowed sufficient excitation of fluo-3 for the \([\text{Ca}^{2+}]_i\) measurements. After the \([\text{Ca}^{2+}]_i\) measurements, the glomeruli were stained with the general cell marker calcine/AM (5 μM for 20 min at room temperature). Then, z-stacks (z-distance, 0.8 μm) of high-resolution images (1024 × 1024 pixels) of the calcine-labeled glomeruli were recorded. With both dyes, all fluorescence emission below 680 nm was collected without using a pinhole in front of the fluorescence detector (13).

**Calibration of the FDIM \([\text{Ca}^{2+}]_i\)**

Calibration of the FDIM \([\text{Ca}^{2+}]_i\) measurements was performed with ionomycin at the end of the experiment, as described (11). No correction for background levels or autofluorescence was necessary in the FDIM or two-photon microscopy experiments, because the two parameters together exhibited a maximal pixel intensity of 5. The results of the successful calibrations were pooled and used for calculation of the \([\text{Ca}^{2+}]_i\) values (mean ± SEM), according to the method of Grynkiewicz et al. (14). The averages of the pixel-by-pixel ratios (fura-2) or the raw fluorescence intensities (fluor-3) of user-selected areas, usually one or two for the FDIM experiments (emission at >470 nm with 345/380-nm excitation) and 15 to 20 for the two-photon images (emission at <680 nm with 780-nm excitation), were used for the data analyses. No \([\text{Ca}^{2+}]_i\) calibration of the fluorescence signal could be performed for the confocal \([\text{Ca}^{2+}]_i\) measurements. The glomerular structure is significantly altered by the application of ionomycin during the calibration procedure. These changes preclude reliable single-cell calibration, which would be necessary for single-wavelength dye calibration.

**Immunohistochemical Analyses**

**Fixation and Preparation of Tissue for Immunohistochemical Analyses.** The distribution of muscarinic receptor subtype M1, M3, and M5 proteins in the kidney was determined by immunocytochemical analyses, using respective polyclonal antisera (IgG fraction rabbit anti-muscarinic M1, M3, and M5 receptor sera; Biotrend, Köln, Germany). In addition, for detection of the M3 receptor, two different polyclonal goat IgG antibodies (N-18, epitope-mapping at the amino terminus of the M3 receptor; and C-20, epitope-mapping at the carboxyl terminus of the M3 receptor; Santa Cruz Biochemicals, Santa Cruz, CA) were used. M3 receptor staining was also tested in HT29 colon carcinoma cells, which were cultured as described previously (15).

Rats were anesthetized with Trapanal (5 to 7 mg/kg body weight; Byk Gulden, Konstanz, Germany). The kidneys were perfused at low pressure, through the artery, with 5 ml of cold (4°C) standard solution and then with approximately 5 ml of 4% paraformaldehyde solution. The kidneys were removed from the rats, decapsulated, and incubated for 24 h at 4°C in 4% paraformaldehyde solution.

**Sectioning and Immunolabeling.** Small pieces (4-mm thickness) of the kidneys were embedded in paraffin and cut into thin slices (5 or 7 μm). The slices were deparaffinized in xylol for 45 min and gradually hydrated through graded alcohols (100 to 70%). The slices were rehydrated with phosphate-buffered saline (PBS), and antigen unmasking was performed by heating the slices in a sodium citrate buffer (1 mM) for 4 min, in a pressure cooker. To reduce nonspecific binding, sections were incubated for 20 min with blocking sera (10%) from the respective animals (Biotrend) and throughout the labeling procedure, background-reducing buffer from DAKO (Copenhagen, Denmark), which also contained 1.5% of the respective blocking sera, was used. Sections were incubated for 24 h, in a humidified chamber at 4°C, with antibodies against M3, M1, or M2 receptors (M1, 1:500; M3, 1:400; M5, 1:500 or 1:800; Biotrend) and in addition, for double-labeling of podocytes, with a mouse monoclonal antibody against synaptopodin (1:2; Progen, Heidelberg, Germany). The slices were washed extensively with PBS and incubated for 45 min with the secondary antibody, Alexa Fluor 488 goat anti-rabbit Ig antibody (1:200 to 1:800; Mobitec, Göttingen, Germany). For double-immunostaining of synaptopodin, Cy3 goat anti-mouse Ig antibody (1:200 to 1:400; Dianova, Hamburg, Germany) was also used. Finally, nuclei were stained with HOE 33342 (10 μM; Mobitec) for 10 min. Slices were washed twice with PBS and twice with distilled water and were mounted with Glycergel (Dako). Sections were examined with conventional confocal microscopy (Zeiss LSM 510), using sequential image acquisition after excitation at 361 nm (HOE33342; emission, 385 to 470 nm), 488 nm (Alexa Fluor 488; emission, 505 to 550 nm), and 543 nm (Cy3; emission, >560 nm). Images of 1024 × 1024 or 2048 × 2048 pixels were recorded by setting the pinholes in the respective acquisition channels to obtain optical slices with a full-width half-maximum of 0.5 μm. The respective controls for nonspecific staining exhibited no fluorescence signals at the same instrument settings.
Western Blotting

Whole-brain tissue and glomeruli were homogenized in ice-cold Tris-buffered saline containing 2 mM ethylenediaminetetraacetate, 100 mM NaCl, 20 mM Tris, 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 2 mM phenylmethysulfonyl fluoride, and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and were centrifuged (14,000 × g at 4°C). The cell membranes were sonicated in buffer containing 1% Nonidet P-40 (Boehringer Mannheim, Mannheim, Germany). The samples were resuspended in Laemmli sample buffer, boiled (5 min), and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer electrophoresis. The transblots were probed with the primary antibodies (anti-muscarinic M5 receptor; Biotrend), followed by peroxidase-labeled secondary antibodies (donkey anti-rabbit; Amersham Pharmacia Biotech, Piscataway, NJ), and were detected with chemiluminescence detection reagents (Amersham Pharmacia Biotech).

Solutions and Chemicals

The standard solution contained 145 mM NaCl, 1.6 mM K2HPO4, 0.4 mM KH2PO4, 1.3 mM calcium gluconate, 1 mM MgCl2, and 5 mM D-glucose, pH 7.4. The specific compositions of the experimental solutions were recently described (11). Fura-2/AM, fluo-3/AM, and calcein/AM were obtained from Mobitec. All other chemicals were of the highest purity grade available and were obtained from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). All experiments were performed at 37°C.

Statistical Analyses

The data are presented as mean ± SEM values; n refers to the number of experiments. Paired or unpaired t tests were used to compare mean values within or between independent experimental series. A P value of ≤0.05 was considered to indicate statistical significance.

Results

FDIM Measurements of \([Ca^{2+}]_i\)

In FDIM measurements, ACh (0.01 to 100 μM) caused reversible increases in \([Ca^{2+}]_i\), measured as the fura-2 345/380-nm excitation ratio signal, inside the chosen focal plane on the outer lower surface of the glomerulus (n = 6 to 24). An original trace of a ratio recording from a single glomerulus, using different concentrations of ACh, is presented in Figure 1A. ACh at higher concentrations (>1 μM) induced a biphasic \([Ca^{2+}]_i\) response, with a peak and a plateau. The shape of the

Figure 1. (A) Original recording of an acetylcholine (ACh)-induced increase in the fura-2 345/380-nm fluorescence ratio as a measure of the intracellular calcium concentration ([Ca^2+])_i in the glomerulus. The ratio was calculated from the mean pixel-by-pixel ratio values for a user-selected window placed in the central part of the fluorescence ratiometric image of the glomerus. (B) ACh concentration-response curve. The numbers in parentheses refer to the number of experiments. *, statistical significance compared with control.

Figure 2. Original recordings showing that the ACh-induced [Ca^{2+}]_i plateau is totally attributable and the [Ca^2+]_i peak is partially attributable to Ca^{2+} influx from the extracellular space. (A) The [Ca^{2+}]_i plateau is completely suppressed in the presence of a low extracellular Ca^{2+} concentration of 1 μM, and the peak is reduced by almost one-half. (B) A reduction of the extracellular Ca^{2+} concentration from 1.3 mM to 1 μM reversibly abolished the [Ca^{2+}]_i plateau, to a value not different from the resting [Ca^{2+}]_i value.
biphasic transient was quite variable; the decrease to a more or less stable plateau could occur in 4 to 6 min (Figures 2B, 3B, and 4A) but sometimes was much slower (Figure 1A). This heterogeneity could also be observed on the single-cell level, using confocal microscopy (Figure 7). Figure 1B shows the concentration-response curve for the peak \([\text{Ca}^{2+}]_{i}\) responses to ACh. A 10-nM threshold concentration of ACh was required to induce a significant \([\text{Ca}^{2+}]_{i}\) increase. The maximal \([\text{Ca}^{2+}]_{i}\) signal was obtained with 10 \(\mu\text{M}\) ACh. The half-maximal response could be calculated to be 0.3 \(\mu\text{M}\). With an ACh concentration of 1 \(\mu\text{M}\), \([\text{Ca}^{2+}]_{i}\) increased from a resting value of 81 ± 26 nM to 406 ± 82 nM (\(n = 9\)). Desensitization of the ACh receptor was tested at three different ACh concentrations. As shown in Figure 6A, repetitive applications of 10 \(\mu\text{M}\) ACh (up to five applications) did not decrease the magnitude of the peak \([\text{Ca}^{2+}]_{i}\) response. Figure 6B summarizes the results obtained with 0.1 to 10 \(\mu\text{M}\) ACh. For none of the tested concentrations was a significant \([\text{Ca}^{2+}]_{i}\) decrease measured between the first and fifth stimulations. Response heterogeneity was again observed. For example, of the five experiments performed with 1 \(\mu\text{M}\) ACh, three demonstrated small and continuous decreases from stimulation to stimulation, whereas the ACh-induced peak \([\text{Ca}^{2+}]_{i}\) response increased slightly in the two other experiments. To address the issue of the heterogeneity of the ACh-induced \([\text{Ca}^{2+}]_{i}\) response on the single-cell level, we used confocal microscopy with the Ca\(^{2+}\) dye fluo-3 and ACh concentrations of 1 and 100 \(\mu\text{M}\). In a total of six experiments, 47 cells responded with \([\text{Ca}^{2+}]_{i}\) increases after stimulation with 1 \(\mu\text{M}\) ACh. The same 47 cells and not more also responded to 100 \(\mu\text{M}\) ACh. The mean peak increases (measured as the mean fluorescence pixel intensity) were not significantly different between 1 \(\mu\text{M}\) (104 ± 10% increase) and 100 \(\mu\text{M}\) (93 ± 10% increase).

Evidence that the ACh-Induced \([\text{Ca}^{2+}]_{i}\) Increase Is Attributable to Intracellular Ca\(^{2+}\) Release and Ca\(^{2+}\) Influx

Figure 2A shows the effects of ACh on \([\text{Ca}^{2+}]_{i}\) in the presence of a reduced extracellular Ca\(^{2+}\) concentration. Re-
duction of the external Ca$^{2+}$ concentration significantly reduced resting [Ca$^{2+}$] from 92 ± 6 nM to 45 ± 7 nM ($n = 9$). The ACh (10 mM)-induced [Ca$^{2+}$], plateau was abolished (52 ± 8 nM), and the peak [Ca$^{2+}$] increase was reduced. One-half of the experimental series of the type presented in Figure 2A were performed in the reverse order, to avoid effects of receptor desensitization. In seven of nine experiments with 10 mM Ca$^{2+}$, a mean reduction of the peak [Ca$^{2+}$] increase of 47 ± 6% was measured. In the two other experiments, the peak was increased (130 and 157%). We have no explanation for the peak increase under these conditions, because the removal of Ca$^{2+}$ had reduced resting [Ca$^{2+}$] in these two glomeruli to a similar extent, compared with the other glomeruli.

Figure 2B demonstrates that the reduction of the extracellular Ca$^{2+}$ concentration from 1.3 mM to 1 mM completely abolished the maximal [Ca$^{2+}$], plateau and reduced [Ca$^{2+}$], below the resting value measured before the reduction of the external Ca$^{2+}$ concentration. The mean resting [Ca$^{2+}$], in these experiments was 136 ± 31 nM before ACh stimulation, compared with a value of 80 ± 34 nM for ACh stimulation with 1 mM Ca$^{2+}$ ($n = 8$).

To examine whether ACh might activate L-type Ca$^{2+}$ channels in podocytes, experiments were performed with the L-type Ca$^{2+}$ channel blocker nicardipine. Nicardipine (1 mM) did not change the ACh-induced [Ca$^{2+}$], increase ($n = 6$), as shown in a typical experiment in Figure 3A. However, lanthanum (La$^{3+}$) and gadolinium (Gd$^{3+}$), which are nonspecific blockers of Ca$^{2+}$-influx pathways activated after inositol trisphosphate-induced Ca$^{2+}$ store release, efficiently blocked the [Ca$^{2+}$], plateau at 1 mM (Figure 3, B and C). In paired experiments ($n = 11$), La$^{3+}$ was more effective than Gd$^{3+}$. The mean reduc-

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Figure 5. Concentration-response curves for the effects of 4-DAMP (A) and pirenzepine (B) on ACh-mediated [Ca$^{2+}$], increases. Note that low concentrations of pirenzepine and 4-DAMP increased, whereas higher concentrations of the antagonists inhibited, the ACh-induced [Ca$^{2+}$], responses. The numbers in parentheses refer to the number of experiments. *, significant differences between the effect of ACh in the presence or absence of 4-DAMP and pirenzepine, respectively.

Figure 6. Repetitive application of ACh (up to five times) did not significantly decrease the magnitude or alter the pattern of the [Ca$^{2+}$], response. (A) Original [Ca$^{2+}$], recording showing five repetitive stimulations with ACh (10 mM). (B) Summary of the experimental series. None of the tested ACh concentrations produced a significant decrease in the peak [Ca$^{2+}$], response. The numbers in parentheses represent the number of experiments for each concentration.
tion of the plateau obtained with 1 μM Gd3+ was 29 ± 11%, compared with −14 ± 8% obtained with 1 μM La3+, which is below the resting [Ca2+]i before ACh stimulation. The effects of La3+ and Gd3+ were not always fully reversible, even after a washout period of 15 min. Possible effects of La3+ and Gd3+ on the [Ca2+]i peak were measured in another experimental series, in which La3+ or Gd3+ (both at 1 μM) was added 2 min before stimulation with ACh. The ACh-induced peak [Ca2+]i values obtained during the application of La3+ or Gd3+ were not significantly different from the ACh-induced [Ca2+]i peaks measured before and after the application of La3+ (98 ± 4%, n = 4, data not shown) or Gd3+ (109 ± 6%, n = 5, data not shown).

Effects of Muscarinic Antagonists on the ACh-Induced [Ca2+]i Increase

Figure 4 presents original recordings of the effects of different concentrations of the muscarinic M3/M5 receptor antagonist 4-diphenylacetoxy-N-methylpiperidinemethiodide (4-DAMP) (Figure 4A) and the muscarinic M1 receptor antagonist pirenzepine (Figure 4B) (16). The summary of these types of experiments (Figure 5), as well as the original recording (Figure 4B), shows that low concentrations (10 and 100 nM) of pirenzepine augmented, whereas higher concentrations inhibited, the [Ca2+]i response to ACh (IC50 of approximately 1 μM). Similar results were obtained with 4-DAMP (Figure 5A); however, the concentration-response curve was shifted to the left by approximately 2.5 decades, compared with pirenzepine (IC50 of approximately 5 nM, compared with 1 μM).

Measurement of [Ca2+]i with Two-Photon Laser Scanning Microscopy

To investigate whether the ACh-mediated [Ca2+]i increase was attributable to a [Ca2+]i increase in podocytes, [Ca2+]i measurements were performed using two-photon laser scanning microscopy (n = 10). [Ca2+]i was measured confocally on the single-cell level with fluo-3, in a confocal slice near the lower surface of the decapsulated glomerulus. Typical fluo-3

Figure 7. Assessment of ACh-induced [Ca2+]i increases using confocal two-photon fluo-3 fluorescence imaging. (A to C) Approximately six to eight cells responded to ACh stimulation with an increase in fluo-3 fluorescence intensity as a measure of [Ca2+]i (arrows). (D) Original trace of fluorescence intensity as a measure of [Ca2+]i recorded from the cells marked in the confocal images.
fluorescence images (recorded at 780-nm excitation) are presented in Figure 7, A through C. Cells that responded to ACh (10 μM) with a [Ca$^{2+}$]$_i$ increase are shown in Figure 7B. Only approximately one-third to one-half of the cells in the image responded. The fluo-3 fluorescence traces from the marked responding cells are presented in Figure 7D. Using stepwise changes in the focus, it was possible to identify podocytes because of their typical structure and location in the glomerulus. To facilitate identification, in some experiments the glomeruli were incubated with calcein/AM, a general marker of living cells. Calcein yielded much brighter and less noisy images, compared with fluo-3, and allowed clear identification of podocytes on the basis of their characteristic morphologic features and location in relation to the capillary network. Figure 8 presents four images from a z-stack of calcein fluorescence images through a glomerulus. Podocyte foot processes located on the basal membrane around the capillaries could be clearly observed. From the confocal two-photon images, it can be concluded that podocytes respond to ACh stimulation with a [Ca$^{2+}$]$_i$ increase.

**Fluorescence Immunohistochemical Staining**

**Demonstrating M$_5$ Receptor Expression in Podocytes**

M$_3$ receptors could not be detected in glomerular cells but were found in colon carcinoma cells, which are known to express M$_3$ receptors (17) (data not shown). Figure 9A demonstrates the positive immunoreactivity for synaptopodin, a protein that, within the glomerulus, is specifically expressed in podocyte foot processes (18). In comparison with synaptopodin, M$_5$ receptors demonstrated similar immunoreactivity localization but were also detected in cell bodies of podocytes (Figure 9B). The specific localization of M$_5$ receptors in podocytes was also demonstrated by double-immunostaining of the glomeruli with antibodies against synaptopodin and the M$_5$ receptor (Figure 9C). Figure 9D shows that staining of M$_1$ receptors could be detected only in the parietal sheet of Bowman’s capsule and not in podocytes. Negative control slides incubated only with secondary antibodies demonstrated no appreciable staining (data not shown).

Figure 10 shows that the expression of muscarinic M$_5$ re-
Receptors in rat glomeruli could be confirmed by Western blot analysis (n = 3).

**Discussion**

Within the glomerulus, the three different resident cell types form a complex architecture. The physiologic responses of each cell type and possible interactions between the cells are poorly understood. It is well known that many vasoactive hormones regulate different glomerular functions but, because of their unique anatomic locations, it is difficult to detect the particular glomerular cell type involved in a given hormonal response. It was reported that ACh increased \( [\text{Ca}^{2+}]_i \) in parietal cells of Bowman’s capsule and in glomerular cells but, because of the limitations of the conventional fluorescence technique, it was unclear which glomerular cell type was the target cell for the response to ACh (8). In this study, fluorescence measurements made using the video-imaging approach demonstrated that ACh increased \( [\text{Ca}^{2+}]_i \) in the glomerulus. By using two-photon laser scanning microscopy, we were further able to demonstrate that single podocytes in glomeruli increased \( [\text{Ca}^{2+}]_i \) in response to ACh. In the confocal studies, ACh did not increase \( [\text{Ca}^{2+}]_i \) in all cells that could be identified as podocytes on the basis of calcein staining. This observation might be attributable to the relatively low excitability of fluo-3 with the two-photon wavelength of 780 nm, resulting in smaller fluorescence responses even with maximal ACh stimulation. However, it is also possible that not all podocytes express functional active muscarinic receptors.

The \( [\text{Ca}^{2+}]_i \) response to ACh was biphasic, with a peak and a plateau. The \( [\text{Ca}^{2+}]_i \) peak was reduced by approximately 50% in a solution with a reduced extracellular \( \text{Ca}^{2+} \) concentration, indicating that the peak was partly attributable to a release of \( \text{Ca}^{2+} \) from intracellular stores. The subsequent \( [\text{Ca}^{2+}]_i \) plateau was mainly the result of \( \text{Ca}^{2+} \) influx from the extracellular space, as supported by the finding that low extracellular \( \text{Ca}^{2+} \) concentrations completely abrogated the plateau. Nicardipine, a blocker of L-type \( \text{Ca}^{2+} \) channels, did not inhibit the \( [\text{Ca}^{2+}]_i \) plateau, which is in line with our recent results showing that depolarization by high extracellular K\(^+\) concentrations did not affect \( [\text{Ca}^{2+}]_i \) (11). The data suggest that the

Figure 9. Immunofluorescence studies of the expression of synaptopodin (A), the M₅ receptor (B), synaptopodin and the M₅ receptor (double-immunostaining) (C), and the M₁ receptor (D) in rat glomeruli. Note that there is strong staining for synaptopodin and the M₅ receptor in podocytes, whereas staining for the M₁ receptor is observed only in the parietal cells of Bowman’s capsule. Two additional experiments yielded similar results.
produced strong desensitization of the AT1 receptor; after a recently demonstrated that, in contrast to ACh, angiotensin II response or only a small response was observed (11). Therefore, ACh- and angiotensin II-induced signaling in podocytes might affect their respective receptors in different ways.

To date, five subtypes of muscarinic receptors (M₁ to M₅), with unique distributions, have been identified by molecular cloning (19). M₂ and M₄ receptors are preferentially coupled to G₁ proteins and inhibit adenylate cyclase, whereas M₁, M₃, and M₅ receptors are preferentially coupled to phospholipase C activation (19). On the basis of pharmacologic criteria, only four subtypes of muscarinic receptors can be distinguished using selective antagonists, because clear discrimination between M₃ and M₅ receptors is currently not possible (16,20).

To determine which muscarinic receptor subtype is coupled to calcium mobilization in glomeruli, the muscarinic receptor antagonists 4-DAMP (an M₁ receptor antagonist) and pirenzepine (an M₁ receptor antagonist) were used (16). Both compounds inhibited the [Ca²⁺]ᵢ response to ACh, but 4-DAMP was approximately 2.5 orders of magnitude more potent than pirenzepine, suggesting that the ACh-mediated [Ca²⁺]ᵢ response is primarily dependent on M₃/M₅ receptor activation.

An unexpected result was that both 4-DAMP and pirenzepine, at concentrations lower than required for inhibition, significantly stimulated the ACh-induced [Ca²⁺]ᵢ response. We currently have no explanation for this observation.

To test for the existence of M₁, M₃, and M₅ receptor protein expression in glomeruli, fluorescence immunohistochemical studies were performed with specific antibodies against the respective receptors. These studies indicated that podocytes express M₅ receptors. These receptors could be detected on the cell surface of podocytes (Figure 9B) and were also colocalized with synaptopodin, a protein that, within the glomerulus, is expressed only in podocyte foot processes (17) (Figure 9, A and C). By using Western blot analyses, appropriate bands for the muscarinic M₅ receptor (approximately 65 kD) were identified in isolated rat glomeruli. Brain tissue served as a positive control sample. Two glycosylation sites have been identified in this receptor (21). The lower band observed for glomeruli might thus be explained by the presence of nonglycosylated M₅ receptors.

M₁ receptors were present only in the parietal sheet of Bowman’s capsule (Figure 9D), suggesting that ACh-induced [Ca²⁺]ᵢ increases in Bowman’s parietal cells, which have been reported by Lebrun et al. (8), are mediated via M₁ receptors. Using three different antibodies against the M₃ receptor, we could not detect this receptor in glomerular cells, indicating that glomerular cells do not express M₃ receptor protein. In positive control experiments, the antibodies against the M₃ receptor stained colon carcinoma cells, which are known to express M₃ receptors (18).

This study demonstrates that podocytes express functionally active M₅ receptors in vivo. M₅ receptors are known to be predominantly expressed in the brain (striatum, hippocampus, midbrain, pons, medulla, and cerebellum), where they might play a role in dopaminergic transmission. There are few data concerning the expression and function of M₅ receptors in peripheral tissues. M₅ receptor expression in rat salivary gland tissue and in a melanoma cell line has been suggested (for review, see reference 20). In the melanoma cell line, activation of M₅ receptors inhibited cloning capacity, indicating that M₅ receptors might have antioncogenic functions in this cell type (22).

This study indicates that M₅ receptors in podocytes might play a role in the regulation of podocyte function and thus might participate in the regulation of the renal glomerular filtration process. Further studies must demonstrate the ultrastructural localization of M₅ receptors in podocytes and clarify their precise role in podocyte function. Because podocyte foot processes contain contractile machinery, it might be speculated that the effect of ACh on [Ca²⁺]ᵢ in podocytes contributes to ACh-induced glomerular contraction.

Figure 10. Results of Western blotting analysis of the muscarinic M₅ receptor. The expected size for this receptor is 60 to 70 kD. M₅ receptor expression of the appropriate size was found in isolated glomeruli. Brain tissue served as a positive control sample. Two additional experiments yielded similar results.
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