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Essential role of Ca^{2+} release channels in angiotensin II-induced Ca^{2+} oscillations and mesangial cell contraction

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The increased resistance of the glomerulus as a result of contractile dysfunction of mesangial cells (MCs) is associated with reduction of glomerular filtration rate and development of glomerulosclerosis. Evidences show MCs contraction changes with intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Here, we explore the mechanism of angiotensin II (AngII)-induced Ca^{2+} oscillations and MCs contraction. Primary MCs from 3-month-old and 28-month-old rats were used for detection of Ca^{2+} oscillations and MC planar area with confocal microscopy. AngII could induce typical Ca^{2+} oscillations and contraction of MCs. This process was abolished by thapsigargin, 2-aminoethoxydiphenyl borate, or 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine, and partially inhibited by ryanodine, but could not be inhibited in the absence of extracellular Ca^{2+}. Ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate ($InsP_3$) receptors displayed a strong colocalization, which may contribute to the amplification of Ca^{2+} response. MLC_{20} phosphorylation and MC planar area were associated with AngII-induced Ca^{2+} oscillations. The frequency of Ca^{2+} oscillations was dependent on the AngII concentration and correlated with the MCs' contractive extent, which could be attenuated by KN-93. The amplitude reduction of oscillations correlated with the decrease in aging-related contraction. In conclusion, $[Ca^{2+}]_i$ response of MCs to AngII is characterized by repetitive spikes through the following repetitive cycles: Ca^{2+} release by phospholipase C –$InsP_3$ pathway, Ca^{2+} amplification by Ca^{2+}-activated RyRs and Ca^{2+} reuptake by the endoplasmic reticulum. MCs contraction can be modulated by oscillations not only in an AngII-induced frequency-dependent mode but also in an aging-related, amplitude-dependent mode.

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Mesangial cells (MCs) are contractile cells that abut and surround the glomerular capillaries. MCs regulate renal filtration surface area as well as intraglomerular blood volume by responding to vasoactive agonists such as angiotensin II (AngII) and nitric oxide. For renal diseases and aging-related renal inadequacy, it is well established that the elevation of local AngII plays an important role in the reduction of glomerular filtration rate and in the development of glomerulosclerosis.[1–3] Early studies using cultured MCs showed that MC's contractive reaction and smooth muscle cell's reaction to vasoactive hormones were similar.[4–6] Contractile dysfunction of MCs contributes to the increased resistance of glomeruli and is associated with the reduction of glomerular filtration rate.

AngII leads to MCs contraction mainly through the calcium signal transduction pathway.[7] Since intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a highly important signal for regulation of the cellular processes, it is reported to have a self-feedback control mechanism during its quiescence state and activation state.[8] Agonist-evoked Ca^{2+} oscillations indicate the existence of such a regulation mechanism. During prolonged stimulation, $[Ca^{2+}]_i$ is reported to set up regular Ca^{2+} oscillations that have been implicated in control of many specific cellular processes in different cells, such as activation of enzymes,[9] gene expression,[10] and exocytosis,[11] contributing to cellular contraction of vascular smooth muscle[12] and release of cytokines from renal epithelial cells.[13] Other experiments also show that a single mesangial cell can respond to stimulation with Ca^{2+} oscillations.[14]

For other cell types, the initiation and the propagation of Ca^{2+} oscillations are induced by the second messenger inositol 1,4,5-trisphosphate ($InsP_3$)[15] or Ca^{2+} coming from endocytoplasmic reticulum (ER) via ryanodine receptors (RyRs).[16] However, for MCs what leads to periodic changes in Ca^{2+} concentration is still unclear. In addition, it is generally supported that the amplitude and frequency of the Ca^{2+} signal are important parameters modulating the

cellular responses to agonists linked to Ca^{2+}.[17,18] Such parameters are also believed to be sensitive to agonist concentration, Ca^{2+}-related kinase activity, and the basal $[Ca^{2+}]_i$ level.[19] We hypothesize that the change of amplitude and frequency in the MCs oscillations is related to the stimulation of different concentrations of AngII, calcium/calmodulin kinase II (CAMKII) activity, and aging of MCs.

In this study, we explore whether AngII stimulation in rat MCs can induce intracellular Ca^{2+} oscillations, how such a process is regulated, and whether the frequency-dependent and/or the amplitude-dependent Ca^{2+} oscillations induced by agonists will subsequently mediate MCs contraction.

RESULTS

Confocal observation of AngII-induced Ca^{2+} oscillations in MCs

Figure 1a depicts the representative change of $[Ca^{2+}]_i$ stimulated by 10^{-7} M AngII in a single MC from a 3-month-old rat. It showed an initial large increase of $[Ca^{2+}]_i$, which was followed by continuous $[Ca^{2+}]_i$ spikes. Figure 1b shows a snapshot of two Ca^{2+} oscillations in a selected region. Traveling in cell and the corresponding intensity profile could be seen from this figure. Figure 1c shows the results of Fast Fourier transform analysis of Ca^{2+} oscillations. The frequency and the power of peak value of Fast Fourier transform magnitude spectrum represent the frequency (0.11 Hz, which means 6.67 min^{-1}) and the mean amplitude value (0.040, denoted by 'power') of this MC's Ca^{2+} oscillations, respectively. With the increase of AngII concentration, the frequency of Ca^{2+} oscillations also increased, but the power of Ca^{2+} oscillations decreased and reached a plateau at the concentration of 10^{-8}–10^{-6} M AngII (Figure 1d). Meanwhile, the number of the oscillations-positive cells, which existed sustained intracellular Ca^{2+} oscillations, was calculated to 81.1% of total MCs. We calculated the equilibration of Ca^{2+} oscillations in MCs through Gaussian curve fitting model with IDL software to determine the related basal sustained level for different AngII concentrations. Figure 1e shows that, with the increase of AngII concentration, the basal sustained level of $[Ca^{2+}]_i$ increased.

The source of MCs Ca^{2+} oscillations

As a control, initial parameters of sustained MCs Ca^{2+} oscillations induced by 10^{-7} M AngII (Figure 2a) were as follows: frequency = 5.90 ± 1.62 min^{-1}, and power = 0.031 ± 0.008 (arbitrary unit, a.u.). After 30 min, the frequency was 6.67 ± 0.98 min^{-1} and the power was 0.023 ± 0.005 ($P > 0.05$, $n = 25$). This indicates that Ca^{2+} oscillations in MCs may exist continuously.

Then, to explore whether the source of Ca^{2+} oscillations derived either from the external medium or from the internal stores, MCs were exposed to 10^{-7} M AngII in a Ca^{2+}-free solution containing 5 mM ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate (EGTA) or in a 2 μM thapsigargin (TG). TG is a highly specific inhibitor of ER Ca^{2+}-

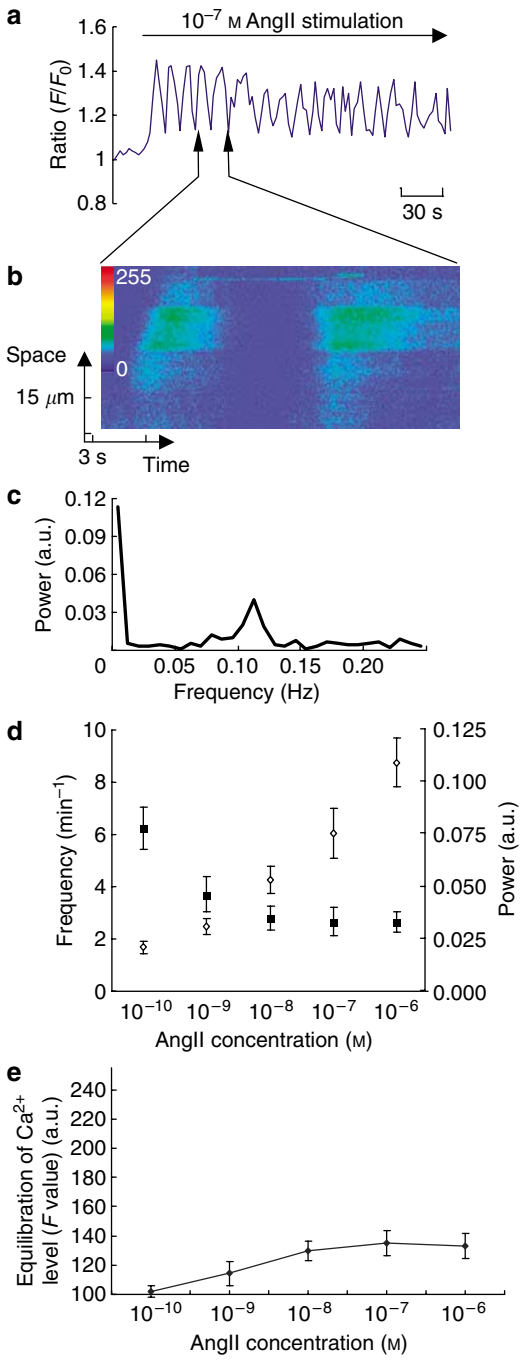

Figure 1 | Induction of Ca^{2+} oscillations with AngII in primary MCs. (**a**) Typical Ca^{2+} oscillations traces in an MC from a 3-month-old rat stimulated with 10^{-7} M AngII. (**b**) Line scanning of two Ca^{2+} spikes in a selected region. Oscillations travel from left to right. The pseudocolor map represents the estimated fluorescent intensity of Ca^{2+} concentrations. (**c**) The Fourier transform analysis results for the frequency and the power of Ca^{2+} oscillations indicated in (**a**). (**d**) Concentration–response curve for the frequency and the power of Ca^{2+} oscillations in MCs stimulated with AngII. (**e**) Concentration–response curves for the equilibration of Ca^{2+} oscillations in MCs induced by AngII. These were measured through the Gaussian curve fitting model using IDL software. Each point represents the mean ± s.d. from 25 different cells from four rats. (F: the actual fluorescence intensity of $[Ca^{2+}]_i$, F_0: the basal fluorescence intensity of $[Ca^{2+}]_i$; arbitrary units (a.u.) represent ratio values corresponding to $[Ca^{2+}]_i$ changes.)

ATPases. Under such conditions with no external Ca^{2+}, AngII still induced the Ca^{2+} oscillations ($n=25$; Figure 2b) with a frequency of 6.10 ± 0.04/min and a power of 0.030 ± 0.006, this was no different than the Ca^{2+} oscillations stimulated in the presence of external Ca^{2+}. However, when ER Ca^{2+} stores were depleted by incubation with TG ($n=25$; Figure 2c), AngII-induced Ca^{2+} oscillations were completely inhibited, implying that Ca^{2+} oscillations were driven from the internal Ca^{2+} store.

Regulation of Ca^{2+} release from the ER occurs mainly via $InsP_3$ receptors ($InsP_3Rs$) or via RyRs. To separate these two potential pathways, 2-aminoethoxydiphenyl borate (2-APB) was introduced as a specific inhibitor of $InsP_3Rs$.[20] The data suggest that low concentrations (up to 20 μM) of 2-APB preferentially inhibit $InsP_3Rs$.[21] We found that AngII-induced Ca^{2+} oscillations were abolished in the majority of treated cells ($n=25$; Figure 2d). Moreover, 100 μM ryanodine, which is enough to completely inhibit the RyRs in the ER,[22] was used to treat the MCs. We found that the power and the frequency of AngII-induced Ca^{2+} oscillations were reduced from 0.029 ± 0.003 and 6.00 ± 1.21/min to 0.018 ± 0.005 ($P<0.05$) and 1.89 ± 0.79/min ($P<0.05$) ($n=25$; Figure 2e). These results indicate that the release of Ca^{2+} via $InsP_3Rs$ is an essential contributor to the Ca^{2+} oscillations triggered, and the release of Ca^{2+} via RyRs is an associate contributor to the Ca^{2+} oscillations triggered in MCs.

Next, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (Et-18-OCH_3, 50 μM), a selective phospholipase C inhibitor,[23] was used. We found that the Ca^{2+} oscillations in Et-18-OCH_3-treated MCs were also abolished ($n=25$; Figure 2f), suggesting that phospholipase C –$InsP_3$ pathway is responsible for the generation of Ca^{2+} oscillations.

Correlation between AngII-induced MCs contraction and Ca^{2+} oscillations

MC planar area change driven by AngII was utilized as a direct measurement of cell contraction. The Ca^{2+} signaling in the MCs was correlated with their contraction by analyzing the changes in the MC planar surface area that were visible in

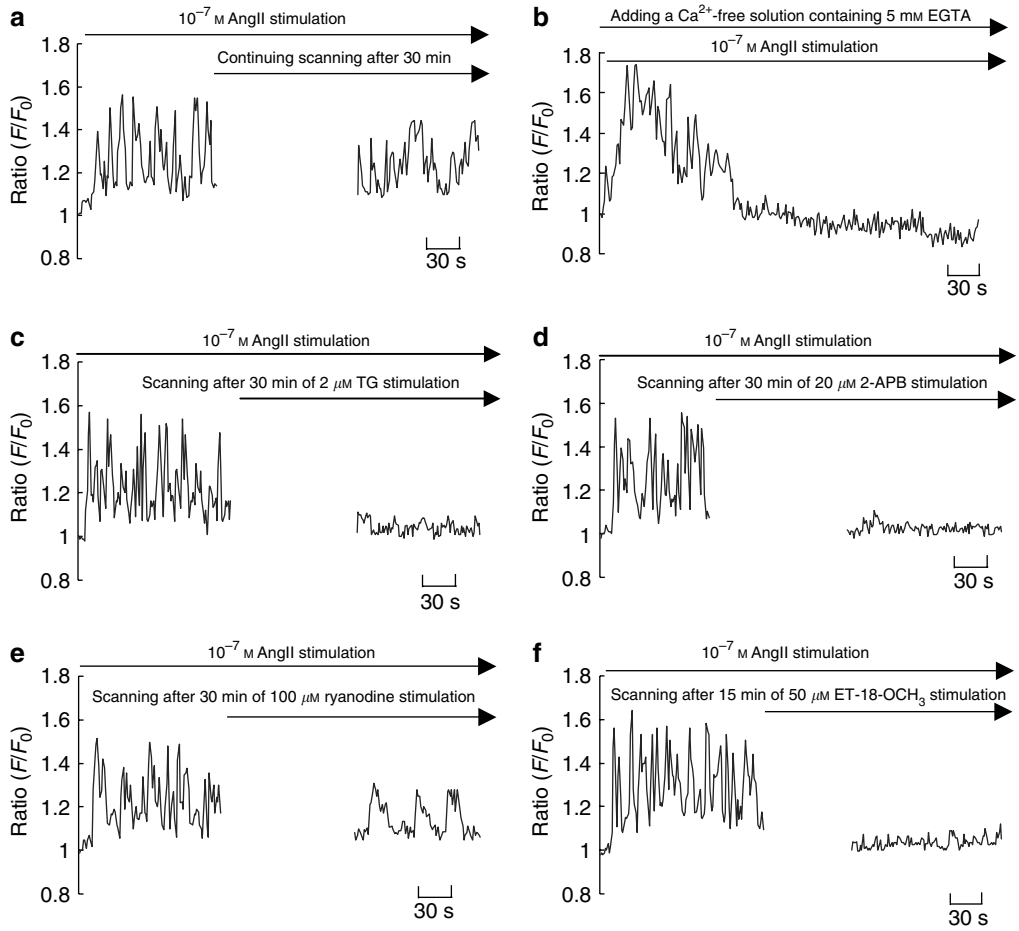

Figure 2 | Mechanisms of Ca^{2+} oscillations induction by AngII in MCs. (**a**) AngII induced representative sustained Ca^{2+} oscillations as a control. (**b**) AngII induced sustained Ca^{2+} oscillations during a long time in the absence of extracellular Ca^{2+}. (**c**) TG (2 μM) blocked completely Ca^{2+} oscillations induced by AngII. (**d**) 2-APB (20 μM) blocked completely Ca^{2+} oscillations induced by AngII. (**e**) Ryanodine (100 μM) partly inhibited Ca^{2+} oscillations induced by AngII ($P<0.01$). (**f**) ET-18-OCH_3 (50 μM) abolished Ca^{2+} oscillations induced by AngII. Representative traces of at least 25 different cells from three rats.

the confocal images simultaneously with the changes in MC fluorescence.

The initial Ca^{2+} oscillation was associated with the initiation of MCs' contraction. The following Ca^{2+} oscillations correlated with the maintenance of contraction (Figure 3a and b). However, for the same experiment settings, a $[Ca^{2+}]_i$ increase could also induce the contraction of Ca^{2+} oscillations-negative MCs (Figure 3c and d). And further, the baseline intensity value (F_0) of $[Ca^{2+}]_i$ in oscillations-positive cells was similar to those in oscillations-negative cells (77.52 ± 15.21 vs 81.63 ± 16.69, NS $n = 25$; Figure 3e). Compared with the oscillations-negative cells, the contractive percentage of planar area in oscillations-positive MCs is much higher: 62.5 ± 4.2 vs $89.1 \pm 6.5\%$ of the original planar area ($n = 16$, $P < 0.01$; Figure 3f). The data imply that agonist-induced Ca^{2+} oscillations are required for cell contraction when contribution of the average $[Ca^{2+}]_i$ level change to the cell contraction is not considered.

Then, we examined the stimulatory effect of AngII on MCs of 3-month-old rats at 0, 2, 5, 10, and 20 min by testing the change of myosin light chain (MLC_{20}) phosphorylation induced by AngII. As shown in Figure 4a, the increase in MLC_{20} phosphorylation was time dependent. At time zero, the densitometry was $2.24 \pm 1.35\%$ (moles of MLC phosphorylated/total moles of MLC, %). It reached to a maximum value the Densitometry $= 40.00 \pm 6.51\%$ at 10 min.

MLC_{20} phosphorylation was abolished after the addition of TG, 2-APB, or ET-18-OCH_3. However, adding ryanodine only reduced MLC_{20} phosphorylation from 58.51 ± 9.66 to $25.20 \pm 4.32\%$ ($P < 0.01$; Figure 4b). Figure 4c demonstrated that, in ET-18-OCH_3 group, 2-APB group, TG group, ryanodine group, and AngII group, the planar area of MCs changed to 98.2 ± 4.3, 101.7 ± 5.6, 100.8 ± 5.8, 81.2 ± 4.5, and $64.1 \pm 5.6\%$ of the original planar area ($^{*}P < 0.05$, $^{**}P < 0.01$ vs ET-18-OCH_3 group, 2-APB group, TG group; $^{\#}P < 0.05$ vs ryanodine group, $n = 25$) at 10 min after 10^{-7} M AngII stimulation. These results further confirm that inhibition of Ca^{2+} oscillations was related to the decrease of MLC_{20} phosphorylation in MCs, suggesting that Ca^{2+} oscillations might have some role in mediating the MCs contraction.

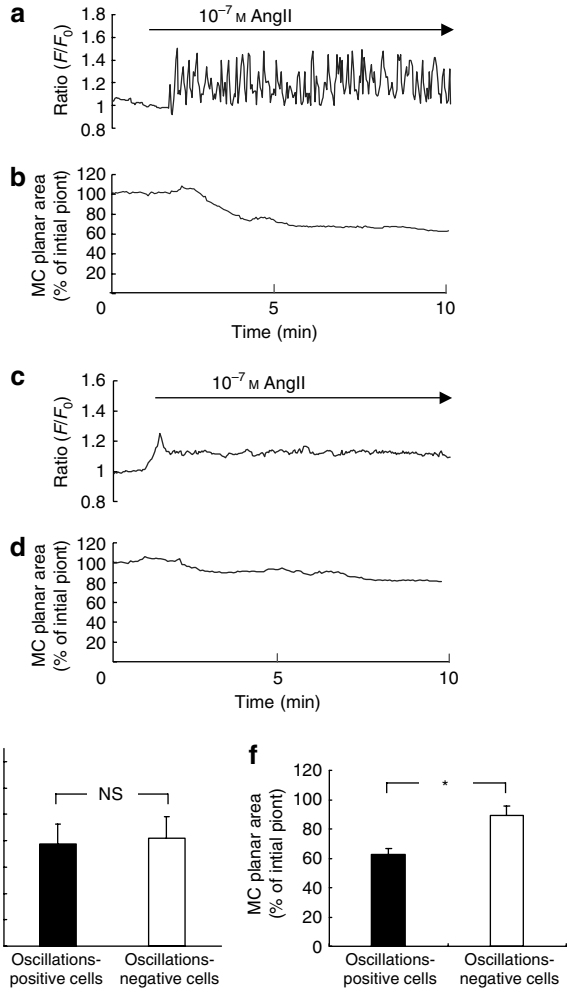

Figure 3 | Correlation of AngII-induced Ca^{2+} signaling and MCs' contraction. ROIs were defined in a single MC, and the representative Ca^{2+} changes in response to 10^{-7} M AngII were expressed as a fluorescence ratio (F/F_0) (**a**) in oscillations-positive cells or (**c**) in oscillations-negative cells. The change in the MC planar area (% of initial point) was measured and displayed simultaneously (**b**) in oscillations-positive cells or (**d**) in oscillations-negative cells. (**e**) The basal intensity value of the $[Ca^{2+}]_i$ level (F_0) in oscillations-positive cells or -negative cells ($n = 25$). (**f**) MC planar area (% of initial point) in oscillation-positive cells compared with negative cells induced by AngII ($n = 16$). Data represent the mean $\pm$ s.d. from 25 different cells from four rats (NS: no significant different; $^{*}P < 0.01$).

Colocalization of $InsP_3R$ and RyR subtypes in MCs

As the Ca^{2+}-induced Ca^{2+} release channels in the ER, RyRs can be activated by the internal Ca^{2+} signal. The results shown above (Figures 2d, e and 4b, c) strongly suggest that there may be a coupling relationship between $InsP_3R$ and RyR subtypes. We observed the localization of $InsP_3R$ and RyR subtypes in MCs by means of confocal immunofluorescence studies. It was found that type-I $InsP_3Rs$ and types-I RyRs were both abundantly expressed in the cytoplasm close to the cell membrane, while there was little expression of types-II, III $InsP_3Rs$ and type-II, III RyRs in MCs (Data not shown). Figure 5 demonstrated that there existed a strong colocalization between type-I $InsP_3Rs$ and types-I RyRs. The spatial colocalization indicated that Ca^{2+} released by $InsP_3Rs$ may further activate neighboring RyRs and enhance the Ca^{2+} oscillations.

MCs contraction can be modulated in a frequency-dependent mode and an amplitude-dependent mode of Ca^{2+} oscillations

AngII concentration from 10^{-8} to 10^{-6} M increased the frequency of Ca^{2+} oscillations but the power and the basal sustained level of Ca^{2+} oscillations did not significantly increase. We replotted the data from three different concentration–response curves at the different frequencies of the Ca^{2+} oscillations (from 4 to 12 spikes/min) and the different contractility data for AngII. It was obvious that the

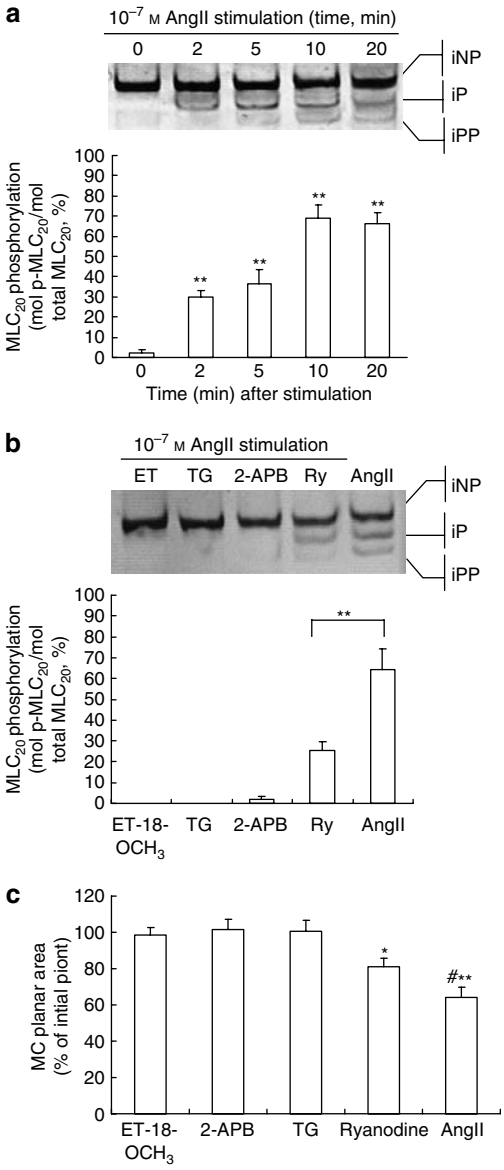

Figure 4 | Contribution of different Ca^{2+} antagonists to the MLC_{20} phosphorylation levels and cellular contractions induced by AngII. (**a**) A time-dependent response for differentially phosphorylated forms of MLC_{20} stimulated by AngII. (**$P<0.01$ vs time 0, $n=4$). (**b**) The MLC_{20} phosphorylation level of MCs pretreated with TG, 2-APB, ET-18-OCH_3, and ryanodine in the presence of AngII or only with AngII at 10 min point. (iNP: non-phosphorylated MLC_{20} level, iP: mono-phosphorylated MLC_{20} level, iPP: di-phosphorylated MLC_{20} level; **$P<0.01$, ryanodine group vs AngII only, $n=4$). (**c**) The MC planar area (% of initial point) pretreated with TG, 2-APB, ET-18-OCH_3, and ryanodine in the presence of AngII or only with AngII at 10 min point (*$P<0.05$, **$P<0.01$, vs ET-18-OCH_3 group, TG group, 2-APB group; $^{\#}P<0.05$, vs ryanodine group; $n=25$).

increased Ca^{2+} oscillations frequency associated with the increased MCs contraction. This suggests that Ca^{2+} oscillations frequency can mediate the MCs contraction ($n=20$; Figure 6).

Moreover, it is well known that CAMKII is directly sensitive to the frequency of Ca^{2+} oscillations.[9] Here, we used KN-93, which is a selective CAMKII inhibitor, and KN-92, which is a negative control of KN-93, to examine the relationship of the Ca^{2+} oscillations frequency and the MLC_{20} phosphorylation level of MCs. Figure 7 illustrates that MLC_{20} phosphorylation could respond to AngII in the presence of 30 μM KN-93 and 30 μM KN-92. KN-93 could markedly inhibit MLC_{20} phosphorylation compared with KN-92 and control group ($P<0.01$), but it did not significantly differ from KN-92 and control group ($P>0.05$). These results imply that MCs contraction can be modulated in a Ca^{2+} oscillations frequency-dependent mode.

In addition, the typical traces of Ca^{2+} oscillations responding to 10^{-7} M AngII in aging MCs (derived from 28-month-old rats) and young MCs (derived from 3-month-old rats) are depicted in Figure 8a and b, and the mean powers of those Ca^{2+} oscillations were 0.008 ± 0.002 and 0.038 ± 0.008, respectively ($P<0.01$, $n=25$; Figure 8c). There were no significant differences of the oscillations frequencies between them (6.06 ± 1.70 vs 4.92 ± 0.30/min, NS, $n=25$; Figure 8d). Moreover, the baseline intensity value (F_0) of $[Ca^{2+}]_i$ from aging cells was higher than the young ($P<0.05$, $n=25$; Figure 8e).

Furthermore, it was found that MLC_{20} phosphorylation in young cells was lower than that of aging cells before stimulation (2.24 ± 1.02 vs $10.3\pm3.51\%$, $P<0.01$). However, 10 min after stimulation, the level of MLC_{20} phosphorylation in young cells was significantly higher (63.11 ± 11.4 vs $42.21\pm2.91\%$, $P<0.01$; Figure 9a). For the change of planar area after stimulation, in oscillations-positive cells, the young MC planar area decreased to $65.2\pm4.5\%$, while the aging ones just decreased to $78.5\pm3.8\%$ of the original planar area ($P<0.01$). However, there was no significant difference between the oscillations-negative cells from the young and the aging (89.1 ± 6.5 vs $92.1\pm6.8\%$, $P>0.05$, $n=25$; Figure 9b). The data showed that, for aging cells, the increase of MLC_{20} phosphorylation and MCs contraction were weakened after the stimulation of AngII in oscillations-positive cells. Such data also indicated that the low contractibility to stimulation displayed by the aging-related mesangial cells came from the power reduction of Ca^{2+} oscillations stimulated by AngII.

DISCUSSION

Recent studies[7] show that there is a strong relationship among the action of renal local AngII, the mechanisms regulating mesangial contraction and the regulating rule of glomerular filtration rate. Such a relationship depends on intracellular Ca^{2+} signaling and proper excitation–contraction coupling in MCs. More evidences have showed that, in response to extracellular stimulation, it is the cytoplasmic Ca^{2+} oscillations that regulate the subsequent cellular functions.[8]

In our study, we found that AngII could trigger typical Ca^{2+} oscillations in MCs. And as AngII concentration increased, the frequency of Ca^{2+} oscillations increased too. However, the power of Ca^{2+} oscillations decreased and

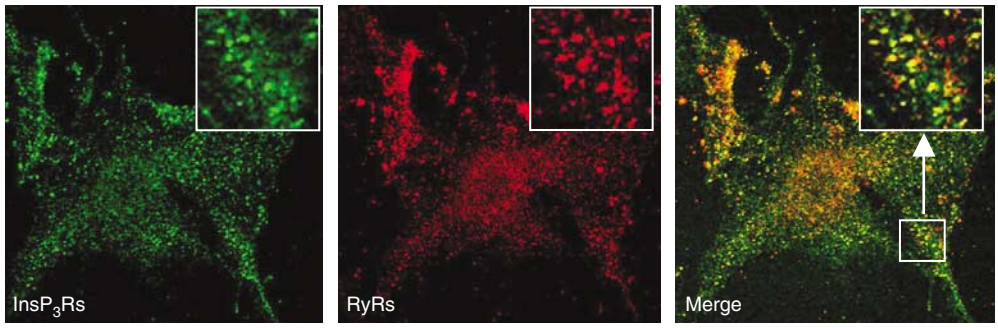

Figure 5 | Confocal colocalization of $InsP_3R$ and RyR subtypes in MCs. Both type-I $InsP_3$ receptors (green color, original magnification, $\times 600$) and type-I RyRs (red color, original magnification, $\times 600$) colocalization as in the merged image (yellow color, original magnification, $\times 600$) in MCs used by confocal microscope (right upper: local original magnification, $\times 2400$).

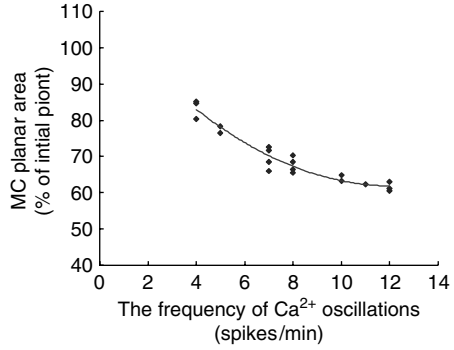

Figure 6 | Relationship between the Ca^{2+} oscillation frequency and the MC planar area (% of initial point) induced by AngII. Data from concentration–response curves for the frequency of Ca^{2+} oscillations (from 4 to 13 spikes/min) and the contractility for AngII (10^{-8}–10^{-6} M) were replotted. Data were fitted with a sigmoidal curve ($n = 20$).

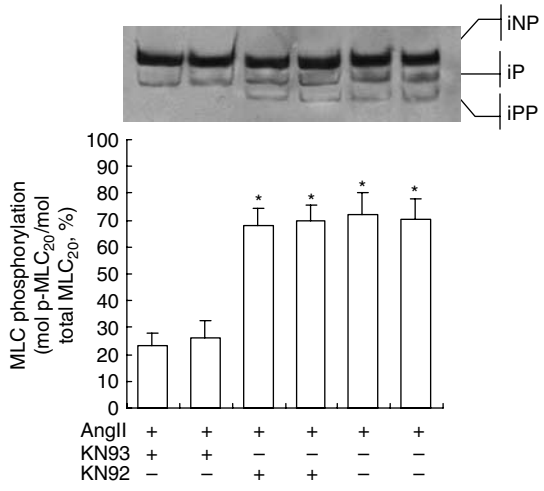

Figure 7 | Inhibitory effect of KN-93 on the MLC_{20} phosphorylation level induced by AngII. MCs were pretreated with AngII in the presence of KN-92, KN-93, or only with AngII. The MLC_{20} phosphorylation levels of MCs were measured after 10 min. (iNP: non-phosphorylated MLC_{20} level, iP: mono-phosporylated MLC_{20} level, iPP: di-phosphorylated MLC_{20} level; $^*P < 0.01$ vs KN-93 group, $n = 4$).

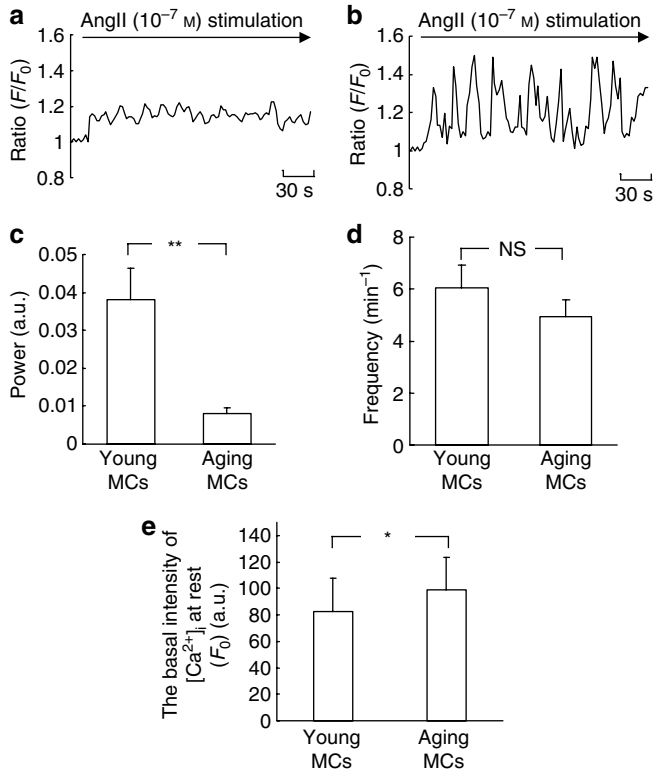

Figure 8 | The traces of Ca^{2+} oscillations induced by AngII in aging MCs compared with young MCs. The typical Ca^{2+} oscillations were induced by AngII (**a**) in an aging MC and (**b**) in a young MC. (**c**) The Ca^{2+} oscillations power compared young with aging MCs used by the Fourier transform analysis ($^{**}P < 0.01$, $n = 25$). (**d**) The Ca^{2+} oscillations frequency compared young with aging MCs used by the Fourier transform analysis (NS, not significant; $n = 25$). (**e**) The basal intensity value of the $[Ca^{2+}]_i$ level (F_0) at rest in young and old oscillations-positive cells ($^*P < 0.05$, $n = 25$).

reached a plateau at 10^{-8}–10^{-6} M AngII in this process. At low frequencies of Ca^{2+} oscillations, the amplitude of such oscillations was high and the basal sustained level was low. With the increase of Ca^{2+} oscillation frequencies, the amplitude of such oscillations was lower and the basal sustained level of $[Ca^{2+}]_i$ increased too. These results may be due to high intracellular $InsP_3$ levels in cells that are

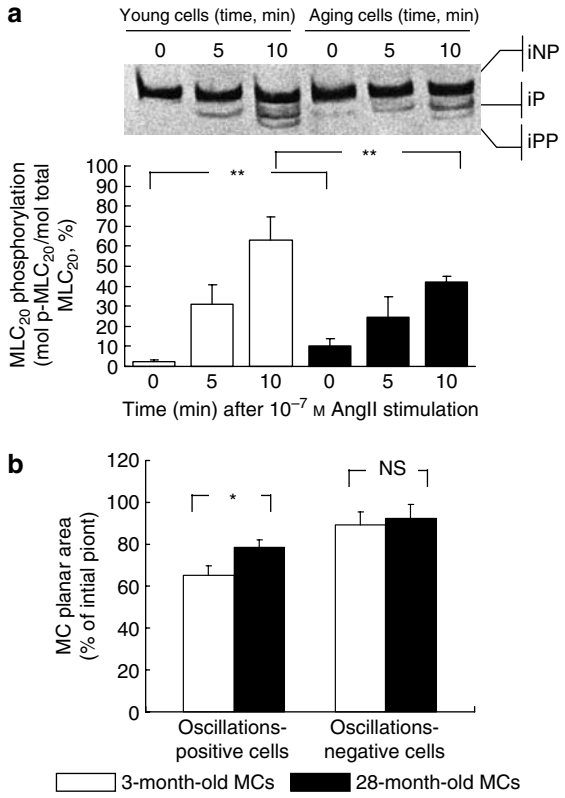

Figure 9 | Reduction of the MLC_{20} phosphorylation level and the cellular contraction of aging MCs compared with young ones. (**a**) The MLC_{20} phosphorylation levels induced by AngII at 0, 5, and 10 min in young and aging cells (**$P<0.01$; $n=4$). (**b**) The MC planar area (% of initial point) induced by AngII in the presence of oscillations or non-oscillations of young and aging cells (*$P<0.05$, NS: not significant, $n=25$). (□; 3-month-old MCs, ■; 28-month-old MCs; iNP: non-phosphorylated MLC_{20} level; iP: mono-phosporylated MLC_{20} level; iPP: di-phosphorylated MLC_{20} level).

generated, at high AngII concentrations. At such a level of $InsP_3$, the repetitive Ca^{2+} oscillations may be generated before the Ca^{2+} pump reuptake can lower the $[Ca^{2+}]_i$ level occurring between each oscillation at near resting levels. At lower AngII ($InsP_3$) levels, there is sufficient time between each oscillation for the pump mechanism to reduce $[Ca^{2+}]_i$ to near resting levels; therefore, the amplitude of each oscillation was higher.[24]

In view of the strong Ca^{2+} oscillations that we observed in primary MCs, it is a surprise that such Ca^{2+} oscillations in response to AngII were not observed by some researchers.[25,26] The possible reasons for this, we presumed, were the low-spatio-temporal resolution and the fluorescent dye sensibility of Ca^{2+} measurements, and the fact that, as we have noted, when the cells are overloaded with the fluorescent dye, the Ca^{2+} oscillations cannot be observed with ease. So by changing the loading conditions (e.g., using less time, lower temperature, or less dye concentration), the Ca^{2+} oscillations can be observed in a majority of the cells.

The source of Ca^{2+} oscillations appears to result from the internal Ca^{2+} release and reuptake into ER store because MCs oscillations were abolished by TG, but not inhibited in the absence of extracellular Ca^{2+}, which is in consistence with the hypothesis proposed by Berridge and Galione.[27] However, the fact that the eventual cessation of the Ca^{2+} oscillations in the absence of extracellular Ca^{2+} suggests the need for extracellular Ca^{2+} influx. Then, our results disclosed that both 2-APB and ET-18-OCH_3 could completely inhibit Ca^{2+} oscillations, indicating that phospholipase C–$InsP_3$ pathway mediates the generation of Ca^{2+} oscillations from extracellular agonists in MCs. Interestingly, our results showed that ryanodine could partly inhibit Ca^{2+} oscillations, not abolish them, indicating that MCs oscillations are augmented by RyRs as well as $InsP_3$Rs in MCs. Further, our subsequent observation showed that $InsP_3$Rs and RyRs could be colocalized, providing evidence that Ca^{2+}-releasing by $InsP_3$Rs may be the spatio-temporal upstream signal of neighboring RyRs in regulating Ca^{2+} oscillatory response of MCs.

In our study, we found that MCs' contraction stimulated by AngII was associated with sustained Ca^{2+} oscillations. To rule out the average $[Ca^{2+}]_i$ increase responding to AngII over the same period of time, we compared the contractive extent of Ca^{2+} oscillations-positive cells with that of Ca^{2+} oscillations-negative cells. Furthermore, both MLC_{20} phosphorylation analysis and the MC planar area measurement demonstrated that oscillations inhibition could also lead to the inhibition of MCs contraction. The results suggest that AngII-induced MCs oscillations are required for the cellular contraction. Some previous studies[7] have demonstrated spontaneous and AngII-induced MCs contraction because of intracellular Ca^{2+} increase, and we in our experiments tried to correlate the extent of contraction with the level of the sustained increase $[Ca^{2+}]_i$, and found that the contractive extent was lower in oscillations-negative cells than in oscillations-positive cells. So our conclusion is that it is the AngII-induced Ca^{2+} oscillations that can increase the extent of contractions in MCs.

As a result of receptor activation, several studies have shown that the specificity of Ca^{2+} oscillations may be encoded in a frequency-dependent mode in addition to, or rather than, an amplitude-dependent mode.[28] A potentially more attractive hypothesis is that Ca^{2+} exerts its action through a frequency-dependent mode.[27,29] With little change in the amplitude of Ca^{2+} oscillations, it also found a good correlation for the relationship between MCs contraction and the frequency of oscillations in our study. Meanwhile, the frequency decrease using CAMKII inhibitor was accompanied with the reduction of MLC_{20} phosphorylation level in MCs. Such a relationship suggests that the size of cells is regulated by the change of Ca^{2+} oscillations frequency.[8]

Under appropriate experimental conditions, it was interesting to note that the frequency of Ca^{2+} oscillations in aging cells did not show any significant changes, and that the basal Ca^{2+} at rest also was not reduced in aging cells either, but the power of Ca^{2+} oscillations decreased and accordingly matched with the decrease of cellular contraction in

oscillation-positive cells. We suggest the reason is that the amplitude reduction in aging cells is insufficient to activate contraction-related proteins, which contributed to a lot to the deduction of contraction.

In conclusion, our results have demonstrated that $[Ca^{2+}]_i$ of individual mesangial cells in response to AngII is characterized by repetitive spikes through repetitive cycles of the internal Ca^{2+} release by the ERs $InsP_3$ receptors and Ca^{2+} amplification by succedent Ca^{2+}-activated RyRs and thereafter Ca^{2+} reuptake by the ER. Furthermore, it has been demonstrated that Ca^{2+} oscillations can be required to modulate MCs contraction not only in an AngII-induced, frequency-dependent mode but also in an aging-related amplitude-dependent mode.

MATERIALS AND METHODS

Materials

Cell culture reagents were obtained from Invitrogen Corp. (Carsbad, CA, USA). Anti-RyR and anti-$InsP_3$ receptor antibodies were generous gifts from Professor Heping Cheng (Beijing University, People's Republic of China). Anti-MLC_{20} antibody and second antibodies were from Santa Cruz Biotechnology Inc. Other reagents were from Sigma-Aldrich Corp. (St. Louis, MO, USA). The normal external solution was Hanks'-buffered saline solution with addition of streptomycin sulfate (1×10^6 U/ml) and the pH was titrated to 7.4.

Culture of primary rat glomerular mesangial cell

MCs isolation and culture of 3-month-old or 28-month-old male Wistar rats were performed as described previously.[30] MCs between passage 5 and 6 were used for the experiments.

Confocal imaging of intracellular Ca^{2+}

MCs were cultured to confluence in special glass-bottom microwell dishes (MatTek Corporation, Ashland, MA, USA), and then incubated with 2.5 μM fluo-3 (Molecular Probes, Eugene, OR, USA) plus 0.02% Pluronic F-127 (Molecular Probes) in Hanks'-buffered saline solution. The fluorescent images of cells were collected every second for fast signals and analyzed frame by frame with confocal microscope (Bio-Rad Laboratory Inc., Hercules, CA, USA). For the specific MCs Ca^{2+} oscillations, line-scanning method was made over a single horizontal position consisting of 1000 lines at 20 ms intervals. The Ca^{2+} level was expressed as a pseudo-ratio value (F/F_0) of the actual fluorescence intensity (F) divided by the basal intensity of the $[Ca^{2+}]_i$ at rest (F_0).[31,32]

Experimental protocol

(1) MCs were scanned for at least 30–40 min after adding AngII at different concentrations (10^{-10}–10^{-6} M); (2) MCs were scanned for 10 min and then the following agents were added: Ca^{2+}-free solution containing 5 mM ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate, 2 μM TG (exposed for 30 min), 20 μM 2-APB (exposed for 30 min), 50 μM ET-18-OCH_3 (exposed for 15 min), or 100 μm ryanodine (exposed for 30 min), and the same cells were scanned for another 20–30 min.

Mesangial cell planar area measurements

MCs were cultured as shown above. Images of the same cells with and without Ca^{2+} oscillations were captured serially using confocal phase-contrast light transmission program through a second photomultiplier tube at the same time that the Ca^{2+} signaling were collected. The planar area was automatically calculated by LaserPix software. The change in planar surface area compared to original size was calculated and expressed as a percentage of the initial value for each cell. The planar area expressed as mean $\pm$ s.d. was determined at each time point.

Analysis of MLC_{20} phosphorylation

Phosphorylation of the 20-kDa MLC_{20} was measured with the method from Dlugosz *et al.*[33] and Persechini *et al.*[34] Densitometry was performed using AlphImage 2000 (Alpha Innotech Comp., San Leandro, CA, USA), which quantified the intensity of each band associated with non-phosphorylated (denoted by iNP), mono-phosphorylated, (iP) and di-phosphorylated (iPP) MLC_{20} forms. The fraction of the MLC_{20} phosphorylated form (i.e. moles of MLC_{20} phosphorylated to total moles of MLC_{20}) was calculated as the total phosphorylated MLC_{20} (equal to iP + (2 × iPP)) divided by the total MLC_{20} (equal to iP + iPP + iNP).[35,36]

Double immunofluorescence in MCs with confocal microscopy

Indirect immunofluorescence double staining was performed using a routine procedure.[37]

Data analysis and statistics

To describe and quantitatively analyze the characteristics of Ca^{2+} oscillation in young and aging MCs, computer programs were made using Interactive Data Language, IDL (RSI company). Confocal images were processed with Fast Fourier transform so that we can achieve the frequency and power spectrum of Ca^{2+} oscillation.[38,39] The results were presented as means $\pm$ s.d. and evaluated statistically with Student's *t*-test or analysis of variance for significant difference.

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