Dihydropyridine-sensitive Ca\(^{2+}\) channels in human glomerular mesangial cells

DAVID A. HALL, PAMELA K. CARMINES, AND STEVEN C. SANSOM
Department of Physiology and Biophysics, University of Nebraska Medical Center, Omaha, Nebraska 68198-4575

Hall, David A., Pamela K. Carmines, and Steven C. Sansom. Dihydropyridine-sensitive Ca\(^{2+}\) channels in human glomerular mesangial cells. Am. J. Physiol. Renal Physiol. 278: F97–F103, 2000.—In mesangial cells (MC), the response of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) to a contractile agonist is biphasic with a large transient increase in [Ca\(^{2+}\)]\(_i\) followed by a smaller but sustained elevation as Ca\(^{2+}\) flows into the cell from the extracellular fluid. It has been postulated that membrane depolarization precedes opening of Ca\(^{2+}\) channels in the plasma-lemmal membrane. However, a role for voltage-gated Ca\(^{2+}\) channels (VGCC) in human MC has been controversial, and their existence has not been verified with single-channel analysis. We used fura 2 fluorescence and patch-clamp techniques to determine the properties of the Ca\(^{2+}\) entry pathway responsible for the sustained response of [Ca\(^{2+}\)]\(_i\) in human MC. We found that ANG II at 10 nM, 100 nM, and 1 µM increased [Ca\(^{2+}\)]\(_i\) to sustained levels of 22%, 35%, and 49%, respectively, above baseline. The sustained response to 1 µM ANG II was attenuated by diltiazem and was reduced to a value less than baseline in the absence of external Ca\(^{2+}\). None of the peak responses (due to release of intracellular stores of Ca\(^{2+}\)) were affected by removal of external Ca\(^{2+}\) or addition of diltiazem. Upon elevating the extracellular [K\(^+\)] from 5 mM to 75 mM, [Ca\(^{2+}\)]\(_i\) reached a sustained level of 48% greater than baseline. This effect of high K\(^+\) was attenuated by either Ca\(^{2+}\) removal or addition of diltiazem. In the presence of 75 or 140 mM K\(^+\), the dihydropyridine agonist BAY K 8644 (1 µM and 10 µM) initiated sustained [Ca\(^{2+}\)]\(_i\) responses averaging 18% and 25%, respectively, greater than baseline. With <10 mM Ca\(^{2+}\) in the external solution, BAY K 8644 did not significantly affect [Ca\(^{2+}\)]. In separate patch-clamp experiments, barium-selective channels were found in cell-attached patches with 90 mM BaCl\(_2\) and 10 µM BAY K 8644 in the pipette solution. The single-channel conductance was 11.2 pS, and the open probability increased steeply at membrane potentials between ~30 mV and 0 mV. It is concluded that human glomerular MC contain dihydropyridine-sensitive Ca\(^{2+}\) channels responsible for the voltage-regulated entry of Ca\(^{2+}\) into the cell during an agonist-induced contraction.

diltiazem; BAY K 8644; voltage-gated calcium channel; fura 2; patch clamp

MESANGIAL CELLS (MC) are contractile cells that surround the glomerular capillaries and regulate rates of renal filtration by responding to vasoactive agonists such as angiotensin II (ANG II) and nitric oxide. The electrophysiology of MC, studied by several investiga-

METHODS

MC cultures. Human MC, originally isolated by the laboratory of Hanna Abboud, were subcultured to no more than 10
generations by standard methods (13). Beyond 10 generations, MC lose the phenotypic shape that is characteristic of smooth muscle cells. Cells were cultured in DMEM media (pH 7.0) supplemented with 10 mM HEPES, 2.0 mM glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 µg/ml streptomycin, and 17% fetal bovine serum. For patch-clamp experiments, cells were grown on coverslips (22 × 22 mm; Fisher, Pittsburgh, PA) and maintained in a humidified tissue culture incubator at 37°C, 5% CO2 (IR Autoflow; Nuaire, Plymouth, MN).

Patch-clamp procedure. Patch-clamp experiments were performed with the pipette attached to the membrane (cell attached). The bath solution contained (in mM) 140 KCl, 10 HEPES, 2 MgCl2, and 1 CaCl2, pH 7.4. The pipette solution contained 90 mM BaCl2 and 10 mM HEPES, pH 7.4. The free Ca2+ concentration of the bath was adjusted to 1.0 µM by buffering with 1.08 mM EGTA, according to the Ca2+ state (S) divided by the total time of the recording. When multiple channels occupied a patch, the channel activity was calculated with Po = ΣnPn, where Pn is the probability of finding n channels open. Currents were recorded and analyzed using the Axoscope acquisition program and pClamp program set 6.02 (Axon Instruments, Foster City, CA).

Fura 2 measurements of [Ca2+]. Measurements of [Ca2+]i in human MC using fura 2 was performed as previously described (5). Dual-excitation wavelength fluorescence microscopy micrographs were obtained on individual MC. The perfusion chamber (Warner RC-20H) was mounted on the stage of an inverted microscope (Nikon Diaphot 300). Cells attached to the coverslip floor of the chamber were illuminated alternately with light at 340- and 380-nm wavelengths (3-nm bandwidths) provided by a Deltascan dual monochromator system (Photon Technology International, Monmouth Junction, NJ). An adjustable optical sampling window was positioned to allow emission fluorescence (510 nm, 20-nm band pass) from a single cell to be detected by a photon-counting photomultiplier. Background-corrected data were collected at 5 points/s, stored, and processed using the FELIX software package (Photon Technologies). Calibration of the fura 2 signal was performed according to established methods (9), as described in detail previously (5). Cells were fura 2 loaded with 60-min incubation (37°C) in Waymouth culture media containing 7 µM fura 2-AM, 0.09 g/dl DMSO, and 0.018 g/dl Pluronic F-127 (Molecular Probes, Eugene, OR). The bathing solution contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, and 1 CaCl2. In some experiments, bath [Ca2+]i was reduced to less than 10 nM by addition of EGTA, and [K+]i was increased to 75 or 140 mM by substituting for Na+. Diltiazem and BAY K 8644 were purchased from Sigma Chemical (St. Louis, MO). Differences among groups of data were determined using the one-way ANOVA plus Student-Newman-Keuls test. P < 0.05 was considered statistically significant. Data are reported as means ± SE; n = number of cells.

RESULTS

Measurements of [Ca2+]i. Figure 1 illustrates the impact of VGCC blockade and external [Ca2+]i on the responses of human MC to ANG II. Figure 1A depicts representative [Ca2+]i responses to 1 µM ANG II and the effects of VGCC blockade and removal of bath Ca2+ on this response. In the presence of 1 mM Ca2+ (top, Fig. 1A), ANG II evoked a transient increase in [Ca2+]i from 39 nM (baseline) to 730 nM (peak), subsequently subsiding to a sustained value (68 nM) that was ~75% greater than baseline in this example. Neither diltiazem nor the nominally Ca2+-free bath significantly influenced baseline [Ca2+]i; however, the pattern of the response to ANG II was modified by these treatments. In the nominally Ca2+-free bath (middle, Fig. 1A), [Ca2+]i transiently increased from 41 to 950 nM in response to ANG II; however, [Ca2+]i subsequently declined to a value less than baseline despite continued exposure to ANG II. During treatment with 5 µM diltiazem in the presence of 1 mM Ca2+ (bottom, Fig. 1A), ANG II still elicited a large transient increase in [Ca2+]i (from 53 to 1,239 nM in this example), but the sustained response was reduced to a value approximating baseline. There was no significant difference in the peak response to ANG II in the presence (Δ = 420 ± 73 nM, n = 7) or absence of Ca2+ (Δ = 609 ± 99 nM, n = 6) or with addition of diltiazem to the Ca2+-containing bath (Δ = 723 ± 225 nM, n = 6). As shown in the summary bar graph of Fig. 1B, ANG II increased the sustained [Ca2+]i response in a dose-dependent manner at peptide concentrations of 10 nM, 100 nM, and 1 µM in the presence of external Ca2+. The sustained [Ca2+]i response evoked by 1 µM ANG II was significantly greater than the response to 10 nM ANG II. The sustained [Ca2+]i response to 1 µM ANG II achieved a value significantly below baseline in the absence of external Ca2+ and was abolished by addition of diltiazem to the Ca2+-containing bathing solution.

To determine whether a voltage-dependent mechanism can drive Ca2+ entry into these cells, some cells were subjected to membrane depolarization achieved by increasing bath [K+] from 5 to 75 mM. Typical responses are illustrated in Fig. 2A. The top tracing in Fig. 2A illustrates that K+-induced membrane depolarization increased [Ca2+]i from 31 nM to a peak value of 571 nM in the presence of 1 mM Ca2+, subsequently achieving a sustained plateau at ~41 nM in this example. In the nominally Ca2+-free bath (middle, Fig. 2A) or in the presence of both 1 mM Ca2+ and 5 µM diltiazem (bottom, Fig. 2A), K+-induced membrane depolarization did not evoke any change in [Ca2+]i. Results from these experiments are summarized in Fig. 2B, indicating that the significant increase in [Ca2+]i evoked by 75 mM K+ (in the presence of 1 mM Ca2+) was abolished by diltiazem treatment or removal of bath Ca2+.

At depolarizing potentials, the dihydropyridine agonist BAY K 8644 promotes Ca2+ influx through VGCC
in several cell types. Accordingly, further experiments assessed the impact of BAY K 8644 on \([Ca^{2+}]_i\) in human MC studied during \(K^+\)-induced membrane depolarization. Figure 3A presents representative tracings illustrating the critical involvement of \(Ca^{2+}\) influx in evoking the \([Ca^{2+}]_i\) response to BAY K 8644 in solutions containing 140 mM \(K^+\). Exposure to 10 µM BAY K 8644 (top, Fig. 3A) increased \([Ca^{2+}]_i\), from 48 nM to a maximal value of 565 nM, with a subsequent sustained plateau at 65 nM (35% above baseline in this example). In contrast, exposure of depolarized MCs to 10 µM BAY K 8644 under nominally \(Ca^{2+}\)-free conditions failed to elicit any change in \([Ca^{2+}]_i\) (bottom, Fig. 3A). The impact of BAY K 8644 on mesangial \([Ca^{2+}]_i\) is summarized in Fig. 3B. In the presence of 75 mM \(K^+\), 1 and 10 µM BAY K 8644 evoked sustained increases in \([Ca^{2+}]_i\) that averaged 18 and 25% above baseline, respectively. Sustained responses to 10 µM BAY K 8644 were not significantly enhanced by further membrane depolarization (140 mM \(K^+\)) but were abolished in the absence of extracellular \(Ca^{2+}\). These observations document the presence of a dihydropyridine-sensitive \(Ca^{2+}\) influx mechanism in human MC studied under depolarizing conditions.

Table 1 shows a summary of the absolute baseline and sustained response values for \([Ca^{2+}]_i\) evoked by the various experimental conditions of this study. In groups which contained 1 mM external \(Ca^{2+}\), basal levels of \([Ca^{2+}]_i\) varied from 32.9 ± 3.0 to 46.7 ± 9.0 nM. In groups in which external \([Ca^{2+}]_i\) was reduced to less than 10 nM with EGTA, baseline values ranged from 27.3 ± 3.8 to 30.2 ± 3.5 nM. None of these baseline values was significantly different from the other values when using ANOVA plus Student-Newman-Keuls test. However, the baseline \([Ca^{2+}]_i\) for the combined groups with <10 nM external calcium (27.0 ± 1.9, n = 24) was significantly less (\(P < 0.001\)) than the combined groups with 1 mM external calcium (39.4 ± 2.1, n = 63) when compared using the unpaired \(t\)-test.

Patch-clamp experiments. Single-channel current analysis (Fig. 4) using the patch-clamp technique provided direct evidence for the existence of L-type \(Ca^{2+}\) channels in MC. Figure 4A shows typical tracings of single-channel currents in the cell-attached configuration with 90 mM BaCl₂ in the presence and absence of 10 µM BAY K 8644. In the absence of BAY K 8644, inward currents of this amplitude were never observed. In the presence of 10 µM BAY K 8644, single-channel currents were observed at potentials as low as -20 mV. As shown in Fig. 4B, the open probability increased in an exponential manner from 0.03 at

![Fig. 1. Effects of extracellular \([Ca^{2+}]_i\) and diltiazem on mesangial cell (MC) intracellular \(Ca^{2+}\) concentration (\([Ca^{2+}]_i\)) (plotted on log scale) responses to ANG II. A: representative responses to 1 µM ANG II observed in presence of 1 mM \(Ca^{2+}\) (top), in the nominally \(Ca^{2+}\)-free bath (middle), and upon addition of 5 µM diltiazem to the \(Ca^{2+}\)-containing bath (bottom). B: average sustained \([Ca^{2+}]_i\) responses to ANG II, expressed as percentage change from baseline. Bath concentrations of ANG II, diltiazem (Dltzm), and \(Ca^{2+}\) are specified for each experiment. *\(P < 0.05\) vs. baseline; †\(P < 0.05\) vs. 1 µM ANG II + 1 mM \(Ca^{2+}\) (0 Dltzm); n = 5–7 cells.
and saturated at 0 mV at a $P_o$ of 0.87. Figure 4C shows a summary current-voltage plot of five separate Ca$^{2+}$ channels. The single-channel conductance was 11.2 pS, and the extrapolated reversal potential was 33 mV. Assuming $[K^+] = 120$ mM, we estimated the selectivity for Ba$^{2+}$/K$^+$ at 4.2.

**DISCUSSION**

The results of this study provide evidence for a role for a VGCC in the sustained entry of Ca$^{2+}$ into MC during an agonist-induced contraction. The ANG II-induced sustained increase in [Ca$^{2+}$], is mimicked by depolarizing the cell with 75 mM K$^+$, indicating that the Ca$^{2+}$ entry pathway is voltage dependent. That this VGCC is of the L-type variety is supported by the effects of diltiazem, which attenuated the sustained [Ca$^{2+}$] responses to ANG II and K$^+$-induced depolarization, and of BAY K 8644, which evoked an increase in sustained [Ca$^{2+}$]. This contention is further supported by the ability of BAY K 8644 to enhance single-channel barium currents in a voltage range consistent with the L-type VGCC.

Analysis of the sustained increase in [Ca$^{2+}$]. The results of this study are consistent with earlier studies that demonstrated an elevation of [Ca$^{2+}$] in rat GMC upon depolarizing the membrane potential with high-K$^+$ solution (25, 31). However, other studies indicated that human MC do not possess VGCC (19). This latter study showed that an increase in [Ca$^{2+}$], resulting from raising external Ca$^{2+}$, was not potentiated by membrane depolarization with 50 mM K$^+$. This result may have been partly due to analyzing the average [Ca$^{2+}$] of several cells in a monolayer rather than focusing on individual cells. Because some cells have a delayed or cyclical response to elevating external Ca$^{2+}$, the average change in [Ca$^{2+}$] would appear as a large sustained elevation with extreme variability, which would make it difficult to discern a significant difference in a sustained response. Moreover, it is well known that MC in culture are phenotypically heterogeneous, with some cells not having the ability to contract. It has been observed by this laboratory that these cells, which are “star”-shaped or “diamond”-shaped do not contract in culture and do not have an ANG II-evoked sustained [Ca$^{2+}$] response. In the present study, only spindle-shaped cells, known to contract in vitro, are used in the measurements of [Ca$^{2+}$]. The response of [Ca$^{2+}$] to depolarizing potentials would be blunted if these cells were included in the analysis.

The effects of diltiazem on the sustained increase in [Ca$^{2+}$] evoked by ANG II are consistent with the view

---

**Fig. 2.** Effects of extracellular [Ca$^{2+}$] and diltiazem on MC [Ca$^{2+}$] responses to membrane depolarization, achieved by increasing bath [K$^+$] from 5 to 75 mM. A: representative responses to 75 mM K$^+$ observed in presence of 1 mM Ca$^{2+}$ (top), in the nominally Ca$^{2+}$-free bath (middle), and upon addition of 5 µM diltiazem to the Ca$^{2+}$-containing bath (bottom). B: average sustained [Ca$^{2+}$] responses to 75 mM K$^+$, expressed as percentage change from baseline. Bath concentrations of Ca$^{2+}$ and diltiazem (Dltzm) are specified for each experiment. *P < 0.05 vs. baseline; †P < 0.05 vs. 1 mM Ca$^{2+}$ (0 Dltzm); n = 7–9 cells.
that Ca\(^{2+}\) enters the cell through an L-type VGCC (26). Diltiazem abolished the sustained \([Ca^{2+}]_i\) increase but did not reduce it to the level obtained with removal of external Ca\(^{2+}\), which was 25% less than the original baseline. These results suggest the presence of an additional Ca\(^{2+}\) influx pathway, distinct from and in parallel with the VGCC that is activated during agonist-induced contraction. It is possible that this channel is a capacitative calcium entry pathway as described for lymphocytes and other nonexcitable cells (3, 14). Méné et al. (19) previously provided evidence for the existence of store-operated Ca\(^{2+}\) channels in human MC in culture (19). The existence of a Ca\(^{2+}\) influx pathway normally identified in lymphocytes is interesting since MC can acquire an immunocyte phenotype in culture and in vivo.

L-type VGCC are prototypically identified by sensitivity to dihydropyridines such as BAY K 8644 (20, 22). At 1 µM, BAY K 8644 caused a significant sustained elevation in \([Ca^{2+}]_i\) that was eliminated when Ca\(^{2+}\) was removed from the bathing solution. This observation provides firm evidence for an L-type VGCC that is notably activated by BAY K 8644 in the presence of depolarizing potentials (2, 11). However, it is not understood why further depolarization of the membrane potential with 140 mM K\(^+\) instead of 75 mM K\(^+\) failed to enhance the effect of BAY K 8644. A possible explanation is that membrane depolarization by 75 mM K\(^+\) maximizes the combination of \(P_o\) and electrochemical driving force for Ca\(^{2+}\) entry, whereas further depolarization to \(-10\) mV by 140 mM K\(^+\) may not enhance the \(P_o\) sufficiently to overcome the reduced electrical driving force.

Single-channel properties of the mesangial VGCC. Activation of a Ca\(^{2+}\) influx pathway by high K\(^+\) and BAY K 8644 strongly suggests the presence of VGCC of the L-type variety in human MC in culture. Therefore, patch-clamp experiments were performed to determine the biophysical properties of these channels. Although several types of VGCC have been described in excitable cells (22), the pharmacological and biophysical properties determined with single-channel analysis in this study are most consistent with those defined for L-type channels. The voltage-dependent activation range of the mesangial VGCC was from \(-30\) mV to a maximum of 0 mV. L-type channels in a variety of cell types are activated in the range beginning from \(-40\) to \(-20\) mV to a maximal \(P_o\) at potentials from 0 to 20 mV (2, 8, 29, 22).

### Table 1. Baseline and sustained response values of \([Ca^{2+}]_i\)

<table>
<thead>
<tr>
<th>Agent</th>
<th>([Ca^{2+}]_o)</th>
<th>Diltiazem</th>
<th>(n)</th>
<th>Baseline</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG II, 10 nM</td>
<td>1 mM</td>
<td>0</td>
<td>7</td>
<td>40.8 ± 8.0</td>
<td>51.0 ± 11.8</td>
</tr>
<tr>
<td>ANG II, 100 nM</td>
<td>1 mM</td>
<td>0</td>
<td>7</td>
<td>42.7 ± 6.7</td>
<td>55.5 ± 6.8</td>
</tr>
<tr>
<td>ANG II, 1 µM</td>
<td>1 mM</td>
<td>0</td>
<td>8</td>
<td>42.9 ± 9.0</td>
<td>64.3 ± 14.8</td>
</tr>
<tr>
<td>ANG II, 1 µM</td>
<td>&lt;10 nM</td>
<td>0</td>
<td>6</td>
<td>27.3 ± 3.8</td>
<td>20.7 ± 2.9</td>
</tr>
<tr>
<td>ANG II, 1 µM</td>
<td>1 mM</td>
<td>5 µM</td>
<td>6</td>
<td>46.7 ± 6.0</td>
<td>46.5 ± 5.4</td>
</tr>
<tr>
<td>K(^+), 75 mM</td>
<td>1 mM</td>
<td>0</td>
<td>6</td>
<td>41.4 ± 4.7</td>
<td>53.2 ± 5.0</td>
</tr>
<tr>
<td>K(^+), 75 mM</td>
<td>&lt;10 nM</td>
<td>0</td>
<td>9</td>
<td>23.7 ± 2.9</td>
<td>22.2 ± 3.7</td>
</tr>
<tr>
<td>K(^+), 75 mM</td>
<td>1 mM</td>
<td>5 µM</td>
<td>6</td>
<td>33.0 ± 6.4</td>
<td>34.3 ± 7.4</td>
</tr>
<tr>
<td>BAY K, 1 µM</td>
<td>1 mM</td>
<td>0</td>
<td>7</td>
<td>32.9 ± 3.0</td>
<td>38.4 ± 3.2</td>
</tr>
<tr>
<td>BAY K, 10 µM</td>
<td>1 mM</td>
<td>0</td>
<td>12</td>
<td>33.6 ± 4.6</td>
<td>39.6 ± 5.3</td>
</tr>
<tr>
<td>BAY K, 10 µM</td>
<td>&lt;10 nM</td>
<td>0</td>
<td>9</td>
<td>30.2 ± 3.5</td>
<td>29.3 ± 4.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\) = no. of cells. \([Ca^{2+}]_i\) and \([Ca^{2+}]_o\), intracellular and external Ca\(^{2+}\) concentrations, respectively. BAY K, BAY K 8644.
Although an in depth kinetic analysis was not performed on these channels, the bursting pattern of channel gating is consistent with two closed and one open state previously described for L-type channels in chick sensory neurones (8), cardiac cells (11), and cerebral arteries (29).

Elevating the bath \([K^+]\) to 75 mM would be expected to depolarize the cell membrane potential to approximately \(-210\) to \(-30\) mV, an increase high enough to evoke a sustained increase in \([Ca^{2+}]_i\). It is not understood why L-type \(Ca^{2+}\) channels were not evident in cell-attached patches in the absence of BAY K 8644 in the pipette. However, in the fura 2 experiments, conditions were set to observe a response of \([Ca^{2+}]_i\), immediately after changing the solution to high \(K^+\). In the patch-clamp experiments, the cells were depolarized in high-\(K^+\) solution for at least 30 min before observing the channel. It is possible that during this time of sustained depolarization the L-type channel in the patch downregulated via the effects of an inactivating enzyme such as a phosphatase.

The single-channel conductance of 11.2 pS is smaller than most L-type \(Ca^{2+}\) channels, which are normally 20–25 pS. However, similar channels of \(~9–12\) pS with L-type properties have been described in VSM cells from cerebral (29) and mesenteric arteries (28). Moreover, a 10-pS L-type channel has been described in renal proximal tubule cells (32). These smaller L-type channels are often found in the same cell preparations that exhibit larger conductance L-type channels (29). It is not known whether the small channel is a splice variant of the larger channel or whether it originates from a distinct gene.

In summary, the results of this study are consistent with the notion that membrane depolarization activates L-type VGCC, causing \(Ca^{2+}\) influx into human MC. Functional expression of this electrophysiological mechanism involving L-type \(Ca^{2+}\) channels for maintaining tone is further confirmation that the contractile phenotype of MC is very similar, if not identical, to that of VSM cells.

We are grateful to Dr. Hanna Abboud of the University of Texas Health Science Center at San Antonio for providing us with cultures of human mesangial cells. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-49561 (to S. C. Sansom).
REFERENCES


