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P2X Receptor Stimulated Calcium Responses in Preglomerular Vascular Smooth Muscle Cells Involves 20-HETE

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d) **Abbreviations:** CYP450, Cyrochrome P450; HETE, hydroxyeicosatraeonic acid; DDMS, N-methylsulfonyl-12,12-dibromododec-11-enamide; HEDE, hydroxyeicosa-6(Z), 15(Z)-dienoic acid.

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ABSTRACT

The current study tested the hypothesis that endogenous 20-HETE contributes to the increase in intracellular calcium ($[Ca^{2+}]_i$) elicited by P2X receptor activation in renal microvascular smooth muscle cells. Vascular smooth muscle cells obtained from rats were loaded with fura-2 and studied using standard single cell fluorescence microscopy. Basal renal myocyte $[Ca^{2+}]_i$ averaged 96 ± 5 nM. ATP (10 and 100 μM) increased vascular smooth muscle cell $[Ca^{2+}]_i$ by 340 ± 88 nM and 555 ± 80 nM, respectively. The CYP450 hydroxylase inhibitor, N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS), or the 20-HETE antagonist, 20-hydroxyeicosa-6(Z), 15(Z)-dienoic acid (20-HEDE), significantly attenuated the peak myocyte $[Ca^{2+}]_i$ responses to 10 and 100 μM ATP. ATP (100 μM) increased vascular smooth muscle cell $[Ca^{2+}]_i$ by 372 ± 93 nM and 163 ± 55 nM in the presence of DDMS or 20-HEDE, respectively. The P2X receptor agonist, α,β-methylene ATP (10 μM) increased myocyte $[Ca^{2+}]_i$ by 78 ± 12 nM and this response was significantly attenuated by DDMS (40 ± 15 nM). In contrast, the vascular smooth muscle cell $[Ca^{2+}]_i$ evoked by the P2Y agonist, UTP (100 μM), was not altered by DDMS or 20-HEDE. The effect of 20-HETE on $[Ca^{2+}]_i$ was also assessed and the peak increases in $[Ca^{2+}]_i$ averaged 62 ± 12 and 146 ± 70 nM at 20-HETE concentrations of 1 and 10 μM, respectively. These results demonstrate that 20-HETE plays a significant role in the renal microvascular smooth muscle cell $[Ca^{2+}]_i$ response to P2X receptor activation.

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ATP induces vasoconstriction by activating P2 receptors on preglomerular microvascular smooth muscle cells (Eltze and Ullrich, 1996; Inscho et al., 1998; Inscho et al., 1995). This vasoconstriction appears to depend on activation of P2X and P2Y receptors. ATP-mediated afferent arteriolar vasoconstriction is largely dependent on the influx of extracellular Ca^{2+} and the sustained vasoconstriction is maintained by Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (Navar et al., 1996; Inscho and Cook, 2002, Inscho et al., 1999b; Inscho et al., 1995). Activation of P2X and P2Y receptors on microvascular smooth muscle cells stimulates an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by distinct Ca^{2+} signaling pathways (Inscho et al., 1999a; Inscho et al., 1999b). P2X receptors are described as having 2 membrane-spanning domains and function as ligand-gated channels that allow influx of extracellular cations including Ca^{2+} (Abbracchio and Burnstock, 1994; Dubyak and El-Moatassim, 1993; El-Moatassium et al., 1992; Evans et al., 1998, Fredholm et al., 1994; Ralevic and Burnstock, 1998; White et al., 2001). Interestingly, inactivation of P2X receptors on preglomerular microvessels inhibits autoregulatory behavior (Inscho et al., 1996; Mitchell and Navar, 1993; Majid et al., 1999; Inscho et al., 2003). In contrast, P2Y receptors have 7 membrane-spanning domains coupled to G proteins and increase $[Ca^{2+}]_i$ in part by stimulating mobilization of Ca^{2+} from intracellular stores (Abbracchio and Burnstock, 1994; Dubyak and El-Moatassim, 1993; El-Moatassium et al., 1992; Evans et al., 1998, Fredholm et al., 1994; Ralevic and Burnstock, 1998). Nevertheless, the cell signaling mechanisms responsible for P2X and P2Y mediated increases in renal microvascular smooth muscle cells are not well known.

20-Hydroxyeicosatraeonic acid (20-HETE), a metabolite of the arachidonic acid cytochrome P450 (CYP450) pathway, plays an important role in the regulation of renal vascular and tubular function (Imig et al., 1996; Ma et al., 1993; Hercule and Oyekan, 2000; Harder et al.

1994). 20-HETE inhibits vascular smooth muscle potassium channels resulting in membrane depolarization and subsequent activation of L-type Ca^{2+} channels leading to vasoconstriction of the afferent arteriole (Imig et al., 1999; Imig et al., 2000; Gebremedhin et al., 1998; Lange et al., 1997). Interestingly, P2X receptor inactivation or CYP450 hydroxylase inhibition significantly attenuates pressure-mediated afferent arteriolar vasoconstrictor responses (Imig et al., 1999; Inscho et al., 2003). Our previous studies showed that either CYP450 inhibition or 20-HETE antagonism attenuated the initial vasoconstriction and abolished the sustained vasoconstriction evoked by the P2 receptor agonist ATP (Zhao et al., 2001). In addition, N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS) and 20-hydroxyeicosa-6(Z), 15(Z)-dienoic acid (20-HEDE), also attenuated the initial vasoconstrictor response and abolished the sustained vasoconstrictor response to the P2X receptor agonist, α,β-methylene-ATP (Zhao et al., 2001). These data suggest that the CYP450 metabolite 20-HETE participates in the afferent arteriolar response to activation of P2X receptors. Based on these studies, we hypothesized that endogenous 20-HETE contributes to the increase in $[Ca^{2+}]_i$ elicited by P2X receptor activation in renal microvascular smooth muscle cells.

MATERIALS AND METHODS

Tissue Preparation and Renal Microvascular Smooth Muscle Cell Isolation. All studies were performed in compliance with the guidelines and practices dictated by the Medical College of Georgia Advisory Committee for Animal Resources. Suspensions of preglomerular microvascular smooth muscle cells were prepared with a modification of the method previously described (White et al., 2001). Male Sprague-Dawley CD-VAF rats (250-375 g; Charles River Laboratories; Wilmington, MA) were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), and the abdominal cavity was exposed to permit cannulation of the abdominal aorta via the superior mesenteric artery. Ligatures were placed around the abdominal aorta at sites proximal and distal to the left and right renal arteries, respectively. The kidneys were cleared of blood by perfusion of the isolated aortic segment with an ice-cold, low-calcium physiological salt solution (PSS; pH 7.35) of the following composition (in mmol/L); 125 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 glucose, 20.0 HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 0.1 $CaCl_2$, and 6% bovine serum albumin. After the kidneys were rinsed of blood, the perfusate was changed to a similar solution containing 1% Evans blue in low-calcium PSS.

The kidneys were resected from the animal and decapsulated, and the renal medullary tissue was removed. The cortical tissue was pressed through a sieve (180 μm), and the sieve retentate was washed several times with ice-cold low-calcium PSS. The vascular tissue remaining on the sieve was transferred to an enzyme solution containing 0.075% collagenase (Boehringer Mannheim Corp), 0.02% dithiothreitol (Sigma Chemical Co), and 0.1% bovine serum albumin dissolved in low-calcium PSS, and this mixture was incubated at 37^{o}C for 30 minutes. The vascular tissue was removed from the enzyme solution and transferred to a nylon mesh (70 μm) where it was vigorously rinsed with ice-cold low-calcium PSS. The mesh

containing the retained vascular tissue was transferred to a Petri dish containing ice-cold low-calcium PSS. Segments of interlobular artery with attached afferent arterioles were collected by microdissection using a stereoscope and transferred to a 10 mL dissociation flask. The rinse solution was decanted from the selected vascular segments and replaced with an enzyme solution containing 0.075% papain (Sigma Chemical Co) and 0.02% dithiothreitol (Sigma Chemical Co) in low-calcium PSS. The tissue was incubated at 37^{o}C for 15 minutes before being collected by centrifugation (2000 g for 50 seconds). The tissue pellet was transferred to an enzyme solution containing 0.3% collagenase (Boehringer Mannheim Corp) and 0.2% soybean trypsin inhibitor (type 1-S, Sigma Chemical Co) in low-calcium PSS at 37^{o}C. After a 15-minute incubation period, the mixture was gently triturated and quickly centrifuged (500 g for 5 minutes) to collect the dispersed cells. The supernatant was discarded, and the cells were gently resuspended in 1.0 mL Dulbecco's minimum essential medium (DMEM; Sigma Chemical Company) supplemented with 20% fetal calf serum (Whittaker Bioproducts) and 100 U/mL penicillin and 200 μg/mL streptomycin (Sigma Chemical Co). Cell suspensions were stored on ice until use.

Fluorescence Measurements in Single Microvascular Smooth Muscle Cells. Experiments were performed using a standard microscope-based fluorescence spectrophotometry system (Photon Technology International) as previously described (White et al., 2001). The excitation wavelengths were set at 340 and 380 nm, and the emitted light was collected at 510 ± 20 nm (Photon Technology Intl). Measurements of fluorescence intensity were collected at 5 data points per second, and analyzed with the aid of the Photon Technology International software. Calibration of the fluorescence data was accomplished as previously described (White et al., 2001).

Measurement of $[Ca^{2+}]_i$ in single microvascular smooth muscle cells was performed as described previously (White et al., 2001). Suspensions of freshly isolated renal microvascular cells were loaded with the calcium sensitive fluorescent probe, fura 2 acetoxymethyl ester (fura2-AM; 4.0 μmol/L; Molecular Probes). An aliquot of cell suspension was transferred to the perfusion chamber (Warner Instrument Corporation) and mounted to the stage of a Nikon Diaphot inverted microscope. The cells were continuously superfused (1.3 mL/min) with a 1.8 mM calcium PSS solution of the following composition (in mM): 125 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 glucose, 20.0 HEPES, 1.8 $CaCl_2$, and 0.111 g/L bovine serum albumin. For each experiment, a single microvascular cell was isolated in the optical field by positioning the adjustable sampling window directly over the cell of interest. Neighboring cells and debris are thus excluded from the sampling field, allowing fluorescence emission to be measured only from the background subtraction, and a new coverslip of cells was used for each experiment.

Experimental Approach. **Series 1**. Experiments were performed to assess the role of 20-HETE in the microvascular smooth muscle cell response to ATP. For these experiments, cells were challenged with ATP while being bathed in a PSS solution containing the CYP450 hydroxylase inhibitor, N-methylsulfony-12,12-dibromododec-11-enamide (DDMS) (Wang et al., 1998; Imig et al., 1999), or the 20-HETE antagonist, 20-hydroxyeicosa-6(Z), 15(Z)-dienoic acid (20-HEDE) (Alonso-Galicia et al., 1999; Zhao et al., 2001). Fura 2 fluorescence was monitored in these cells under control conditions (0-100 s), during exposure to 25 μM DDMS in the presence of 1.8 mM Ca^{2+} (100-150 s), during subsequent exposure to ATP (10 or 100 μM) in combination with DDMS and 1.8 mM Ca^{2+} (150-350 s), and during the recovery period, during which ATP and DDMS was removed from the bathing solution (350-600 s). Identical studies were performed by

using 3 μM 20-HEDE instead of DDMS. These responses were compared with responses obtained from similar cells challenged in normal-calcium PSS without CYP450 hydroxylase inhibition or 20-HETE antagonism.

Series 2. Studies were performed to further determine the effect of CYP450 hydroxylase inhibition and 20-HETE antagonism on the increase in $[Ca^{2+}]_i$ induced by the P2X receptor agonist, α,β-methylene-ATP. Fura 2 fluorescence was monitored in these cells under control conditions (0-100 s), during exposure to DDMS or 20-HEDE in the presence of 1.8 mmol/L Ca^{2+} (100-150 s), during subsequent exposure to α,β-methylene-ATP (10 μM) in combination with DDMS or 20-HEDE and 1.8 mmol/L Ca^{2+} (150-350 s), and during the recovery period (350-600 s). These responses were compared with responses obtained from similar cells challenged with α,β–methylene-ATP alone.

Series 3. Experiments were performed to assess the role of 20-HETE in the renal myocyte response to the P2Y receptor agonist UTP. Fura 2 fluorescence was monitored in these cells under control conditions (0-100 s), during exposure to DDMS or 20-HEDE in the presence of 1.8 mmol/L Ca^{2+} (100-150 s), during subsequent exposure to UTP (100 μM) in combination with DDMS or 20-HEDE and 1.8 mmol/L Ca^{2+} (150-350 s), and during the recovery period (350-600 s). These responses were compared with responses obtained from similar cells challenged with UTP alone.

Series 4. Additional experiments were performed to assess the direct effect of 20-HETE on $[Ca^{2+}]_i$ in renal microvascular smooth muscle cells. Fura 2 fluorescence was monitored in these cells under control conditions (0-150 s), during exposure to 20-HETE (1, 10 μM) in 1.8 mmol/L Ca^{2+} (150-350 s), and during the recovery period (350-600 s).

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Statistical Analysis. Data are presented as means ± SE. Within-group comparisons of peak $[Ca^{2+}]_i$ with baseline $[Ca^{2+}]_i$ were analyzed using ANOVA for repeated measures. Differences between treated and untreated groups of cell $[Ca^{2+}]_i$ values were analyzed by ANOVA followed by Newman-Keuls multiple-range test. Statistical probabilities of <0.05 (p<0.05) are considered significantly different.

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Results

CYP450 Hydroxylase Inhibition and 20-HETE Antagonism on Renal Microvascular Smooth Muscle Cell $[Ca^{2+}]_i$ Response to ATP. To test the hypothesis that 20-HETE may be involved in the microvascular smooth muscle cell response to ATP, we investigated the effect of DDMS and 20-HEDE on the $[Ca^{2+}]_i$ response evoked by ATP. Figures 1 and 2 present representative traces depicting the changes in $[Ca^{2+}]_i$ elicited by ATP (10 and 100 μM) in the presence or absence of DDMS or 20-HEDE. Consistent with previous data, exposure to ATP evoked a rapid peak response followed by a steady-state plateau. DDMS (25 μM) or 20-HEDE (3 μM) significantly attenuated the peak renal myocte $[Ca^{2+}]_i$ responses to 10 and 100 μM ATP. Figure 3 presents the average responses in series 1 experiment. Baseline was similar among all these groups and basal microvascular smooth muscle cell $[Ca^{2+}]_i$ averaged 96 ± 5 nM (n=70). ATP (10 and 100 μM) increased renal myocyte $[Ca^{2+}]_i$ by 340 ± 88 nM and 555 ± 80 nM, respectively. The administration of 25 μM DDMS or 3 μM 20-HEDE had no effect on baseline $[Ca^{2+}]_i$, but significantly attenuated the peak renal myocyte $[Ca^{2+}]_i$ responses to 10 and 100 μM ATP. In the presence of DDMS, the peak renal myocyte $[Ca^{2+}]_i$ responses to 10 and 100 μM ATP were 172 ± 77 nM and 372 ± 93 nM. In addition, 20-HEDE decreased the peak renal myocyte $[Ca^{2+}]_i$ responses to 10 and 100 μM ATP to 131 ± 44 and 163 ± 55 nM. These data demonstrate that CYP450 hydroxylase inhibition and 20-HETE antagonism attenuated the initial $[Ca^{2+}]_i$ response to P2 receptor activation.

CYP450 Hydroxylase Inhibition and 20-HETE Antagonism on Renal Microvascular Smooth Muscle Cell $[Ca^{2+}]_i$ Response to P2X Receptor Activation. The second series of experiments were performed using the P2X receptor-selective ATP analog α,β-methylene-ATP to further examine the contribution of 20-HETE to P2 receptor mediated calcium signaling and

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the results of those studies were presented in Figures 4 and 5. Figure 4 shows typical traces depicting the changes in $[Ca^{2+}]_i$ evoked by α,β-methylene-ATP in the presence or absence of DDMS or 20-HEDE. Exposure of microvascular smooth muscle cells to 10 μM α,β-methylene-ATP evoked an increase in $[Ca^{2+}]_i$ that typically included a rapid peak response followed by a gradual return to steady-state level similar to baseline. DDMS significantly attenuated the renal myocyte $[Ca^{2+}]_i$ response to α,β-methylene-ATP. Figure 5 presents the average responses in these experiments. α,β-methylene ATP (10 μM) increased vascular smooth muscle cell $[Ca^{2+}]_i$ by 80 ± 12 nM (n=18 cells). DDMS significantly reduced this response to 40 ± 15 nM (n=19 cells, $P<0.05$); however, 20-HEDE decreased this response by only 13%.

CYP450 Hydroxylase Inhibition and 20-HETE Antagonism on Renal Microvascular Smooth Muscle Cell $[Ca^{2+}]_i$ Response to P2Y Receptor Activation. The P2Y receptor agonist, UTP, was used to assess the role of 20-HETE in the renal myocyte $[Ca^{2+}]_i$ response to P2Y receptor activation. The results of series 3 experiments are presented in Figures 6 and 7. UTP (100 μM) caused a rapid increase in $[Ca^{2+}]_i$ that reached a peak (822 ± 170 nM, n=18 cells) followed by a gradual recovery to a steady-state $[Ca^{2+}]_i$ (65 ± 19 nM, n=18 cells) that is significantly greater than baseline ($P<0.05$). Microvascular smooth muscle cell $[Ca^{2+}]_i$ responses evoked by UTP were not altered by DDMS or 20-HEDE. The peak increases in $[Ca^{2+}]_i$ elicited by UTP were 691 ± 172 (n=15 cells) and 964 ± 156 nM (n=16 cells) in the presence of DDMS or 20-HEDE (Figure 7), respectively. These data suggest that 20-HETE is not involved in the P2Y receptor-mediated renal microvascular smooth muscle cell calcium signaling.

Renal Microvascular Smooth Muscle Cell $[Ca^{2+}]_i$ Response to 20-HETE. Figure 8 presents a representative trace depicting the change in $[Ca^{2+}]_i$ elicited by 10 μM 20-HETE. Exposure of renal myocytes to 20-HETE evoked an increase in $[Ca^{2+}]_i$. The peak $[Ca^{2+}]_i$ elicited

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by 20-HETE (1 and 10 μM) averaged 62 ± 12 and 146 ± 70 nM, respectively, and was significantly different from their respective baseline calcium concentrations.

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Discussion

The current studies were performed to determine whether CYP450 hydroxylase metabolite, 20-HETE, is involved in the $[Ca^{2+}]_i$ response in the renal microvascular smooth muscle cells to purinoceptor activation. Consistent with our previous studies (Inscho et al., 1999a; White et al., 2001), ATP and UTP elicited a biphasic response including a peak response followed by a steady-state plateau, whereas α,β–methylene-ATP evoked a transient increase in $[Ca^{2+}]_i$ that quickly returned to baseline. The CYP450 hydroxylase inhibitor, DDMS, and the 20-HETE antagonist, 20-HEDE, had no effect on baseline renal microvascular smooth muscle cell $[Ca^{2+}]_i$; however, the addition of DDMS or 20-HEDE, significantly attenuated the initial $[Ca^{2+}]_i$ responses evoked by 10 and 100 μM ATP. In addition, DDMS also markedly reduced the $[Ca^{2+}]_i$ response induced by the P2X receptor agonist α,β–methylene-ATP. CYP450 hydroxylase inhibition and 20-HETE antagonism did not alter the renal myocyte $[Ca^{2+}]_i$ responses to the P2Y receptor agonist UTP. These data demonstrate that 20-HETE plays a role in the $[Ca^{2+}]_i$ response evoked by P2X receptor activation.

Renal hemodynamic control is accomplished by local adjustments in intrarenal vascular resistance. The majority of these resistance adjustments are preglomerular and occur at the level of the afferent arterioles (Arendshorst and Navar, 1993). Myogenic and tubuloglomerular feedback-mediated adjustments in preglomerular resistance are the major contributors to renal blood flow autoregulation. Tubuloglomerular feedback is believed to be a major regulatory system coupling changes in distal tubular flow with preglomerular resistance through the actions of the macula densa. ATP, released from the macula densa, serves as the chemical messenger linking the macula densa with regulation of afferent arteriolar resistance. Regulation of afferent arteriolar resistance involves ATP-dependent activation of P2X receptors that are heavily

expressed along the preglomerular, but not the postglomerular, microvasculature (Chan et al., 1998; Inscho, 2001; Inscho, 1999; Navar et al., 1996; Bell et al., 2003; Inscho et al., 1996; Mitchell and Navar, 1993). Inactivation of P2 receptors on preglomerular microvessels inhibits autoregulatory behavior (Inscho et al., 1996; Majid et al., 1999). More recent data suggest a specific role for $P2X_1$ receptors in the afferent arteriolar autoregulatory response. Pressure-dependent afferent arteriolar diameter responses were abolished by pharmacological blockade of P2X receptors in rats and deletion of $P2X_1$ receptors in mice (Inscho et al., 2003). Taken together, these studies strongly support the postulate that $P2X_1$ receptor activation plays a critical role in mediating afferent arteriolar autoregulatory adjustments.

The cellular signaling mechanisms responsible for P2 receptor vasoconstriction are not well defined. ATP-mediated afferent arteriolar vasoconstriction is largely dependent on the influx of extracellular Ca^{2+} and the sustained vasoconstriction is maintained by Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (Inscho and Cook, 2002; Inscho et al., 1999a; Navar et al., 1996; Inscho et al., 1995). P2 receptor activation results in the release of arachidonic acid from membrane phospholipids in glomerular messangial cells and rat astrocytes (Bolego et al., 1997; Pfeilschifter, 1990; Schulze-Lohoff et al., 1992). Our previous studies showed that CYP450 inhibition or 20-HETE antagonism significantly attenuated both the initial and sustained afferent arteriolar constrictor responsiveness to ATP (Zhao et al., 2001). These data demonstrate that the CYP450 metabolite 20-HETE participates in the afferent arteriolar response to P2 receptor activation. In order to determine whether 20-HETE contributes to the afferent arteriolar response to P2 receptor activation by influencing the microvascular smooth muscle cell calcium signaling, we further investigated the effect of DDMS or 20-HEDE on the renal myocyte $[Ca^{2+}]_i$ response to ATP. Our studies showed that both DDMS and 20-HEDE

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significantly attenuated the initial $[Ca^{2+}]_i$ responses to ATP. These data suggest that 20-HETE is involved in the renal microvascular smooth muscle cell $[Ca^{2+}]_i$ response to P2 receptor activation.

P2 receptors were first defined by Burnstock in 1978 (Burnstock, 1978). Since then, P2 receptors have grown into a large family of receptors divided into two major categories classified as P2X and P2Y (Abbracchio and Buenstock, 1994; Fredholm et al., 1994; Ralevic and Burnstock, 1998; Fredholm et al., 1997). P2X receptors are described as having 2 membrane-spanning domains and function as ligand-gated channels (Abbracchio and Buenstock, 1994; Ralevic and Burnstock, 1998). P2Y receptors have 7 membrane-spanning domains and function as G protein-coupled receptors (Abbracchio and Buenstock, 1994; Ralevic and Burnstock, 1998). Previous studies have shown that renal microvascular smooth muscle cell $[Ca^{2+}]_i$ responses to P2 receptor stimulation with ATP involve the activation of both P2X and P2Y receptor subtypes (Inscho et al., 1999a; White et al., 2001). Each receptor type activates different calcium signaling pathways. Exposure of renal myocytes to the P2X-selective agonist α,β–methylene-ATP results in an elevation of $[Ca^{2+}]_i$ through activation of calcium influx pathways (White et al., 2001). The magnitude and time course of the response to α,β–methylene-ATP are markedly different from those evoked by an equimolar concentration of ATP (White et al., 2001). The average peak response elicited by 10 μM α,β–methylene-ATP was ~34% of the response obtained with 10 μM ATP. The response to α,β–methylene-ATP was transient whereas the response to ATP exhibited a sustained elevation of $[Ca^{2+}]_i$. In the current study, DDMS markedly reduced the $[Ca^{2+}]_i$ response to the P2X receptor agonist α,β–methylene-ATP. The inability of 20-HEDE to significantly decrease the renal microvascular smooth muscle cell Ca^{2+} response to α,β-methylene ATP is perplexing. These data are not consistent with the afferent arteriolar diameter

responses to ATP and α,β-methylene ATP or the renal myocte Ca^{2+} response to ATP in the presence of 20-HEDE. Even though there was a slight trend for a decreased α,β-methylene ATP response in cells treated with 20-HEDE, we have no real compelling explanation for the lack of a 20-HEDE effect in this experimental setting. Overall, the majority of afferent arteriolar and renal myocyte experiments would suggest that endogenous 20-HETE may contribute to the P2X receptor-mediated afferent arteriolar vasoconstriction by influencing calcium influx in preglomerular microvascular smooth muscle cells.

P2Y receptor activation stimulates vascular smooth muscle cell $[Ca^{2+}]_i$ in a strikingly different way. The P2Y receptor agonist UTP is purported to interact primarily with G protein-regulated P2Y receptors and is reported to activate phospholipase C (Abbracchio and Burnstock, 1994; Dubyak and El-Moatassim, 1993; Conigrave and Jiang, 1995). Previous studies showed that the mechanisms by which UTP and ATP elevate $[Ca^{2+}]_i$ appear to be substantially different, although they stimulate similar increases in $[Ca^{2+}]_i$ overall (Inscho and Cook, 2002; Inscho et al., 1999a). ATP utilized both calcium influx and calcium mobilization, whereas the response to UTP appears to arise almost exclusively from the release of calcium from intracellular stores. Removal of calcium from the extracellular medium or blockade of calcium influx through L-type calcium channels had no perceptible effect on the magnitude or time course of UTP-mediated increases in $[Ca^{2+}]_i$. This suggests that binding of UTP to its receptor stimulates a signal transduction cascade designed to access stored calcium. On the basis of findings generated by other investigators, UTP-mediated activation of the phospholipase C/inositol trisphosphate/diacylglycerol pathway represents the most likely signal transduction mechanism. In our current study, stimulation of the cells with UTP induced a rapid increase in intracellular Ca^{2+} level, followed by a slow decrease to basal levels, or followed by cytoplasmic Ca^{2+}

oscillation. CYP450 hydroxylase inhibition and 20-HETE antagonism did not alter the initial microvascular smooth muscle cell $[Ca^{2+}]_i$ response to UTP. Whether CYP450 hydroxylase inhibition or 20-HETE antagonism affects the oscillatory response is still not clear. Taken together, these data suggest that 20-HETE is not involved in the P2Y receptor activated calcium signaling pathway, which is consistent with our previous finding that 20-HETE is not involved in the P2Y receptor-mediated preglomerular vasoconstriction.

Previous studies have implicated the arachidionic acid metabolite, 20-HETE, as one paracrine factor mediating tubuloglomerular feedback and autoregulatory related signals to the afferent arterioles. 20-HETE is produced by renal vascular smooth muscle cells and is a potent constrictor that depolarizes vascular smooth muscle cells by blocking calcium-activated potassium channels (Imig et al., 1996; Maier and Roman, 2001). Inhibition of 20-HETE formation blocks the myogenic response of isolated renal arterioles, in vitro (Maier and Roman, 2001). In our experiments, we observed the influence of DDMS and 20-HEDE on the renal microvascular smooth muscle cell Ca^{2+} response to ATP and found that DDMS or 20-HEDE significantly decreased the peak $[Ca^{2+}]_i$ response. Interestingly, P2 receptor activation liberates arachidonic acid from membrane phospholipids (Pfeilschifter J, 1990), which would provide the substrate for CYP450 hydroxylase enzymes and 20-HETE production. Additional experiments demonstrated that renal microvascular smooth muscle cell Ca^{2+} levels increased in response to 20-HETE. The Ca^{2+} response to 20-HETE was rapid in onset and had a time course and magnitude similar to that of P2X receptor activation. 20-HETE has been previously shown to increase intracellular Ca^{2+} levels in smooth muscle cells from canine renal arteries (Ma et al., 1993). Our laboratory has also demonstrated that the afferent arteriolar constrictor response to 20-HETE involves inactivation of potassium channels that results in smooth muscle cell

depolarization and Ca^{2+} channel activation (Imig et al., 1996). Taken together, these results suggest that CYP450 hydroxylase metabolite, 20-HETE, is produced by renal microvascular smooth muscle cells and contributes to the renal preglomerular microvascular response to ATP.

In summary, both DDMS and 20-HEDE attenuated the initial $[Ca^{2+}]_i$ response evoked by the P2 receptor agonist ATP. In addition, DDMS also attenuated the renal myocyte $[Ca^{2+}]_i$ response to the P2X receptor agonist, α,β-methylene-ATP. In contrast, DDMS and 20-HEDE had no effect on $[Ca^{2+}]_i$ to the P2Y receptor agonist UTP. These data demonstrate that the CYP450 metabolite 20-HETE is involved in the renal microvascular smooth muscle cell calcium response to P2X receptor activation.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1 Response of intracellular Ca^{2+} to 10 μM ATP during CYP450 hydroxylase inhibition with DDMS or 20-HETE antagonism with 20-HEDE. A: a typical response of a renal microvascular smooth muscle cell to 10 μM ATP in the presence and absence of DDMS (25 μM). B: a typical response of cell to 10 μM ATP in the presence and absence of 20-HEDE (3 μM). Solid horizontal bar: period of exposure to ATP.

Figure 2 Response of intracellular Ca^{2+} to 100 μM ATP in the presence of DDMS or 20-HEDE. A: a typical response of a microvascular smooth muscle cell to 100 μM ATP in the presence and absence of DDMS (25 μM). B: a typical response of a cell to 100 μM ATP in the presence and absence of 20-HEDE (3 μM). Solid horizontal bar: period of exposure to ATP.

Figure 3 Effect of DDMS and 20-HEDE on the intracellular Ca^{2+} concentration response to 10 and 100 μM ATP. 10 μM (Fig. 3A) or 100 μM (Fig. 3B) ATP was administered in the absence or presence of 25 μM DDMS or 3 μM 20-HEDE. Peak changes in $[Ca^{2+}]_i$ were determined in the first 150 s of agonist exposure. Data present average changes in $[Ca^{2+}]_i$ obtained from 15-21 cells from 3-9 tissue dissociations for each group. *Significant decrease in $[Ca^{2+}]_i$ compared with respective ATP alone.

Figure 4 Response of intracellular Ca^{2+} to α,β-methylene-ATP during CYP450 hydroxylase inhibition with DDMS (4A) or 20-HETE antagonism with 20-HEDE (4B). The typical responses of microvascular smooth muscle cells to 10 μM α,β–methylene-ATP in the

presence and absence of 25 μM DDMS or 3 μM 20-HEDE. Solid horizontal bars: period of exposure to 10 μM α,β–methylene-ATP.

Figure 5 Effect of DDMS and 20-HEDE on the intracellular Ca^{2+} concentration response to α, β-methylene-ATP. 10 μM α,β-methylene-ATP was administered in the presence or absence of 25 μM DDMS. Peak changes in $[Ca^{2+}]_i$ were determined in the first 150 s of agonist exposure. Data present average changes in $[Ca^{2+}]_i$ obtained from a minimum 19 cells from 5 tissue dissociations for each group. Values are mean ±SE. *Significant difference from control α, β-methylene ATP response.

Figure 6 Response of intracellular Ca^{2+} to UTP during CYP450 hydroxylase inhibition with DDMS or 20-HETE antagonism with 20-HEDE. The typical responses of MVSMC to 100 μM UTP in the presence and absence of 25 μM DDMS (6A) or 3 μM 20-HEDE (6B). Solid horizontal bars: period of exposure to 100 μM UTP.

Figure 7 Effect of DDMS and 20-HEDE on the intracellular Ca^{2+} concentration response to UTP. 100 μM UTP was administered in the presence of 25 μM DDMS or 3 μM 20-HEDE. Peak changes in $[Ca^{2+}]_i$ were determined in the first 150 s of agonist exposure. Data present average changes in $[Ca^{2+}]_i$ obtained from a minimum 19 cells from 5 tissue dissociations for each group. Values are mean ±SE.

Figure 8 Effect of 20-HETE on the intracellular calcium concentration in renal microvascular smooth muscle cells. Characteristic response to 10 μM 20-HETE is shown.

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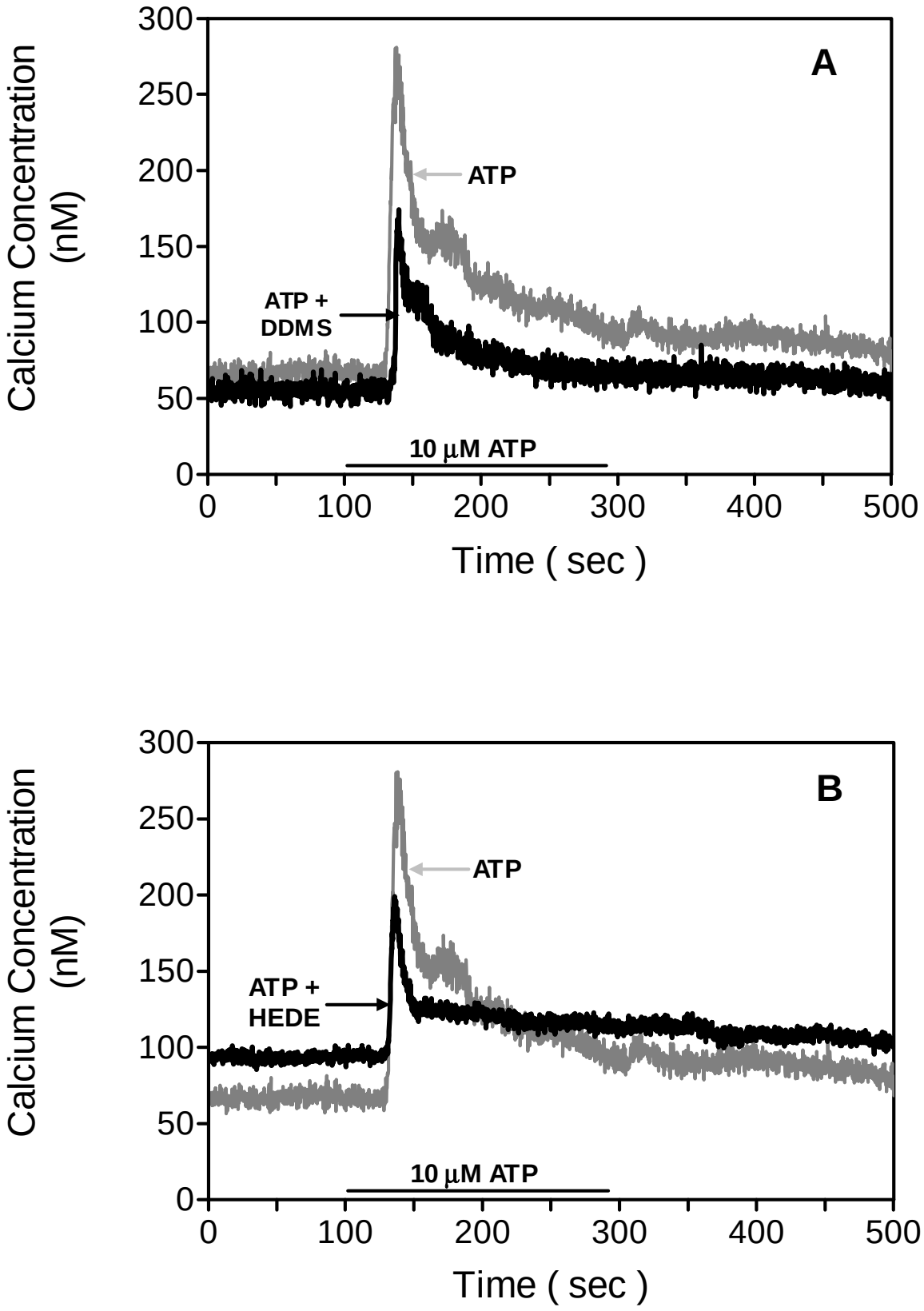

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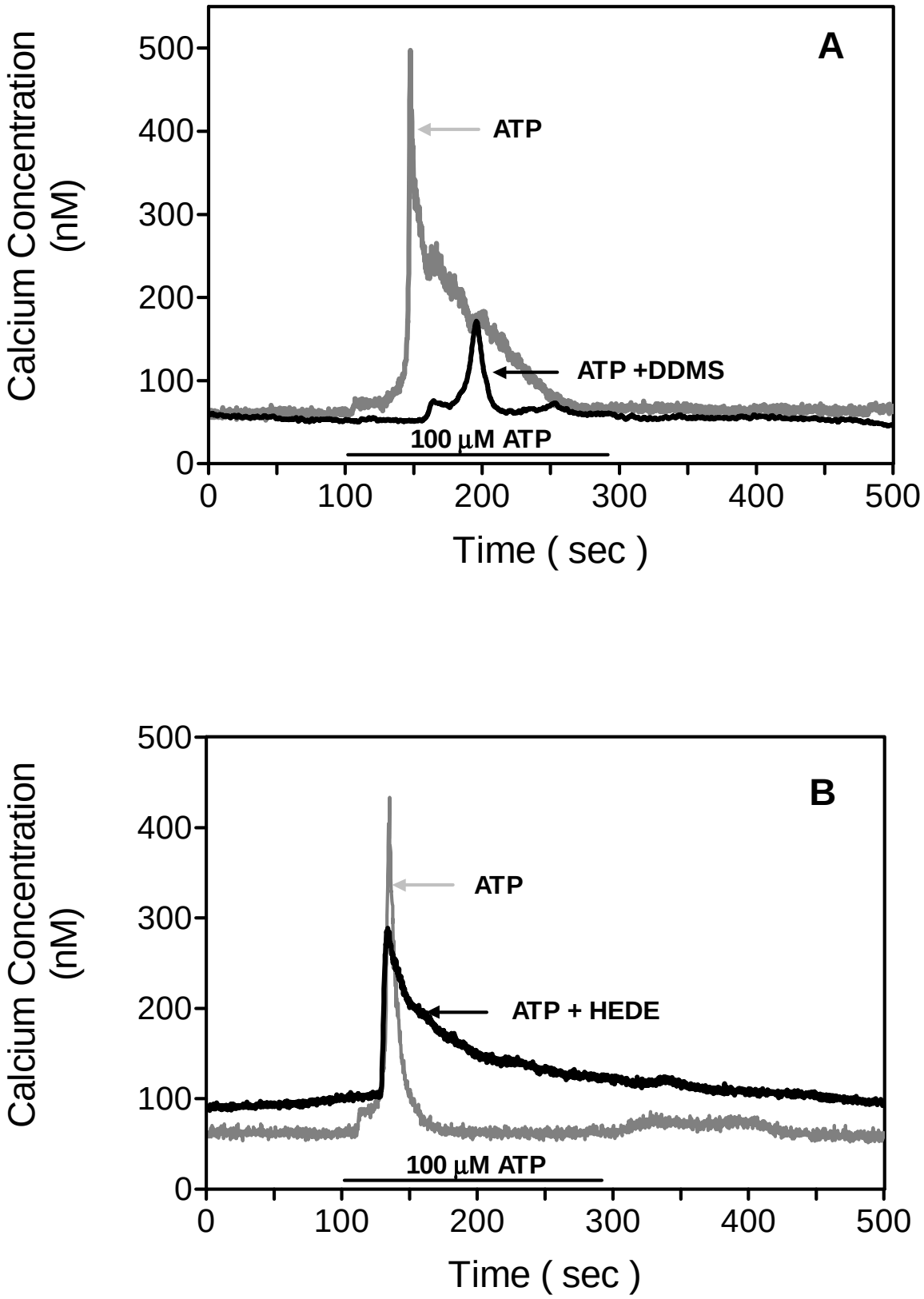

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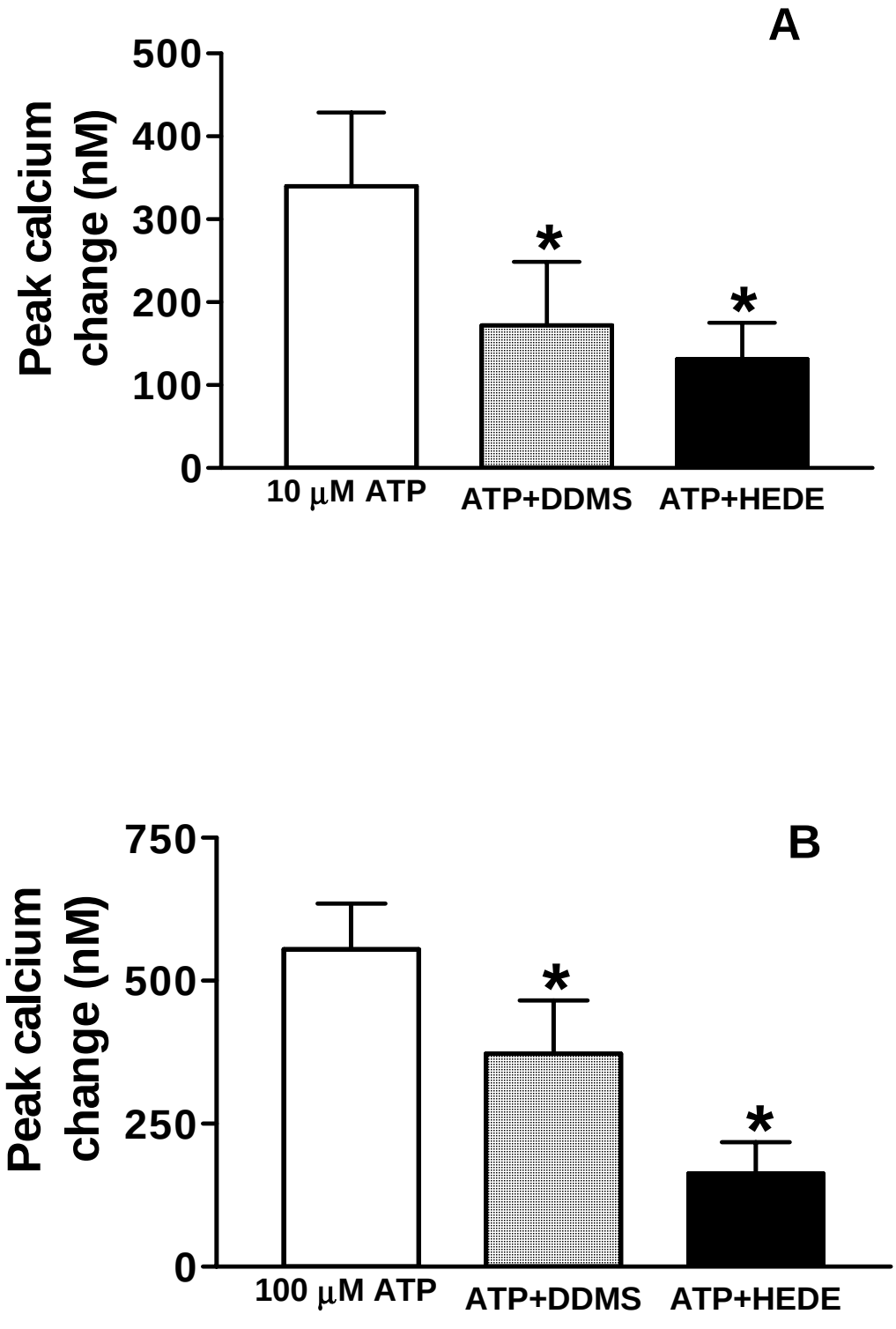

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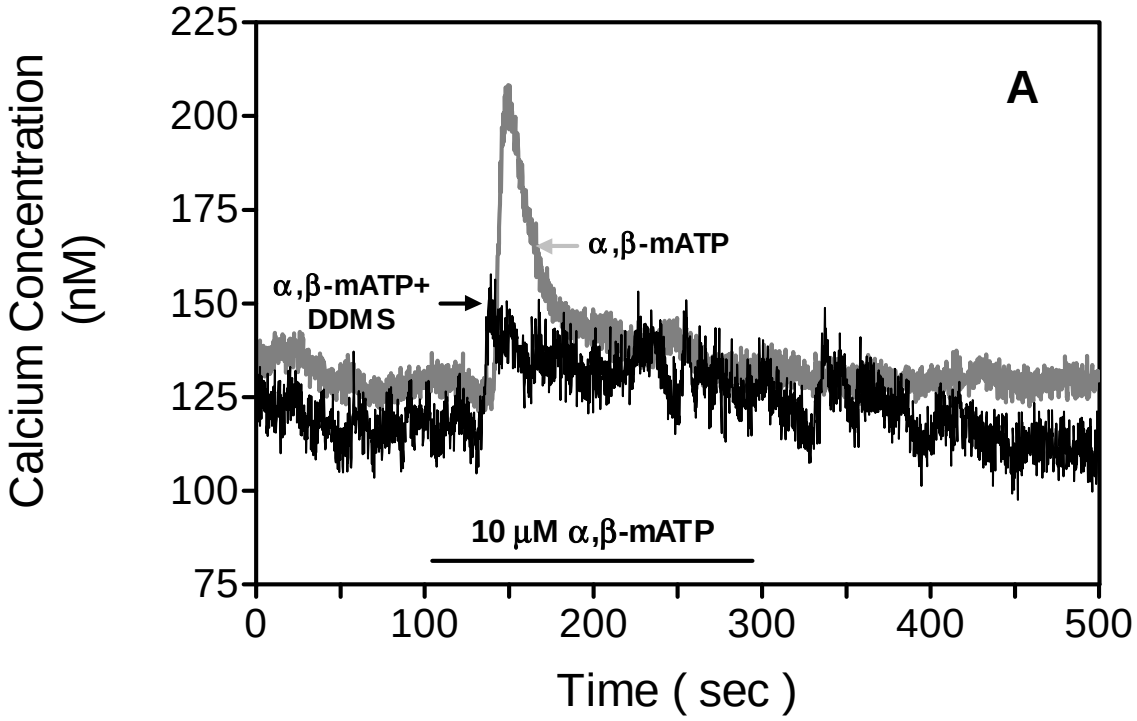

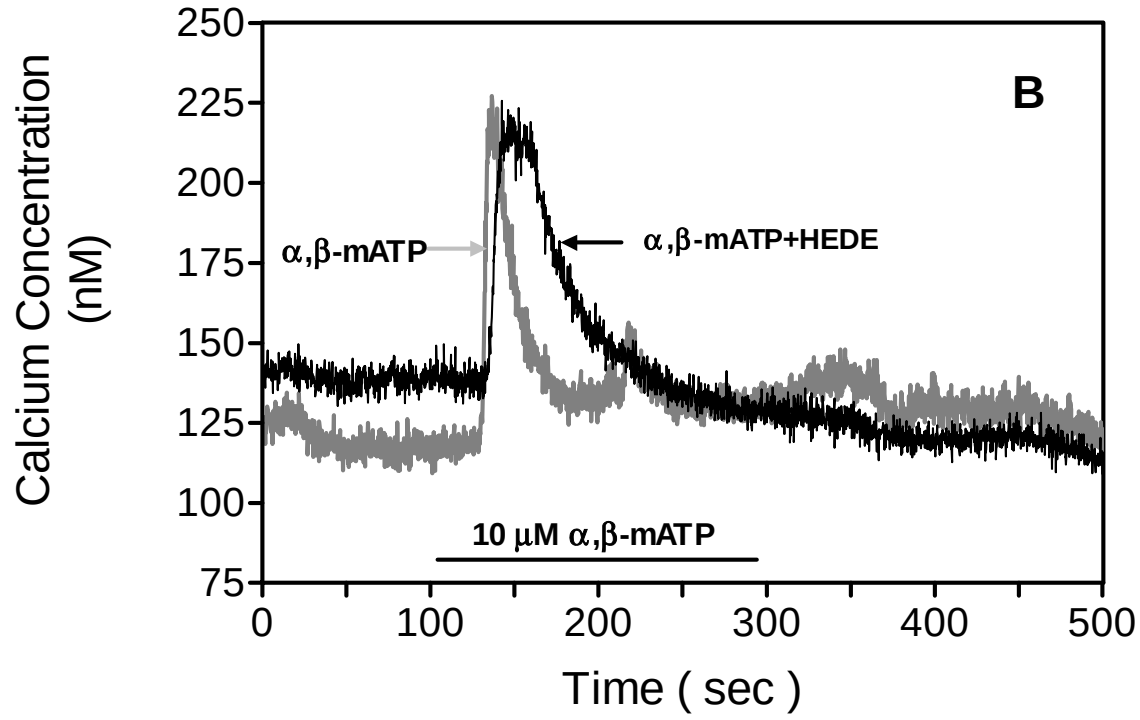

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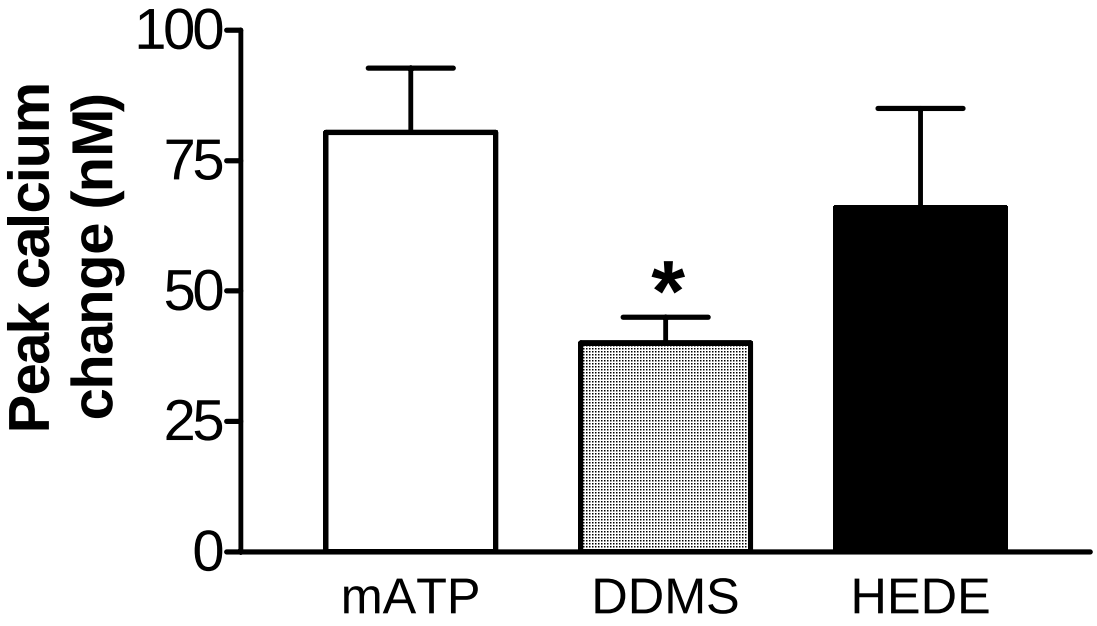

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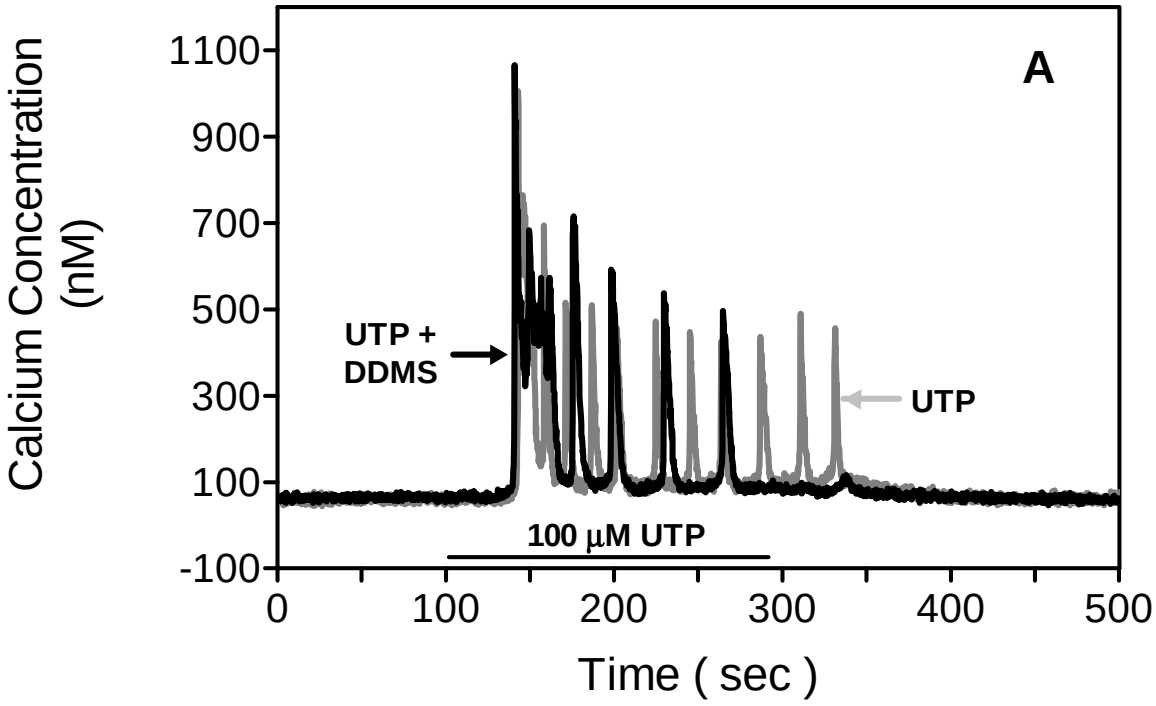

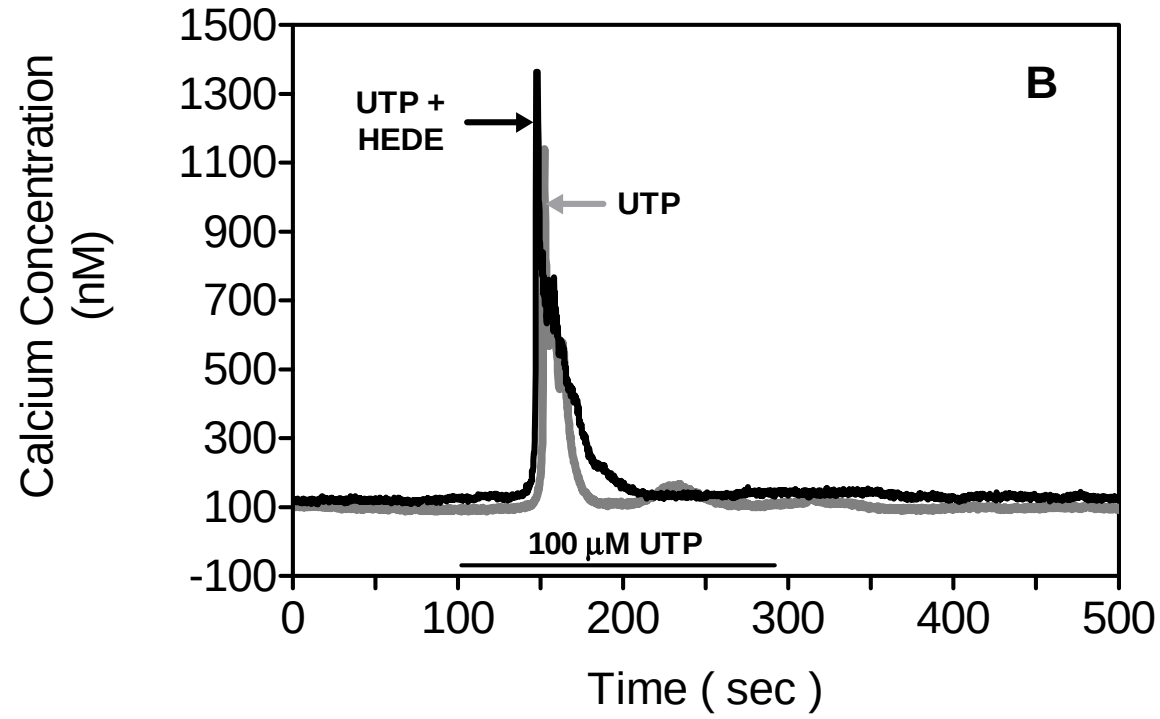

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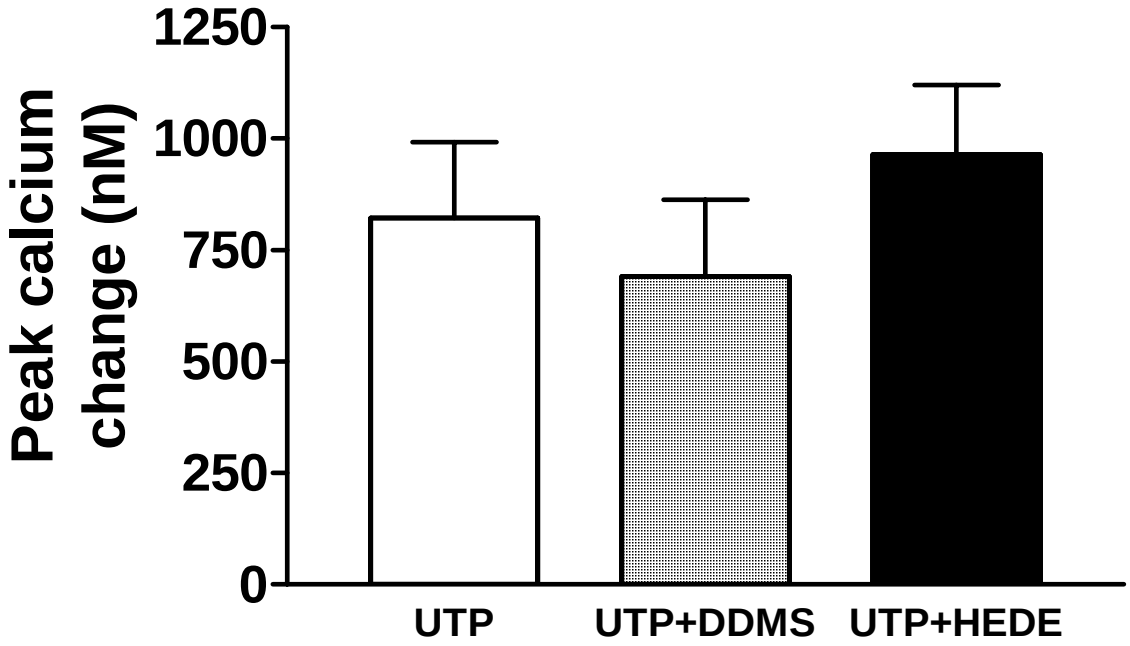

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