Kidney International, Vol. 40 (1991), pp. 1075–1081

Receptor-mediated increase in cytosolic calcium in LLC-PK$_1$ cells by platelet activating factor and thromboxane A$_2$

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Receptor-mediated increase in cytosolic calcium in LLC-PK$_1$ cells by platelet activating factor and thromboxane A$_2$. Several studies indicate an important role of platelet activating factor (PAF) and thromboxane A$_2$ (TXA$_2$) in glomerular pathophysiology. However, the potential role of PAF or TXA$_2$ in renal tubular pathophysiology has received little attention, and the presence of functional receptors for these autacoids in renal tubular epithelium has not been previously studied. We examined the effects of PAF and the TXA$_2$ analogue, ONO11113, on the cytosolic free calcium concentration ([Ca^{2+}]$_i$) in cultured LLC-PK$_1$ cell line using a fluorescent probe, fura-2. In these cells, the addition of PAF or ONO11113 caused a significant increment in [Ca^{2+}]$_i$ in a dose-dependent manner: both agonists (10^{-7} M) increased [Ca^{2+}]$_i$ from 148 ± 16 to 288 ± 39 nM and from 130 ± 8 to 240 ± 18 nM, with the values of EC$_{50}$ for PAF and ONO11113 being 17 ± 4 and 17 ± 2 nM, respectively. These effects were both rapid and transient, returning to baseline in two minutes. The effect of PAF was selectively blocked by PAF receptor antagonist BN50730, but not by TXA$_2$ receptor antagonist L657925. Similarly ONO11113 response was abolished by L657925, but not by BN50730. PAF- or ONO11113-challenged cells did not respond to a second addition of the same agent and showed heterologous desensitization to the other agonist. The initial peaks of [Ca^{2+}]$_i$ as well as the sustained elevations in [Ca^{2+}]$_i$ induced by PAF or ONO11113 were reduced following the chelation of extracellular Ca^{2+} by 10 mM ethylene glycol-bis(β-aminomethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). In the absence of extracellular Ca^{2+} 8-(N,N-dimethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) (which has extensively been used to study the contribution of Ca^{2+} released from intracellular storage sites in the increase in [Ca^{2+}]$_i$), blocked the increment in [Ca^{2+}]$_i$ induced by PAF or ONO11113. These results indicate that LLC-PK$_1$ cells express discrete receptors for PAF and TXA$_2$ that are coupled to an increase in [Ca^{2+}]$_i$ through mobilization of calcium from both intracellular storage sites and extracellular milieu, and suggest the possible importance of PAF and TXA$_2$ in tubular pathophysiology.

A large body of in vitro and in vivo studies have delineated an important role of two endogenously generated lipids, platelet activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) and thromboxane A$_2$ (TXA$_2$) in a variety of inflammatory processes [1–9]. PAF or TXA$_2$, which can be generated by either bone marrow derived cells or resident glomerular cells, have been shown to affect a variety of biological processes of potential importance in the pathophysiology of glomerular disease [1–7, 9, 10]. Not only are some of the biological effects of PAF and TXA$_2$ similar [1, 5, 6, 10, 11], but both in vivo and in vitro studies have demonstrated interactions between these mediators [1, 7–10]; for example, PAF is also capable of stimulating the biosynthesis of TXA$_2$ from mesangial cells or other renal tissue [1, 7, 8, 10]. In vivo studies have also implicated these autacoids in several models of glomerular injury, including adriamycin-induced nephrotic syndrome [6], acute serum sickness [2], and anti-GBM antibody disease [12]. Taken together, these studies indicate an important role of these mediators in glomerular pathophysiology. However, the potential role of PAF or TXA$_2$ in renal tubular pathophysiology has received little attention, and the presence of functional receptors for these autacoids in renal tubular epithelium has not been previously studied.

Based on the potential importance of intracellular calcium as second messenger modulating cellular function of tubules [13–19] and its possible importance in tubular injury [20], as well as the ability of PAF [21–28] or TXA$_2$ [29–32] to increase intracellular cytosolic free calcium ([Ca^{2+}]$_i$) in other tissues, the present studies were undertaken to determine the effects of PAF and a stable TXA$_2$ analogue, ONO 11113, on [Ca^{2+}]$_i$ in the LLC-PK$_1$ cell line. LLC-PK$_1$ cells, a widely studied, established cell line with some transport characteristics of proximal tubule [33], have been shown in several recent studies to be useful for studying cellular calcium metabolism [34, 35]. We also examined whether the effects of PAF or TXA$_2$ were receptor mediated, using selective receptor antagonists for PAF [36] or for TXA$_2$ [11].

Methods

Culture condition

LLC-PK$_1$ cells were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were regularly grown in 75 cm^2 polystyrene tissue culture flasks (Corning Glass Works, Corning, New York, USA) and maintained in a medium composed of Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, New York, USA), 10% heat-inactivated bovine serum (FBS), 20 mM N-2 hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2 mM L-gluthamine, and 2 mM non-essential amino acids. Cultures were maintained in a humidified incubator gassed with 5% CO$_2$-95% air at 37°C, and fed at intervals of 48

Received for publication April 22, 1991
and in revised form July 19, 1991
Accepted for publication July 30, 1991

to 72 hours. Experiments were performed two to three days post-confluency. Medium was changed 12 to 24 hours before experiments. Experiments were performed on passages between 200 and 220.

Intracellular free calcium concentration measurement

Cells were harvested by trypsinization (for 5 to 10 min) with 0.05% trypsin and 0.53 mM EDTA (Gibco). The treatment of the cells with trypsinization for a short term period has been shown not to affect the total cellular Ca^{2+} content in LLC-PK_1 cells [34]. Isolated cells were washed in a modified Gey's buffer (MGB; composition in mM: 145.0 NaCl, 5.0 KCl, 1.0 Na_2HPO_4, 10.0 HEPES, 5.0 glucose, 1.0 $CaCl_2$, 0.5 $MgSO_4$, adjusted pH to 7.40) by centrifugation for five minutes at 200 × g and resuspended in 10 ml MGB. Cells were incubated with 4 μM fura-2-AM diluted in dimethyl sulfoxide (0.4% final DMSO concentration) for 30 minutes, at 37°C. Cells were then washed three times and resuspended in the MGB to a concentration of 1×10^6 cells/ml. Cell viability after loading fura-2 and at the end of the experiments was assessed by trypan blue exclusion, and exceeded 95%.

Intracellular fura-2 fluorescence was monitored using a LS-5 Fluorescence Spectrophotometer (Perkin-Elmer, Norwalk, Connecticut, USA) with 340 nm excitation (slit width 5 nm) and 500 nm emission (slit width 5 nm). Measurements were made on continuously stirred 1.2 ml cell suspensions maintained at 37°C by a circulating water jacket. Fluorescence measured after the addition of 50 μM digitonin defined the maximum fluorescence (F_{max}), and subsequent addition of 10 mM Tris base and 10mM EGTA defined the minimum fluorescence (F_{min}). We confirmed that the fluorescence reached after addition of either high concentrations of ionomycin (30 μM) or 0.5% Triton X-100 were identical to those attained when cells were permealized with 50 μM digitonin. The fall in fluorescence following 10 mM EGTA added to a fresh sample of cells before each experiment was used to determine background fluorescence due to extracellular fura-2 and was used to correct the responses (cF). Cytosolic free calcium, $[Ca^{2+}]_i$, was calculated from the following equation:

$$[Ca^{2+}]_i = K_d[(cF_{obs} - F_{min})/(F_{max} - cF_{obs})]$$

The K_d value of fura-2 for Ca^{2+} has been determined to be 224 nM [37].

To improve an assessment and accuracy of calcium concentration measurement using fura-2AM, the following was addressed. We determined whether the fura-2-loaded cells were de-esterified completely after loading by the method of Highsmith, Bloebaum and Snowdowne [38]. Cells were incubated with fura-2AM (1 μM) using either Ca^{2+} free (with 1 mM EGTA) or Ca^{2+} replete MGB (Ca^{2+} 1 mM) for 30, 60 and 90 minutes, then washed and resuspended in the same buffer. The cell suspensions were lysed with 0.5% Triton X-100 and centrifuged at 200 × g. Then the supernatants were monitored at excitation between 310 and 410 nm with 510 nm emission. These spectra were compared with the spectra obtained by the fura-2 free acid solution (1 μM) in the same Ca^{2+} free or Ca^{2+} replete MGB. The spectra of the supernatant at the time indicated after loading with fura-2AM was identical completely to that attained by the fura-2 free acid solution, suggesting virtually complete deesterification of fura-2AM in LLC-PK_1 cells in our experiments. Since fura-2AM can be hydrolyzed in mitochondria as well as cytosol [39, 40], the predominant cytosolic localization of fura-2 providing a Ca^{2+}-sensitive signal in our experiments was established as previously described [40]. In brief, the fura-2 loaded cells were resuspended and incubated for five minutes in the MGB containing 1 μM Ca^{2+} and 1.3 μM rhodamine 123, then 20 μM digitonin was added until the leakage of cytosolic fura-2 was complete. The cell suspension was washed and resuspended in the same MGB except for absence of rhodamine, followed by observation of the cells for rhodamine 123 localization at 490 nm excitation, 520 nm emission. The cells treated in this fashion accumulated and retained rhodamine in their mitochondria, but no signal of fura-2 from a compartment of mitochondria was retained. This indicated no significant mitochondrial contribution to the Ca^{2+}-sensitive fura-2 signal of LLC-PK_1 cells in our experiments since 1 μM Ca^{2+} should be enough to retain a mitochondrial potential [41]. Furthermore, a photobleaching was not evident until at least 30 minutes after the exposure of a xenon light (slit width 5 nm) to fura-2 free acid solution in the Ca^{2+} free and Ca^{2+} replete MGB by monitoring the intensity and spectra of fura-2 emission when excited at 340 nm with 510 nm emission as previously described [42]. To calibrate the rate of fura-2 leakage, $MnCl_2$ (0.1 mM) was added to the cell suspensions in Ca^{2+} replete MGB (Ca^{2+} 1 mM), followed by the addition of diethylenetriaminepentaacetic acid (DTPA, 0.2 mM) as previously described [24, 39]. Under the condition of our experiments, the leakage of fura-2, 60 minutes after loading, represented approximately 10% of the total cellular fluorescence. In addition, the magnitude of quenching extracellular and/or leakage of fura-2 by 0.1 mM $MnCl_2$ was identical to that obtained by addition of 10 mM EGTA. However, to minimize fura-2 leakage, cells were used immediately after loading and discarded within one hour.

Reagents

PAF (L-α-phosphatidylcholine, β-acetyl-γ-O-hexadecyl) was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). The stable TXA_2 analogue, ONO11113 (9,11-epithio-11,12-methano-thromboxane A_2) was obtained from Ono Pharmaceutical Co. (Osaka, Japan). The PAF antagonist, BN50730, was obtained from Dr. Braquet (Institute Henri Beaufour, Le Plessis-Robinson, France), and TXA_2 receptor antagonist, L657925, was obtained from Merk-Frosst, Canada. 8-(N,N-dimethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Fura-2, fura-2 AM and ionomycin were purchased from Calbiochem Co. (San Diego, California, USA). All other chemicals used were obtained from Sigma Chemical Co. and were of the highest commercial grade available.

Statistics

Results are expressed as means ±SEM. The EC_{50} was defined as the concentration of agonist producing half-maximal response. EC_{50} values were determined by a log-logit transformation of the concentration-response curve. Statistical differences were determined using the paired t-test. A P value of less than 0.05 was considered statistically different.

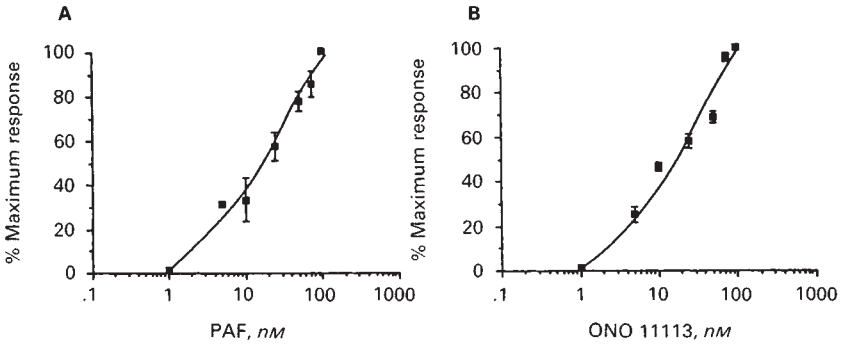

Fig. 1. *Concentration dependent effect of PAF and ONO11113 on the increase in cytosolic free Ca^{2+}.* Fura-2 loaded cells were suspended in modified Gey's buffer (Ca^{2+} 1 mM) as described under **Methods.** Values are mean ± SEM, $N = 4$. EC_{50} values for PAF and ONO11113 are 17 ± 4 nM and 17 ± 2 nM, respectively. EC_{50} values are determined by a log-logit transformation of the concentration-response curve.

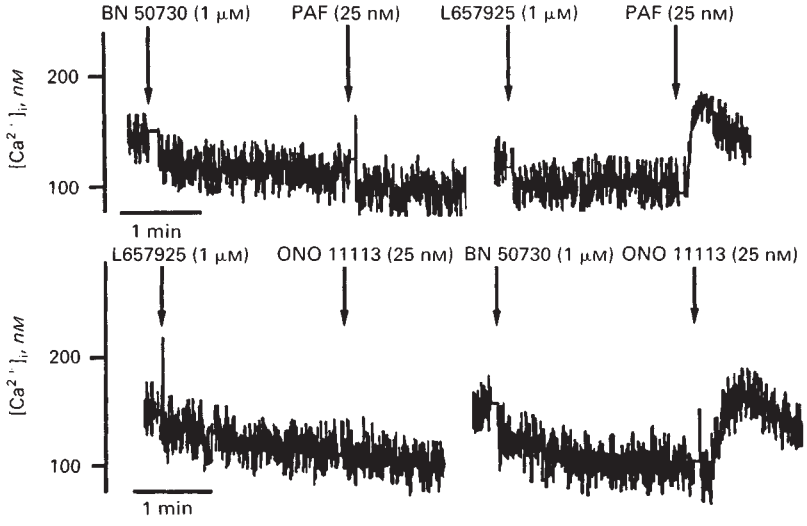

Fig. 2. *Selective inhibition of PAF- or ONO11113-evoked increase in cytosolic free Ca^{2+} with either PAF antagonist, BN50730, or TXA_2 receptor antagonist, L657925.* A suspension of fura-2 loaded cells was assayed in modified Gey's buffer (Ca^{2+} 1 mM). Top: Cells were challenged with 25 nM PAF 2 min after exposure to 1 μM BN50730 (left), or 1 μM L657925 (right). Bottom: Cells were challenged with 25 nM ONO11113 2 min after exposure to the same concentration of BN50730 (left), or L657925 (right). Tracings are representative of three experiments.

Results

PAF- and TXA_2-stimulated increase in $[Ca^{2+}]_i$

A specific molecular species of PAF was utilized in the studies. PAF or the TXA_2 mimetic, ONO11113, elicited a concentration-dependent increase in $[Ca^{2+}]_i$. Both PAF and ONO11113 at 10^{-9} M elicited a small elevation in $[Ca^{2+}]_i$ and a maximum response achieved at 10^{-7} M of each agonist. Higher concentrations of these compounds had no additional effects. PAF or ONO11113 at the concentration of 10^{-7} M increased $[Ca^{2+}]_i$ from 148 ± 16 to 288 ± 39 nM ($N = 8$) and from 130 ± 8 to 240 ± 18 nM ($N = 7$), respectively. Concentration-response curves (Fig. 1) yielded EC_{50} values for PAF and ONO11113 of 17 ± 4 nM and 17 ± 2 nM, respectively ($N = 4$).

PAF and TXA_2 interact with discrete receptors

To examine whether the increases in $[Ca^{2+}]_i$ in response to PAF or TXA_2 mimetic, ONO11113, were mediated through activation of discrete receptors, test samples were incubated for two minutes with either the PAF receptor antagonist, BN50730 [36], or the TXA_2/PGH_2 receptor antagonist, L657925 [11], prior to the addition of agonist. BN50730 (1 μM) completely inhibited the response to PAF (25 nM) but had no effect on the response to ONO11113 (25 nM). L657925 (1 μM) completely inhibited the response to ONO11113 (25 nM) but had no effect on the response to PAF (25 nM). A representative (of 3 experiments) set of tracings is presented in Figure 2. Taken together, these data indicate that PAF and TXA_2 stimulate the increase in $[Ca^{2+}]_i$ via discrete receptors.

The responses to PAF or ONO11113 were rapidly desensitized. At maximum concentration of each agonist (100 nM), a second exposure to the same agonist after the $[Ca^{2+}]_i$ returned to baseline, failed to elicit a second response ($N = 5$, Fig. 3). This desensitization of repeated agonist stimulation was also seen even at submaximal concentrations (data not shown). On the other hand, prior exposure to PAF or ONO11113 resulted in heterologous desensitization of the other agonist. At maximum concentration of each agonist (100 nM), a second exposure to other agonist after the $[Ca^{2+}]_i$ returned to baseline, also failed to elicit a second response ($N = 3$, Fig. 3). However, after exposure to 25 nM ONO11113, the addition of 25 nM PAF to the same sample elicited 54 ± 17% of the PAF control response ($N = 3$), and prior exposure to 25 nM PAF followed by 25 nM ONO11113 resulted in 55 ± 15% of the ONO11113 control response ($N = 4$).

Contribution of extracellular calcium to the increase in $[Ca^{2+}]_i$ induced by PAF and TXA_2

To establish the contribution of influx of extracellular Ca^{2+} to the maximum increase in $[Ca^{2+}]_i$, responses to PAF (100 nM) or ONO11113 (100 nM) were elicited in the absence and presence

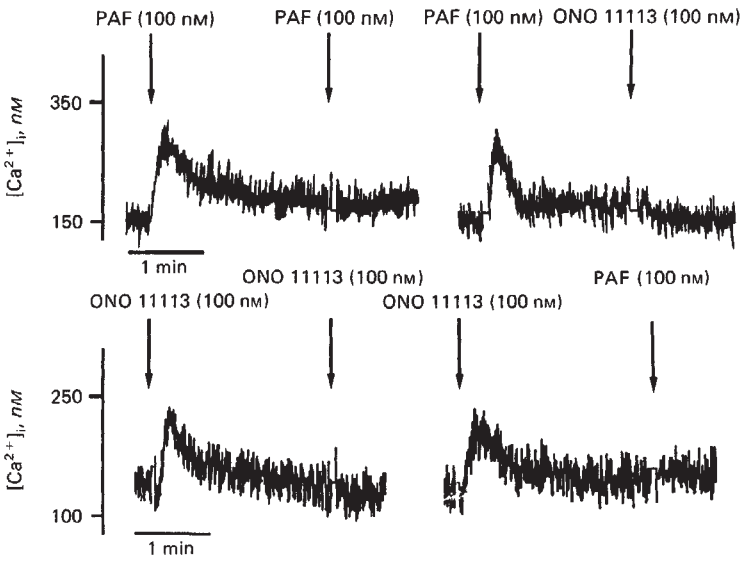

Fig. 3. *Desensitization of PAF- and ONO11113-evoked increase in cytosolic free Ca^{2+}.* Left panel: A second exposure to the same agonist after the cytosolic free Ca^{2+} returned to baseline failed to elicit a second response even at maximum concentrations (each 100 nM). Right panel: At maximum concentration of PAF and ONO11113 (100 nM), a second exposure to other agonist after the cytosolic Ca^{2+} returned to baseline, also failed to elicit a second response. Tracings are representative of three to five experiments.

Table 1. Effect of extracellular calcium on basal and maximum increase in $[Ca^{2+}]_i$ induced by PAF and ONO11113

	Basal $[Ca^{2+}]_i$	Maximum $[Ca^{2+}]_i$	$\Delta [Ca^{2+}]_i$
		nM	
PAF (100 nM, $N = 5$)			
Extracellular Ca^{2+} +	158 ± 16	328 ± 50	170 ± 42
Extracellular Ca^{2+} −	123 ± 14[a]	198 ± 21[b]	71 ± 10[b]
ONO11113 (100 nM, $N = 3$)			
Extracellular Ca^{2+} +	125 ± 8	225 ± 18	100 ± 10
Extracellular Ca^{2+} −	94 ± 2[a]	137 ± 3[b]	48 ± 4[b]

Data are expressed as means ± SEM.
[a] $P \leq 0.005$, and [b] $P \leq 0.025$, when compared with the samples in the presence of extracellular Ca^{2+} (1 mM); extracellular Ca^{2+} was chelated by addition of 10 mM EGTA to a cell suspension.

of 10 mM EGTA (to chelate extracellular Ca^{2+}; Table 1). In the presence of EGTA, the basal $[Ca^{2+}]_i$ in each experiment was stable at least during the period of observation (data not shown), and the maximum response to PAF and ONO11113 was reduced to 51 ± 8% ($N = 5$) and 53 ± 11% ($N = 3$) of that in the absence of EGTA, respectively. In the presence or absence of extracellular Ca^{2+} both agonists elicited a rapid increase in $[Ca^{2+}]_i$ that peaked at ~20 seconds. In the presence of extracellular Ca^{2+} the initial peak of $[Ca^{2+}]_i$ induced by both agonists was followed by a slow decay towards baseline with the sustained elevation at 1.5 to 2 minutes. In the absence of extracellular Ca^{2+}, the response was less sustained than that in the presence of Ca^{2+} and the return toward baseline was more rapid and complete (~1 min). Verapamil (10^{-4} M), a Ca^{2+} channel blocker, failed to block the response induced by PAF or ONO11113 in the presence of extracellular Ca^{2+} even when cells were exposed to the agent for up to 15 minutes ($N = 3$; data not shown).

Effect of TMB-8 on release of intracellular calcium

TMB-8 has been used as a tool to investigate agonist-stimulated release of intracellular Ca^{2+} in other cell types. Although its mechanism and exact site of action are poorly understood, TMB-8 has been shown to block calcium efflux from intracellular organelles in a variety of cellular responses [32, 43, 44]. To investigate the contribution to the overall increase in $[Ca^{2+}]_i$ made by release from intracellular stores, LLC-PK_1 cells were incubated with TMB-8 (10^{-4} M) for 10 min prior to the addition of PAF or ONO11113 utilizing a dose from the previous study [32]. In the presence of 10 mM EGTA chelating extracellular Ca^{2+}, TMB-8 blocked the PAF (25 nM)-induced increase in $[Ca^{2+}]_i$ by 92 ± 5% ($N = 6$, $P \leq 0.0005$) and ONO11113 (25 nM)-induced increase in $[Ca^{2+}]_i$ by 99 ± 1% ($N = 3$, $P \leq 0.0005$) as compared to controls (Fig. 4). The data strongly suggest that PAF and TXA_2 stimulate the release of Ca^{2+} from intracellular storage sites.

Discussion

The present study demonstrates that LLC-PK_1 cells express discrete receptors for PAF and TXA_2 that are functionally coupled to an increase in $[Ca^{2+}]_i$. The presence of functional receptors for these autacoids has not been previously reported in renal tubular epithelial cells. The rapid increase in $[Ca^{2+}]_i$ that we observed with these compounds (~20 seconds) is similar to the time response that has been obtained with vasopressin in LLC-PK_1 cells [35] and prostaglandins E_1 and E_2 in MDCK cells [32]. The increase in $[Ca^{2+}]_i$ that we observed with PAF or ONO11113 is lower than that with vasopressin in LLC-PK_1 cells [35], but similar to that with prostaglandins E_1 and E_2 in MDCK cells [32].

The EC_{50} value of PAF (17 nM) for $[Ca^{2+}]_i$ in LLC-PK_1 cells is higher than that determined in macrophage (0.4 nM) [28], but it is comparable to that in human platelets (10 nM) [21] and the circulating PAF level (4 to 12 nM) in experimental models of serum sickness and immunoglobulin E-induced anaphylaxis in rabbits [2, 3]. The EC_{50} value of ONO11113 in our study (17 nM) is similar to that in human platelets (18 nM) [11]. Although the EC_{50} value of this compound in other cell types is not known, Altin and Bygrave found that the maximal increment in $[Ca^{2+}]_i$ was induced at 20 nM ONO11113 in rat liver cells [31], and

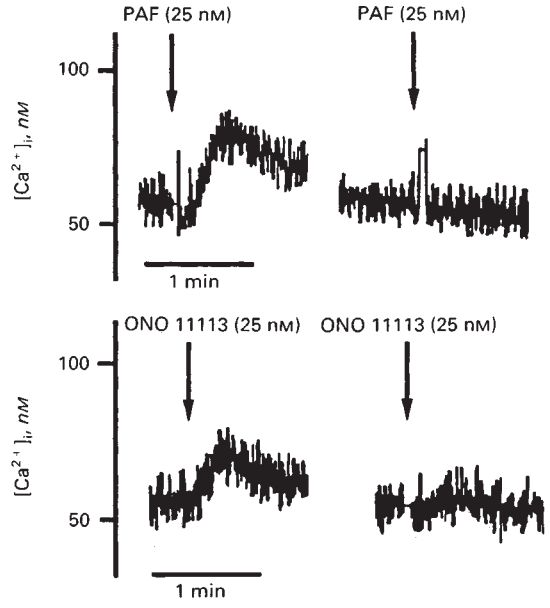

Fig. 4. *Blockade by TMB-8 of PAF- and ONO11113-elicited increase in cytosolic free Ca^{2+}.* In the presence of 10 mM EGTA, chelating extracellular Ca^{2+} cells were exposed to 25 nM PAF (top) or 25 nM ONO11113 (bottom) in the absence (left) or 10 min after addition of 10^{-4} M TMB-8 (right). TMB-8 was diluted in the same buffer and added to the cell suspension. Tracings are representative of three to six experiments.

Mene et al reported, using structurally different TXA_2 analogue, U-46619, that this compound ranging from 10 nM to 1 μM induced $[Ca^{2+}]_i$ increment in a dose-dependent manner in rat and human mesangial cells [29].

Both PAF and ONO11113 evoked an increment in $[Ca^{2+}]_i$ even in the absence of extracellular Ca^{2+}, indicating that at least part of the increase in $[Ca^{2+}]_i$ is due to release from intracellular storage sites. The contribution of intracellular storage sites is further supported by the ability of TMB-8 (10^{-4} M), which has extensively been used to study the contribution of Ca^{2+} released from intracellular storage sites in the increase in $[Ca^{2+}]_i$ [32, 43, 44] to block almost completely the PAF- or ONO11113-induced increment in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. In addition, our data indicate an important contribution of extracellular Ca^{2+} and plasma membrane-bound Ca^{2+} pools to PAF- or ONO11113-induced increase in $[Ca^{2+}]_i$. At maximum response, the increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} is approximately twice of that achieved when extracellular Ca^{2+} is chelated with EGTA. The basal $[Ca^{2+}]_i$ after the addition of EGTA was stable, and the baseline after cellular activation induced by PAF or TXA_2 in the presence of EGTA was the same as that before the addition of these agonists. The lower basal $[Ca^{2+}]_i$ induced by EGTA represents the new baseline achieved after extracellular Ca^{2+} was chelated as previously described [32]. In addition, the increase in $[Ca^{2+}]_i$ was not affected by verapamil, suggesting a verapamil-sensitive Ca^{2+} channel is not mediating the influx. Taken together, these results indicate that the increment in $[Ca^{2+}]_i$ induced by PAF or ONO11113 is derived from both intracellular storage sites and influenced by entry of calcium from the extracellular milieu.

The selective PAF antagonist, BN50730 (1 μM) [36], completely abolished PAF-induced increment in $[Ca^{2+}]_i$, but failed to affect ONO11113-induced increment in $[Ca^{2+}]_i$. In contrast, the potent TXA_2 receptor antagonist, L657925 (1 μM) [11], selectively abolished ONO11113-evoked transient increment in $[Ca^{2+}]_i$, but did not affect the PAF-induced increment in $[Ca^{2+}]_i$. In addition, the PAF- or ONO11113-challenged cells were not responsive to a second bolus of the same agent. These observations demonstrating agonist-specific effect on calcium homeostasis in LLC-PK_1 cells as well as showing PAF- and ONO11113-induced desensitization indicate that LLC-PK_1 cells have the discrete receptors for PAF and TXA_2 and that the increment in $[Ca^{2+}]_i$ induced by both agonists are receptor-mediated phenomenon. Previous in vivo study has demonstrated that a selective TXA_2 antagonist ameliorates the biological effects of PAF and that the PAF effects are mediated by TXA_2 in both glomeruli and tubules [9]. Other investigators have shown that PAF stimulates phospholipase A_2, resulting in the formation of a variety of cyclooxygenase products including TXA_2 metabolites [1, 7, 8]. Our conclusion that LLC-PK_1 cells possess the two different receptors for PAF and TXA_2, however, does not exclude these interelations of the two compounds for their biological effects through discrete receptors.

Although we did not examine the mechanisms by which PAF or TXA_2 mobilize $[Ca^{2+}]_i$ in LLC-PK_1 cells, based on previous studies, it appears likely that PAF- and TXA_2-induced increase in $[Ca^{2+}]_i$ in LLC-PK_1 cells is mediated by the generation of IP3 [26, 30, 31, 45] and mobilization of Ca^{2+} from intracellular stores particularly endoplasmic reticulum [26, 34, 45, 46]. In addition, despite the presence of discrete receptors for PAF and the TXA_2 mimetic, ONO11113, the cross-desensitization of the response to release intracellular Ca^{2+} (similar to that with vasopressin and its analogues in LLC-PK_1 cells [35]) and the release of Ca^{2+} from TMB-8 sensitive sites by both agents suggest a common pathway and/or pool of Ca^{2+} release induced by PAF and TXA_2.

It appears that PAF is not usually synthesized in renal tubular cells [47], and it has been inconclusive whether LLC-PK_1 cells have the capacity for synthesis of prostaglandins [48, 49]. However, in response to various stimuli PAF or TXA_2 can be generated by isolated glomeruli, mesangial cells, as well as bone marrow-derived cells including platelets, monocytes, macrophages, and polymorphonuclear cells [1, 5], which can often be seen in a variety of glomerular and tubulointerstitial diseases. Thus, the exposure of renal tubular cells to PAF or TXA_2 generated in the microenvironment may cause the rapid increase in $[Ca^{2+}]_i$, resulting in alterations of physiologic and inflammatory processes in renal tubular cells.

Previous studies have suggested the importance of intracellular calcium as second messenger modulating cellular function of tubules [13–19] and its possible importance in tubular injury [20]. The rise in intracellular Ca^{2+} mediates in a variety of renal tubular cell function including transport of water, salt and glucose [14, 16, 17] and bicarbonate absorption [15] as well as cell volume regulation [18, 19]. Hebert, Jacobson and Breyer have recently shown that prostaglandin E_2 inhibits sodium transport through increase in intracellular Ca^{2+} in rabbit cortical collecting duct [17]. These observations provide at least some evidence that the intracellular concentration of Ca^{2+} plays a key role in tubular cell function. Although the precise

pathophysiological significance of PAF- or TXA_2-induced increase in intracellular Ca^{2+} in tubular cells remains to be determined, the ability of these autacoids to increase intracellular Ca^{2+}, suggests an important role for these autacoids in tubular pathophysiology.

In summary, the present study demonstrates that LLC-PK_1 cells express discrete receptors for PAF and TXA_2 that are functionally coupled to an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ in response to either agonist resulted from both the release of intracellular Ca^{2+} from TMB-8-sensitive sites and the influx of extracellular Ca^{2+}. The presence of receptors for these autacoids and the ability of these autacoids to increase in intracellular Ca^{2+} in renal tubular epithelial cells suggest the potential role of these autacoids in tubular pathophysiology.

Acknowledgments

Norishi Ueda was a recipient of a post-doctoral fellowship from the Louisiana affiliate of the American Heart Association. This research was supported in part by the National Institutes of Health. The authors thank Ms. Chau Nguyen for technical assistance and Ms. Ellen Satter for secretarial assistance.

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