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Z T Madhun, … , U Hopfer, J G Douglas

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Research Article

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An Epoxygenase Metabolite of Arachidonic Acid Mediates Angiotensin II–induced Rises in Cytosolic Calcium in Rabbit Proximal Tubule Epithelial Cells

Zuhayr T. Madhun, David A. Goldthwait, Deborah McKay, Ulrich Hopfer, and Janice G. Douglas
Division of Endocrinology and Hypertension, Department of Medicine, University Hospitals of Cleveland, and Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Abstract

Previous studies from this and other laboratories have shown that angiotensin II (AII) induces $[Ca^{2+}]_i$ transients in proximal tubular epithelium independent of phospholipase C. AII also stimulates formation of 5,6-epoxyeicosatrienoic acid (5,6-EET) from arachidonic acid by a cytochrome P450 epoxygenase and decreases Na^+ transport in the same concentration range. Because 5,6-EET mimics AII with regard to Na^+ transport, its effects on calcium mobilization were evaluated. $[Ca^{2+}]_i$ was measured by video microscopy with the fluorescent indicator fura-2 employing cultured rabbit proximal tubule. AII-induced $[Ca^{2+}]_i$ transients were enhanced by arachidonic acid and attenuated by ketoconazole, an inhibitor of cytochrome P450 epoxygenases. Arachidonic acid also elicited a $[Ca^{2+}]_i$ transient that was attenuated by ketoconazole. 5,6-EET augmented $[Ca^{2+}]_i$ similar to that seen with AII, but was unaffected by ketoconazole. By contrast, the other regioisomers (8,9-, 11,12-, and 14,15-EET) were much less potent. $[Ca^{2+}]_i$ transients resulted from influx through verapamil- and nifedipine-sensitive channels. These results suggest a novel mechanism for AII-induced Ca mobilization in proximal tubule involving cytochrome P450–dependent arachidonic acid metabolism and Ca influx through voltage-sensitive channels. (*J. Clin. Invest.* 1991. 88:456–461.) Key words: eicosanoids • voltage-sensitive calcium channels • fura-2 • ketoconazole • cytochrome P450

Introduction

A number of studies have been concerned with the signaling mechanisms coupled to angiotensin II (AII)[1] receptors and demonstrated several different transduction pathways, depending on the type of cell, including phospholipase C (PLC), phospholipase A_2 (PLA_2), adenylate cyclase, and direct regulation of ion channels (1–9). PLC is the predominant pathway in the majority of target tissues, e.g., glomerular mesangium (6), vascular smooth muscle (7, 8), and adrenal glomerulosa (9). Activation of PLC results in liberation of diacylglycerol and inositol-tris-phosphate (IP_3) from phosphatidyl-inositol-4,5-bis-phosphate; in turn, IP_3 stimulates the release of Ca from intracellular stores into the cytosol, resulting in a transient increase in cytosolic Ca levels ($[Ca^{2+}]_i$). AII in the concentration range of 10^{-8} to 10^{-6} M also increased $[Ca^{2+}]_i$ in proximal tubule cells from rabbit kidney, but in these cells the AII-induced Ca transients could be disassociated from PLC on the basis of two observations (3): First, AII-induced increases in $[Ca^{2+}]_i$ were dependent on extracellular Ca; and second, AII failed to stimulate hydrolysis of phosphatidyl-inositol-4,5-bis-phosphate. Therefore, other signaling mechanisms coupling AII to elevation of $[Ca^{2+}]_i$ need to be considered.

We recently demonstrated that AII stimulates the production of 5,6-epoxyeicosatrienoic acid (5,6-EET) in proximal tubule cells (10), suggesting that 5,6-EET may play a role in the signal transduction of AII regulation. 5,6-EET is a metabolite of arachidonic acid which is produced by a cytochrome P450 epoxygenase in an NADPH-dependent manner. This cytochrome P450 epoxide formation represents a third major pathway for metabolism of arachidonic acid, besides the reactions mediated by cyclooxygenase and lipoxygenase (11). It may be the major one in the proximal tubule as this area is deficient in cyclooxygenase and lipoxygenase (12–14). Interestingly, 5,6-EET increased $[Ca^{2+}]_i$ in pituitary and parotid cells (15, 16) and was implicated to mediate secretory responses (17–21). The mechanism whereby 5,6-EET increases $[Ca^{2+}]_i$ has not been investigated; other eicosanoids, such as $PGF_{2\alpha}$, PGE_2, 5-hydroxyeicosatetraenoic acid (5-HETE), and leukotriene B_4, are known to induce rises in $[Ca^{2+}]_i$ primarily from intracellular stores through IP_3-dependent mechanisms and, to a lesser extent, through influx of extracellular Ca (22–26).

The present study was designed to test the hypothesis that AII-induced increases in $[Ca^{2+}]_i$ in the proximal tubule arise secondary to release of arachidonic acid and production of 5,6-EET. Tissue cultures of rabbit proximal tubule cells provided a convenient experimental system for evaluation of this hypothesis. The results suggest that indeed a novel signaling mechanism involving cytochrome P450 metabolism of arachidonic acid is functional in cells from the proximal tubule.

Methods

Cell isolation. Renal proximal tubule cells were isolated from male New Zealand White rabbits, (2 kg body wt) as previously described (3, 27). Briefly, the method involves homogenization of the renal cortex and separation of fully dissociated cells on a discontinuous 30–60% Percoll gradient. Cells with a density around 1.026 g/ml were removed and cultured on Costar tissue culture flasks according to Chung et al.

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Address reprint requests to Janice G. Douglas, M.D., Dept. of Medicine, Division of Endocrinology and Hypertension, Case Western Reserve University, School of Medicine, Room W165C, 2040 Adelbert Road, Cleveland, OH 44106.

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1. *Abbreviations used in this paper:* AII, angiotensin II; 5,6-EET, 5,6-epoxyeicosatrienoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; IP_3, inositol-tris-phosphate; PLA_2, phospholipase A_2; PLC, phospholipase C.

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(28). The standard growth medium was a 50:50 mixture of Dulbecco's modified Eagle's (DMEM) and Ham's F12 media supplemented with 15 mM Hepes buffer, pH 7.35, 1.2 mg/ml sodium bicarbonate, 192 IU/ml penicillin, 200 μg/ml streptomycin, 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 5×10^{-8} M hydrocortisone, and 5% FBS. Cells from the 1.026 g/ml Percoll fraction had previously been shown to be derived mainly from the proximal tubule (3, 27, 29, 30). Cells were passaged after 2 wk, when they usually were in a subconfluent state, by dissociation with trypsin and EDTA and replating on No. 1 glass cover slips. They were used for experiments when isolated colonies were discernible. The media was changed at least 12–24 h before any experiment.

Fura-2 loading. First-passage cells on cover slips were washed twice with BSS and once more with BSS plus 10 mM D-glucose and 0.1% albumin. BSS contained (in millimolar) 120 NaCl, 5 KCl, 1.5 $MgCl_2$, 1 $CaCl_2$, and 25 mM Hepes adjusted to pH 7.40 with NaOH. Cells were warmed to 37° for 10 min and then incubated with 1.0 μM fura-2-acetoxymethyl ester (fura-2-AM) for 20 min to allow entrapment and hydrolysis of the fura-2 ester (fura-2-AM). Finally, extracellular fura-2 and fura-2-AM were removed by washing with BSS and cells were incubated for another 10 min at 37°. Cells were washed once more with BSS before mounting the cover slip in the perfusion chamber on the stage of an upright light microscope.

$[Ca^{2+}]_i$ determinations. $[Ca^{2+}]$ measurements depend on the different excitation spectra of free fura-2 and its Ca-complex (31). Cells were illuminated alternately with 340 and 380 nm light through the epifluorescence optics of the microscope. The excitation light was provided by a 75W xenon lamp in conjunction with metallic interference filters (Omega, Brattleboro, VT). The light emitted from fura-2 was passed through a 510-nm cut-off filter, detected with a model C1966-20H microchannel plate intensifier (Hamamatsu Corp., Middlesex, NJ) in series with a saticon camera (Photonics Microscopy Inc., Oakbrook, IL), and quantitated with a model MVP-AT frame grabber board (Matrox, Quebec, Canada) mounted internally in a host 286 computer. Normalization of the fluorescent signal was achieved by the ratio method (31) implemented with the Image-1/Fl software (Universal Imaging, Media, PA). In this method a ratio is calculated based on the emission intensity after excitation at 340 and 380 nm. This ratio is a function of the $[Ca^{2+}]$ and independent of fura-2 concentration or amount in any particular microscope field, provided the fluorescence signal is within the detection limits of the system and appropriate background setting are applied. Usually 16 video frames of fluorescence images were averaged (i.e., ~ 0.5 s) for each excitation wavelength, then the ratio image calculated, and finally stored on a hard disk. Successive ratio images could be stored at a rate of ~ 1 every 5 s.

Conversion of the fura-2 ratio to $[Ca^{2+}]_i$ was accomplished by a calibration curve which was constructed with free fura-2 in a solution consisting of (in millimolar) 0.15 KCl, 10 MOPS pH 7.4, 1 EGTA, and appropriate amounts of $CaCl_2$ to yield final $[Ca^{2+}]$ between 50 and 1,000 nM. The total amounts of $CaCl_2$ necessary to give the desired free Ca^{2+} were calculated with the program by Fabiato (32). Fura-2 concentrations for the calibration curve were adjusted so that the emitted light intensity was appropriate for the particular analogue settings used for experiments with the renal cells. In preliminary experiments, it was shown for our settings that the ratio at a given free $[Ca^{2+}]$ was independent of the fura-2 concentration. Usually, 10 μM fura-2 was employed for the calibration.

Chamber and perfusion. To study hormonally regulated changes in $[Ca^{2+}]$ the cover slips with cultured cells were mounted upside-down on a chamber which could be constantly perfused with BSS by gravity feed. The chamber volume was 0.13 ml and the perfusion rate was usually ~ 1.4 ml per min. Hormones and putative messengers were introduced by switching to a different BSS containing the experimental agent. With the dead space of the tubing, the time for new solutions to reach the cells in the chamber was ~ 1.5 min. Access of regulatory agents from the medium to cellular receptors was documented by parathyroid hormone (PTH)-induced $[Ca^{2+}]_i$ transients. Experiments were carried out at 22°.

Phosphoinositide labeling and extraction. Subconfluent first passaged epithelial cells on 9-cm^2 culture dishes were cultured for 2–3 d in serum-free inositol-free DME/Ham's F12 (50:50 mixture) medium, 4 μCi/ml of *myo*-[2-^{3}H(*N*)]-inositol (16.5 Ci/mmol, New England Nuclear, Boston, MA) and other media additives as described earlier. Before stimulation with agonists, monolayers were incubated with BSS containing 10 mM LiCl for 10 min at 37°C. Incubations were performed in triplicate and terminated by the addition of 600 μl methanol and 0.04 M HCl. The monolayers were scraped, extracted with chloroform/phytic acid, the upper phase applied to Dowex columns and eluted with 30–500 mM HCl (33).

Statistical analysis. Results are expressed as mean±SEM. Statistical significance was calculated by Student's *t* test for unpaired data, assuming a significance level of $P < 0.05$. All cells in a microscope view were counted, whether or not they responded, and for each experimental condition at least three cover slips were analyzed. The data generally refer to between 10 and 35 cells for each condition unless otherwise stated.

Materials. Hepes, purified bovine PTH (residues 1–84), bradykinin, nifedipine, and ketoconazole were obtained from Sigma Chemical Co. (St. Louis, MO). Chromatographically pure arachidonic acid was purchased from Nu-Check-Prep (Elysian, MN). 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET, and verapamil were bought from Calbiochem-Behring Corp. (La Jolla, CA). The different epoxides were shipped as methyl esters which were hydrolyzed to the free acids by 10 N NaOH in 90% methanol. AII was purchased from United States Biochemical Corp. (Cleveland, OH). Percoll is a product of Pharmacia Fine Chemicals (Piscataway, NJ). DMEM and Ham's F12 medium were obtained from Gibco (Grand Island, NY). Costar tissue culture flasks were from Costar (Cambridge, MA), No. 1 22 × 22 mm glass cover slip from Fisher Scientific Co. (Pittsburgh, PA), and fura-2 and fura-2-AM from Molecular Probes, Inc. (Eugene, OR).

Results

Baseline Ca concentration. Mean basal $[Ca^{2+}]_i$ of rabbit proximal tubule cells was 126±4 nM (347 cells from 46 fields). Pretreatment of cells with 3 μM arachidonic acid for 3 h or with 100 μM ketoconazole overnight did not significantly change this value (129±6 and 122±5 nM, respectively). These basal Ca levels remained unchanged for at least 30 min perfusion in the microscope chamber.

Signal transduction of AII. AII has been shown to transiently increase $[Ca^{2+}]_i$ in intact proximal tubules, primary cultures, and dispersed cell suspensions (1, 3, 29, 30, 34, 35). The minimal effective AII concentration in these preparations was ~ 10 nM. AII elicited a similar increase in $[Ca^{2+}]_i$ in cells attached to glass cover slips. Fig. 1 *A* illustrates a typical response during perfusion of epithelial cells with AII. After a lag time of ~ 90 s due to the dead space of the perfusion system, $[Ca^{2+}]_i$ rose rapidly and then decayed to baseline over the next 10–15 min. Fig. 2 shows the dose-response curve for AII in terms of peak $[Ca^{2+}]_i$ above basal levels. The minimal AII concentration for which increases in $[Ca^{2+}]_i$ could be detected was ~ 0.1 nM suggesting that either the microscope setup for detecting fluorescence changes was more sensitive than those of previous studies (1, 3, 29, 30, 34, 35) or that the attached, cultured cells are intrinsically more responsive to AII.

Because previous studies (1, 3) had implicated arachidonic acid metabolites in the signaling transduction for AII, the effect of arachidonic acid supplementation of the culture medium was tested. Cells were incubated for 3 h in the usual culture medium plus 3 μM arachidonic acid and then the changes in $[Ca^{2+}]_i$ in response to AII measured. This protocol resulted in a significantly greater response over the entire range of 10^{-9} to

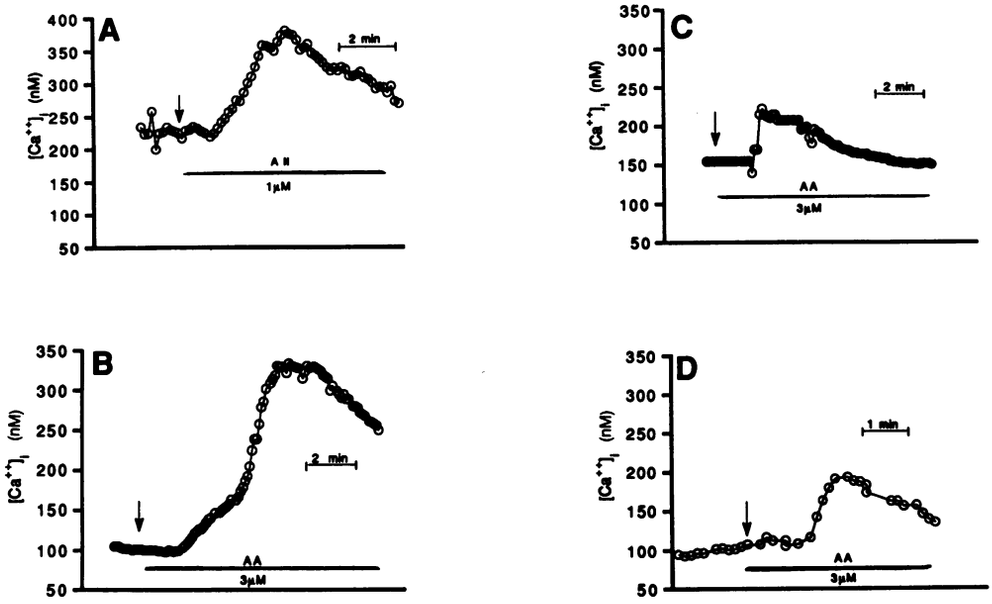

Figure 1. Typical time courses of angiotensin II (*AII*) and arachidonate (*AA*) effects on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in fura-2-loaded cells by video microscopy (see Methods). (*A*) Acute effect of AII; (*B*) acute effect of AA perfused with BSS, which includes 1 mM Ca; (*C*) cell preincubated with 100 μM ketoconazole for 3 h and perfusion with the same solution as in *B*; (*D*) cell treated as in *B*, but without Ca^{2+} in the perfusion solution.

10^{-6} M AII (left shift of dose-response curve) (Fig. 2, *upper trace*). For example, 1 μM AII increased $[Ca^{2+}]_i$ in cells grown in basic medium by 230 nM, whereas the same concentration of AII induced a rise in $[Ca^{2+}]_i$ by 322 nM in arachidonic acid–supplemented cells ($P < 0.05$).

Involvement of an arachidonic acid metabolite in the signaling pathway is also suggested by the results with ketoconazole, an inhibitor of cytochrome P450 epoxygenases. Preincubation of cells in media supplemented with 100 μM ketoconazole greatly inhibited the increases of $[Ca^{2+}]_i$ in response to AII. This inhibition was present regardless whether the medium contained ketoconazole alone or ketoconazole plus arachidonic acid (Fig. 2, *lower trace*). By contrast, ketoconazole failed to abolish bradykin-induced $[Ca^{2+}]_i$ transients, an effect mediated by activation of phosphoinositide specific phospholipase C.

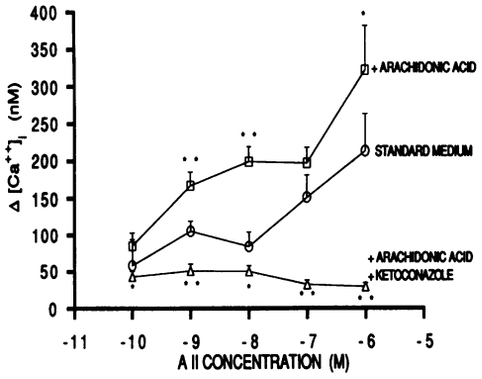

Figure 2. Dose-response relationship of angiotensin II (*AII*) and $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured first in the absence and then in presence of AII in the perfusion medium at the indicated concentrations. Peak $[Ca^{2+}]_i$ minus basal $[Ca^{2+}]_i$ was averaged for cells incubated in standard medium (*circles*); cells incubated in standard medium plus 3 μM arachidonate for 3 h (*squares*); cells incubated in standard medium plus 100 μM ketoconazole and 3 μM arachidonate for 3 h. Data are given as mean±SEM. $^*P < 0.05$, $^{**}P < 0.005$, relative to cells grown in standard medium alone at the same AII concentration.

Under basal conditions, bradykinin induced $[Ca^{2+}]_i$ of 189±28 nM (n = 14) in contrast to 195±21 nM (n = 18, NS) after ketoconazole pretreatment.

Interestingly, acute exposure of cells to arachidonic acid brought about an elevation of $[Ca^{2+}]_i$ (Fig. 1 *B*). Peak changes in $[Ca^{2+}]_i$ for 3 μM arachidonic acid were 371±55 nM (n = 35). This response was actually greater than the peak response to AII and was also largely inhibited by pretreatment of cells with ketoconazole (Fig. 1 *C*). For example, after ketoconazole pretreatment the peak Ca changes elicited by arachidonic acid decreased to 79±7 nM (n = 48). These results suggested that arachidonic acid and one or more of its epoxygenase metabolites were involved in modulating $[Ca^{2+}]_i$.

Regioisomers of EET. To evaluate the involvement of 5,6-EET in the signaling pathway for $[Ca^{2+}]_i$, the effect of acute addition of 5,6-EET on $[Ca]_i$ was measured. Fig. 3 illustrates the dose-dependent increases of $[Ca^{2+}]_i$ that were observed in the range of 10 nM to 1 μM 5,6-EET. At 1 μM, the peak increment of $[Ca^{2+}]_i$ was 100±14 nM. Concentrations of 5,6-EET higher than 1 μM could not be used because of cellular toxicity. Of interest is the fact that the percentage of cells responding increased from 40 to 100% over the dose range employed herein. Pretreatment of the cells with 100 μM ketoconazole to abolish endogenous EET production potentiated the response of acute additions of 5,6-EET (data not shown). In ketoconazole pretreated cells, 1 μM 5,6-EET produced a $[Ca^{2+}]_i$ increase of 168±14 nM, which was significantly ($P < 0.005$) higher than the 100 nM for cells without the inhibitor (see above).

Specificity of the epoxide effect was evaluated by comparing the efficacy of different regioisomers. They were tested at 1 μM with the following results (Table I): 8,9- and 14,15-EET were about one fourth as potent as 5,6-EET, whereas the 11,12-isomer did not elicit any response in the proximal tubule cells. Thus, 5,6-EET was by far the most potent of the four different regioisomers tested.

Source of increases in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ can be elevated by influx of Ca^{2+} either from intracellular stores or from the extracellular medium. To evaluate the contribution of Ca from the extracellular medium, $[Ca^{2+}]_i$ levels were measured in response

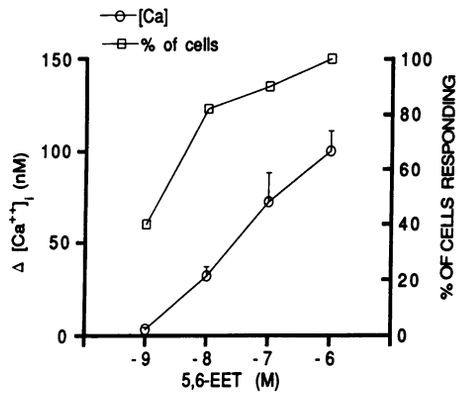

Figure 3. Effects of 5,6-epoxyeicosatrienoic acid (5,6-EET) on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured first in the absence and then in presence of 5,6-EET in the perfusion medium at the indicated concentration (for method see Fig. 1). 5,6-EET was added to the perfusion solution from a ≥ 10 mM stock in 90% methanol–10 N NaOH. The results were analyzed in terms of number of cells responding and average (peak minus basal) $[Ca^{2+}]_i$. Data are given as mean±SEM of 24–36 cells. Note that the number of cells responding and the average peak $[Ca^{2+}]_i$ increased with higher 5,6-EET concentrations. Controls with identical amounts of 90% methanol–10 N NaOH solution, but without epoxide, did not increase $[Ca^{2+}]_i$.

Table II. Effects of Arachidonic Acid and Bradykinin on Phosphoinositide-specific Phospholipase C Presented as ^3H-Inositol Phosphate Accumulation

	Basal	Arachidonic acid (0.1 μM)	Arachidonic acid (1 μM)	Bradykinin (1 μM)
30 s				
IP	1255±42 (6)	ND	1093±96 (3)	5601±24* (3)
IP_2	908±92 (6)	ND	665±10* (3)	1427±50* (3)
IP_3	722±46 (6)	ND	518±3 (3)	1977±42* (3)
15 min				
IP	1753±165 (12)	930±70 (3)	1739±459 (6)	7251±1052* (3)
IP_2	1031±138 (11)	484±37 (3)	737±166 (6)	2168±78* (3)
IP_3	617±51 (12)	515±65 (3)	526±89 (6)	1316±13* (2)

Each value is the mean±SE. No. determinations is in parentheses.
* Significantly different from basal.

to AII and putative intermediates when either no extracellular Ca was present or 50 μM $LaCl_3$ in the medium blocked influx across the plasma membrane. Under both conditions, even maximal doses of 1 μM 5,6-EET did not increase $[Ca^{2+}]_i$, indicating that the 5,6-EET effect depends on influx of extracellular Ca. Similarly, the effects of 3 μM arachidonic acid were attenuated, however, only by about two-thirds: $[Ca^{2+}]_i$ increments of 371 nM in the presence of extracellular Ca dropped to 128±36 nM ($n = 28$, $P < 0.005$) in Ca-free buffer (Fig. 1 *D*) and to 149±22 nM ($n = 19$, $P < 0.01$) in the presence of 50 μM $LaCl_3$ (data not shown).

It is possible that the residual increase of $[Ca^{2+}]_i$ in response to arachidonic acid may have been due to stimulation of phosphoinositide-specific phospholipase C and release of Ca^{2+} from intracellular stores. To evaluate this possibility, bradykinin, a known stimulator of phospholipase C, was compared to arachidonic acid, employing *myo*-inositol labeled epithelial cells. We observed no stimulation of IP, IP_2, or IP_3 with 0.1 or 1 μM arachidonic acid despite brisk stimulation of all three isomers with bradykinin (Table II).

Because voltage-sensitive Ca channels provide one of the influx pathways in other tissues, their participation was probed by measuring $[Ca^{2+}]_i$ in the presence of the blockers verapamil (50 μM) and nifedipine (10 μM). Both blockers essentially abolished the responses to AII and 5,6-EET (Fig. 4, *B* and *C*). This inhibition was specific for the plasma membrane since the release of Ca from IP_3-sensitive, intracellular stores by bradykinin (1 μM) was not changed by the two blockers (Fig. 4, *B* and *C*).

Table I. Effects of EET Regioisomers on $[Ca^{2+}]_i$

Isomer	Peak $\Delta[Ca^{2+}]_i$	No. cells
	nM	
5,6-EET	100±10	36
8,9-EET	23±6	20
11,12-EET	0	36
14,15-EET	27±5	30

All EET isomers were tested at 1 μM.

Discussion

The present study confirms that 0.1 nM to 1 μM AII elicits significant rises in cytosolic Ca in proximal tubule cells. This rise is mediated by arachidonic acid and its metabolites, in particular, 5,6-EET. Three major observations that lead to this conclusion are: First, the epoxygenase inhibitor ketoconazole blocked AII-induced increments in $[Ca^{2+}]_i$. Second, the addition of 5,6-EET by itself caused increases in $[Ca^{2+}]_i$ similar to those achieved with AII. Third, acute additions of arachidonic acid also resulted in increases in $[Ca^{2+}]_i$ which could be partially blocked by ketoconazole. The conclusion is also supported by data from other studies in our laboratory indicating that AII does not activate phosphoinositide-specific PLC (3) and that AII stimulates the conversion of arachidonic acid to 5,6-EET measured by chemical methods (29, 30). The involvement of arachidonic acid is also suggested by the enhanced AII response (left shift of dose-response curve) of cells enriched with arachidonic acid by preincubation. Together these results suggest that AII activates either PLA_2 or phospholipase D pathways that lead to the release of arachidonic acid. The arachidonic acid is then metabolized by a specific cytochrome P450 epoxygenase to an epoxide which acts as third messenger in sequence resulting in increased Ca influx. The most effective epoxide was 5,6-EET.

AII has a biphasic effect on proximal tubular transport wherein pM concentrations stimulate Na absorption, whereas

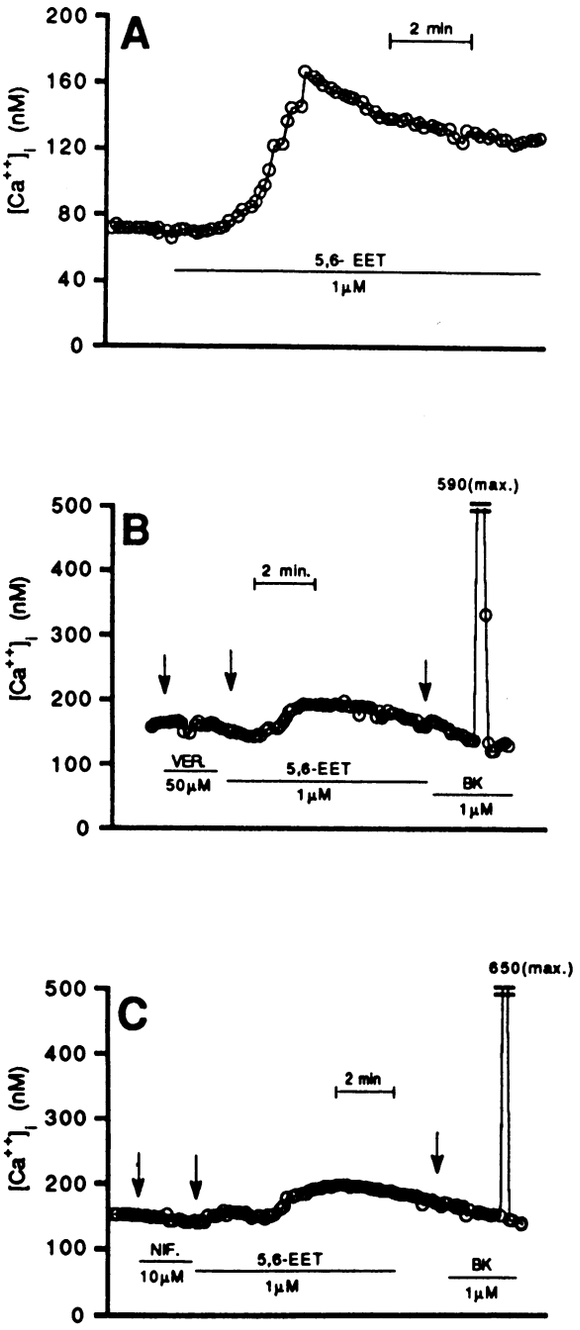

Figure 4. Attenuation of 5,6-EET-induced $[Ca^{2+}]_i$ increases by the Ca channel blockers nifedipine (*NIF*) and verapamil (*VER*). $[Ca^{2+}]_i$ was measured as in Fig. 1. Typical cell responses to 5,6-EET are shown (*A*) and in the presence of 50 μM verapamil (*B*) or 10 μM nifedipine (*C*). Horizontal bars indicate additions to the perfusion medium. Cells were perfused with the blockers for 2 min as indicated by the horizontal bars and then with 1 μM 5,6-EET for an additional 6 min. Subsequently, cells were challenged with 1 μM bradykinin (*BK*) for release of Ca from intracellular IP_3-sensitive stores.

nM concentrations inhibit Na absorption (36, 37). There is general agreement that AII-induced inhibition in cAMP formation stimulates luminal Na/H exchange to facilitate Na and bicarbonate reabsorption (1, 5, 29, 30), however, much less is known about the signal transduction mechanism that mediates AII-induced natriuresis. There are several reasons to believe that cytochrome P450–dependent arachidonic acid metabolism and consequent effects on Ca mobilization mediate the inhibitory effect of AII (5, 10, 29, 30). Romero et al. (10, 29, 30, 38) have recently shown in primary cultures of proximal tubule cells that 5,6-EET mimics the inhibitory effect of AII on Na fluxes from the lumen to the basal compartment. Furthermore, it is likely that these transport effects are mediated by elevation of intracellular $[Ca^{2+}]_i$ because ketoconazole inhibits AII-induced $[Ca^{2+}]_i$ transients and AII-induced natriuresis (29). Sakhrani et al. (39) demonstrated that when $[Ca^{2+}]_i$ of proximal tubular cells is increased, either by calcium loading or Ca ionophore, amiloride-sensitive Na uptake is inhibited by 25–40%. By contrast, 8-(*N,N*-diethylamonia)-octyl-3,4,5-trimethoxybenzoate (TMB-8) that prevents intracellular Ca mobilization blocks the inhibitory effect of AII on sodium and bicarbonate transport (40). These observations support the hypothesis that arachidonic acid and cytochrome P450–dependent epoxygenase metabolites mediate the inhibitory effect of AII on sodium and bicarbonate transport through effects on Ca mobilization. However, the exact mechanism of inhibition by $[Ca^{2+}]_i$ has not been worked out for the proximal tubule and one report actually suggests that the Na^+/H^+ exchanger of brush border membranes is not inhibited, but activated by calcium-dependent protein kinases (41).

The observations of this study and those reported earlier (10, 29, 30, 38) indicate that 5,6-EET fulfills the criteria for a second messenger (42): (*a*) 5,6-EET can be formed by the target tissue, the proximal tubule cells, in response to interaction of AII with its receptor; (*b*) 5,6-EET specifically elicits an increase in cytosolic $[Ca^{2+}]_i$; (*c*) AII-induced $[Ca^{2+}]_i$ transients can be inhibited by compounds that prevent the formation of 5,6-EET (i.e., ketoconazole); (*d*) 5,6-EET mimics the transport effects of AII. Thus, this novel intracellular epoxide metabolite of arachidonic acid is probably an important modulator of $[Ca^{2+}]_i$ in the proximal tubular epithelium which mediates the effect of AII in the concentration range of nanomolar to micromolar.

Formation of 5,6-EET also accounts for the bulk of the effects seen with acute addition of free arachidonic acid. However, with arachidonic acid a substantial residual increase of $[Ca^{2+}]_i$ remained even after ketoconazole treatment or after blocking Ca channels that mediate Ca influx in response to 5,6-EET. This unaccounted portion may be due to arachidonic acid directly activating Ca channels analogous to the activation of potassium channels in smooth muscle cells (43). Recent studies from this laboratory demonstrated that arachidonic acid and other unsaturated fatty acids as well as the inhibitor of eicosanoid biosynthesis, eicosatetraynoic acid mobilize Ca from intracellular stores independent of phosphoinositide-specific phospholipase C (44).

These results indicate that the bulk of the cytosolic Ca increases in response to AII and its putative intermediates results from Ca influx into the cell through Ca transporters that are inhibitable by Ca^{2+} channel blockers and presumably represent voltage-sensitive Ca channels. This conclusion agrees with a previous study in proximal tubule cells (1, 3) which also pointed to influx of extracellular Ca caused by AII. A similar mechanism appears to be operational in adrenal glomerulosa cells where Ca channel agonists potentiate AII-induced Ca influx during the more sustained phase of increased $[Ca^{2+}]_i$ (9).

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Note added in proof: Recent more sophisticated chromatographic analyses of the AII-induced metabolites of arachidonic acid suggest that 5,6-EET may not be the major product, but another one that has a similar chromatographic behavior on reverse HPLC.

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