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Ca^{2+} response of rat mesangial cells to ATP analogues

Antonio M. Gutierrez, Xiaofen Lou, A. Erik, G. Persson, Avi Ring *

Department of Physiology and Medical Biophysics, University of Uppsala, BMC, Box 572, SE-751-23 Uppsala, Sweden

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Abstract

The aim of this investigation was to characterise the effects of ATP analogues and UTP on the single cell intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured rat mesangial cells. Typically, there were two phases in the Ca^{2+} response to the agonists, an initial fast transient peak and a subsequent slower decline, or plateau, phase. For the peak amplitude in $[Ca^{2+}]_i$ the agonists had about equal effect. But when taking in consideration the percentage of responding cells and the integrated Ca^{2+} response over 1 min, the order of efficacy of nucleotide agonists (100 μM) was UTP = ATP > ATPγS > ADP = 2MeS-ATP (2-methylthio-ATP). Adenosine, AMP and β,γ-Me-ATP (100 μM) had no effect. Suramine (100 μM) and reactive blue (50 μM) decreased the number of responding cells. Removing Ca^{2+} from the bath diminished neither the peak in $[Ca^{2+}]_i$ nor the percentage of responding cells, but the average $[Ca^{2+}]_i$ increase in 1 min was significantly reduced. The results indicate that $P2Y_2$ receptors are present in rat mesangial cells but it cannot be excluded that there are receptors distinct from $P2Y_2$ which also mediate a rise in $[Ca^{2+}]_i$. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: ATP; UTP; Nucleotide receptor; Ca^{2+} mobilization; $P2Y_2$ receptor; Mesangial cell

1. Introduction

The general signalling mechanisms of P2 purinoceptors (Burnstock, 1976) have been reviewed (Dubyak and El-Moatassim, 1993; North and Barnard, 1997). P2 receptors have been implicated in the regulation of renal vascular function (Majid and Navar, 1992; Churchill and Ellis, 1993). Adenosine receptors were also shown (Spielman and Arend, 1991) to be involved in the regulation of renal blood flow and recently it was shown that extracellular ATP and extracellular adenosine have different effects on renal blood flow (for review see Inscho et al., 1994; Navar et al., 1996). Extracellular ATP has been shown to have effects on renal epithelial cells (Friedrich et al., 1989), arteriolar smooth muscle (Inscho et al., 1996), mesangial cells (Schultze-Lohoff et al., 1992; Pavenstädt et al., 1993; Huber-Lang et al., 1997) and glomerular endothelial cells (Pavenstädt et al., 1997).

The physiological role of ATP receptors in mesangial cells remains unclear. Nevertheless, it has been shown that extracellular ATP exerts numerous effects on mesangial cells including: phosphatidylinositide hydrolysis and prostaglandin E_2 synthesis (Pfeilschifter, 1990), protein kinase C activation (Pfeilschifter and Merryweather, 1993), proliferation (Schultze-Lohoff et al., 1992), depolarisation, intracellular Ca^{2+} elevation and cell contraction (Pavenstädt et al., 1993; Huber-Lang et al., 1997) and inhibition of cAMP accumulation (Schultze-Lohoff et al., 1995). For most of these effects UTP and ATP were equipotent.

The existence of more than one type of mesangial ATP receptor has been implicated since ATPγS had the strongest mitogenic effects and UTP was much weaker than ATP. Also, the purinergic stimulation of mesangial prostaglandin (prostaglandin E_2) synthesis involves a third distinct purine receptor pathway. Recently, it was shown (Huber-Lang et al., 1997) that UTP gives rise to a sustained depolarisation response which qualitatively differs from that to ATP. In contrast, the $[Ca^{2+}]_i$ responses were similar for both agonists. It has not been clarified whether the influx of extracellular Ca^{2+} is an effect secondary to release from intracellular pools (e.g., Ca^{2+}-dependent activation of protein kinases or $[Ca^{2+}]_i$ activated channels) or due to activation of a distinct receptor pathway. The depolarisation was shown to depend on the activation of Ca^{2+}-activated Cl^- channels but not on inward cation flux from

* Corresponding author. Norwegian Defence Research Establishment, Division of Environmental Toxicology, Box 25, 2007 Kjeller, Norway. Tel.: +47-63807817; Fax: +47-63807811; E-mail: avi.ring@ffi.no

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non-specific cation channels or voltage activated Ca^{2+} channels (Okuda et al., 1986; Pavenstädt et al., 1993). It was suggested that in addition to $P2Y_2$ the mesangial cells also express other P2Y receptors (Huber-Lang et al., 1997).

All pathways for the effects of purine agents on mesangial cells may involve a cascade of effects which raise $[Ca^{2+}]_i$, but not with equal potency. In addition, $[Ca^{2+}]_i$ may be raised both by extracellular influx and by intracellular release. Mesangial cells has become a biochemically well characterised and useful model system for investigations of interactions of second messenger systems (Pfeilschifter and Huwiler, 1996), but no systematic study of the effects of ATP-analogues, including UTP, on $[Ca^{2+}]_i$ has been presented. The present investigation was undertaken in order to characterise the $[Ca^{2+}]_i$-response of first passage single rat mesangial cells to ATP analogues and UTP.

2. Methods

2.1. Cell culture

Rat glomerular mesangial cells were cultured according to Kurtz et al. (1982). Both kidneys from Sprague–Dawley rats (100–200 g) were removed and decapsulated. The renal tissue was minced and forced through a polyester sieve with 100 μm mesh opening. The resulting filtrate was suspended in 50 ml of isolation buffered solution (IBS) containing (in mM): 5 KCl, 2 $CaCl_2$, 130 NaCl, 10 Glucose, 20 Sucrose and 10 Tris, pH adjusted to 7.4 with HCl. This suspension was then filtered through two sieves in series with 150 and 50 μm mesh opening. Isolated glomeruli were collected from the 50 μm sieve (more than 80% are free of capsules; Kreisberg et al., 1978) and digested in 50 ml IBS containing 0.1% collagenase (125 U/mg), 30 min at 37°C, to remove epithelial cells and obtain glomerular cores consisting mostly of mesangium and capillary loops (Striker et al., 1980). Aliquots of the glomeruli were placed in 25 cm^2 tissue flasks (Nunclon) containing 5 ml RPMI 1640 medium. The medium was supplemented with 18% fetal calf serum 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B and 0.66 U/ml insulin. The RPMI 1640 medium contained D-valine instead of L-valine. D-Valine inhibits fibroblast growth (Ausiello et al., 1980). The flasks were incubated at 37°C and 5% CO_2 in a humidified atmosphere in a CO_2-controlled incubator for 3–6 weeks. The medium was exchanged every third day. Confluent cultures of mesangial cells were characterised morphologically (phase contrast microscopy) and immunologically with positive staining for smooth muscle actin, desmin and vimetin. Epithelial and endothelial specific staining (cytokeratin and factor VIII, respectively) were negative, excluding any contamination.

2.2. Measurements of $[Ca^{2+}]_i$

In this study, only cells from the first passage were used. Two days prior to the experiments, the primary culture cells were subcultured by incubating in Ca^{2+} and Mg^{2+} free PBS (phosphate buffered saline, Dulbecco) containing 0.01% EDTA and 0.125% trypsin, for 5 min at 37°C. Trypsin action was stopped by addition of 5 ml growth-medium. After centrifugation the pellet was resuspended in 2 ml medium and aliquots of 0.1 ml were plated onto 24-well dishes, each well containing a glass cover slip of 13 mm in diameter (Menzel-Gläser, Germany). During subculturing and further incubation a concentration of 9% fetal calf serum was used.

$[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator fura-2. The cells were incubated at 37°C with 10 μM amine modified fura-2 ester, (fura-2/AM) in RPMI 1640 with 9% fetal calf serum, 1% dimethyl sulfoxide. The cover slips were then rinsed twice with the standard experimental solution containing (in mM): 145 NaCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 1.6 K_2HPO_4, 0.4 KH_2PO_4, 5 glucose, pH adjusted to 7.4 and osmolality adjusted with sucrose to 290 mosM. For the Ca^{2+}-free experiments $CaCl_2$ was replaced by 5 mM EGTA. The cover slip was then transferred to a chamber on the stage of a Zeiss Axioskop. With a procedure similar to that used with isolated arteriole (Kornfeld et al., 1994), fluorescence excitation light at wavelengths of 340 ± 4 and 380 ± 4 nm was obtained from a single monochromator (UHL, Germany). Wavelengths were switched in less than 10 ms. Emission from the cells was examined with a phase contrast Achroplan 20/0.50 water immersion objective and images were collected every third second with an intensified CCD camera (DAGE-MTI, USA). To get signals of sufficient intensity, an image intensifier was installed prior to the camera in the light path. We analysed the digitised images using the program Image-1/FL (Universal Imaging, USA) which allows online analysis of each cell in the digitised image. The system was calibrated using a Calibration Kit from Molecular Probe, and $[Ca^{2+}]_i$ was calculated on line.

The experiments were performed at 37°C, with a continuous superfusion rate of 7–10 ml/min, driven by gravity pull. An equilibration period of 10 min was allowed for prior to each experiment. All agonists were used at 100 μM and were applied for 2-min periods. The activities of the agonists were, for convenience, compared at this single concentration only since it is expected that the relative potency for the ATP analogues compare well with the activity and maximum response of the agonists for P2 receptors (King et al., 1996; Suh et al., 1997). Others (King et al., 1996) have used similar procedures.

2.3. Statistics

Student's *t*-test for unpaired measurements and assuming unequal variance was used. $P < 0.05$ was considered significant. Values are given as mean ± S.E.M.

2.4. Chemicals

RPMI 1640 medium, fetal calf serum, trypsin-EDTA and PBS were from Biochrom, Germany. Fura-2/AM was from Molecular probes, USA. 2MeS-ATP and Suramin were from ICN Pharmaceuticals. All other chemicals used were purchased from Sigma, USA.

3. Results

Typical responses to ATP are shown in Fig. 1. Some cells responded only with a fast response typical of an Inositol triphosphate (IP_3) mediated intracellular release but in many cases a later and slower phase was also observed (Fig. 1a). A large variability in the plateau phase was noted. No qualitative difference between UTP ($n = 241$) and ATP ($n = 211$) was observed, both exhibiting variability in the time course and the magnitude of the response.

In many cell types the prolonged plateau phase is caused by an influx of Ca^{2+} through voltage activated Ca^{2+} channels or non-specific cation channels. It was therefore interesting to investigate the response in $[Ca^{2+}]_i$ to ATP when the bath Ca^{2+} concentration was low. We find (Fig. 1b) that the response is then qualitatively different and in no case was a plateau phase seen ($n = 140$). It may be noted that the final $[Ca^{2+}]_i$ in this case is below the initial level but when the bath Ca^{2+} concentration is restored (simultaneous with withdrawal of the agonist) the $[Ca^{2+}]_i$ level is restored.

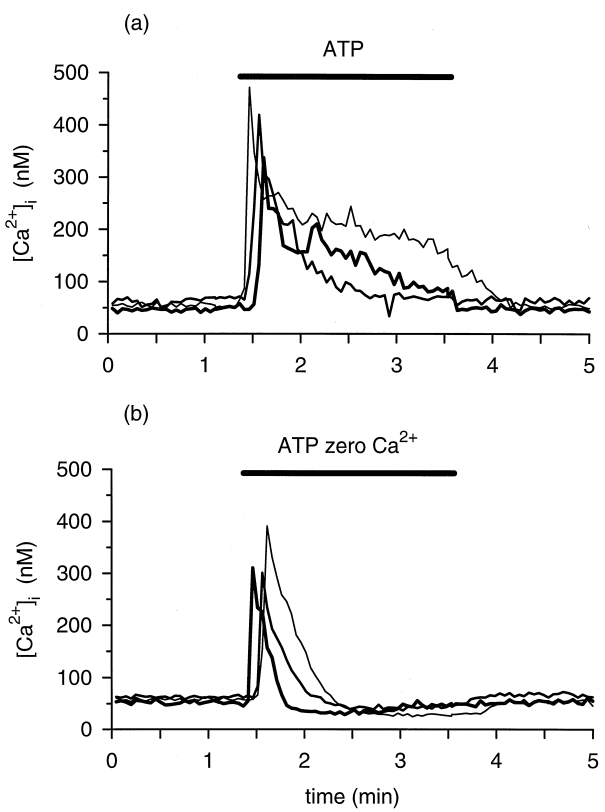

Fig. 1. Typical responses of $[Ca^{2+}]_i$ to ATP. Similar responses were obtained for UTP. (a) Three responses to ATP with normal (1.3 mM) bath Ca^{2+}. (b) Three Response to ATP with calcium-free bath solutions (5 mM EGTA). The bath calcium concentration is restored when ATP is washed out.

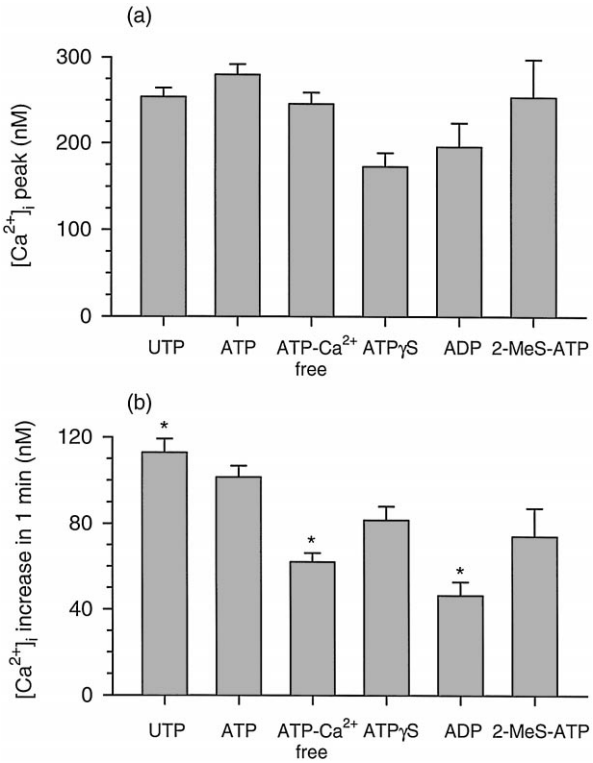

Fig. 2. Summary of $[Ca^{2+}]_i$ responses in all experiments. (a) Average of the amplitude of the $[Ca^{2+}]_i$ peak for the cells that did respond. The amplitude of the $[Ca^{2+}]_i$ peak is the difference between the peak $[Ca^{2+}]_i$ increase and the initial value of $[Ca^{2+}]_i$. (b) Average increase in $[Ca^{2+}]_i$ for the cells that did respond. The average was calculated as the average over 1 min from the start of the peak. $* P < 0.05$ vs. ATP.

In Fig. 2 is shown the amplitude of the $[Ca^{2+}]_i$ peak (Fig. 2a) and the average $[Ca^{2+}]_i$ increase of the responding cells (Fig. 2b). Since the peak response, in contrast to the plateau phase, depends on the IP3 generation it is more closely related to the receptor activation. We therefore chose to compare the peak responses of the purine agents. The average is calculated over 1 min from the start of the peak, i.e., from the beginning of the fast rise in $[Ca^{2+}]_i$. The greatest response is that due to UTP. The relative potency of effects of the ATP response for the $P2Y_2$ has been given as UTP = ATP > ATPγS with ADP and 2-MeS-ATP without effect (North and Barnard, 1997). It is interesting therefore that in the present case both ADP ($n = 152$) and 2-MeSATP ($n = 80$) have significant effects on $[Ca^{2+}]_i$ of a magnitude similar to that of ATPγS ($n = 127$).

Fig. 2 also shows a summary of the responses to ATP when the bath concentration of Ca^{2+} was low. It is clear

that the average $[Ca^{2+}]_i$ increase during 1 min is significantly reduced whereas the amplitude of the peak is not. The selective P1 agonist Adenosine ($n = 142$) and the P2X receptor agonists AMP ($n = 105$) and β,γ-Me-ATP ($n = 127$) had no effect (not shown).

The regulation of the filtration rate in the kidney depends on the response of millions of glomeruli, each of which contains many mesangial cells. The mechanism by which the mesangial cells mediate tubulu-glomerular feedback regulation is not known but contraction of the mesangium has been suggested to be one plausible mechanism (Schlondorff, 1987). The role of the mesangium in regulation of filtration was recently reviewed (Schlondorff, 1996). It may be expected that the physiological responses of the kidney to ATP depend on the percentage of glomeruli and mesangial cells that respond. Fig. 3 shows the percentage of responding cells (Fig. 3a) and the net average rise in $[Ca^{2+}]_i$ (Fig. 3b). Here the average in 1 min includes all the cells in an experiment, i.e., the average response associated with the functional response in a glomerulus. The purinergic antagonists suramine (100 μM) and reactive-blue (RB, 50 μM reduced the percentage of responding cells. The basal concentration of Ca^{2+} prior to application of ATP was not increased in the presence of antagonists, i.e., there was no indication that the antagonists alone reduce the number of viable cells. The control value for ATP alone was 80% and those for ATP applied in the presence of antagonists were Suramine 38% ($n = 98$) and RB 44% ($n = 61$). (Suramine and RB interfere with the 340 fluorescence and the magnitude of the $[Ca^{2+}]_i$ response was therefore not characterised in this case).

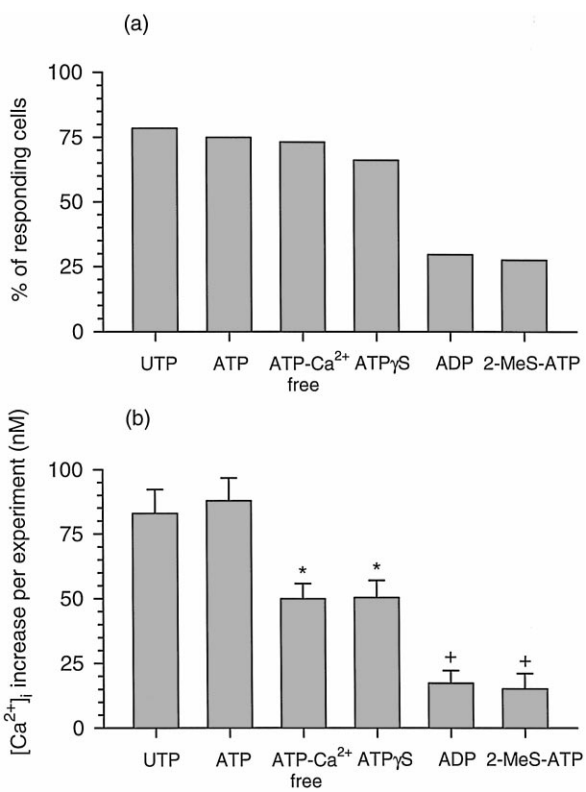

Fig. 3. (a) Percentage of responding cells and (b) the mean Ca^{2+} response for all the cells in each experiment, i.e., including cells which did not respond. For each cell, the Ca^{2+} response is the average $[Ca^{2+}]_i$ increase over 1 min. $^{*}P < 0.05$ vs. UTP and ATP. $^{+}P < 0.05$ vs. ATPγS.

4. Discussion

The presence of a pronounced plateau in the membrane potential response (Pavenstädt et al., 1993; Huber-Lang et al., 1997) in rat cultured mesangial cells has been reported. The variability in response observed here may be inherent to the variability in purine mediated responses. For mesangial cells it was shown (Pavenstädt et al., 1993) that the membrane depolarisation is not due to an influx of cations through such channels, but rather, to $[Ca^{2+}]_i$ dependent activation of Cl^- outflow through Cl^- channels. It is therefore likely that the contribution of Ca^{2+} influx to the membrane depolarisation is small. Nevertheless, a small influx of Ca^{2+} through non-specific cation channels may be sufficient, and necessary, for a prolonged activation of Cl^- channels. The highly variable plateau phase observed in these experiments therefore do not contradict the hypothesis that membrane depolarisation during the late phase of the Ca^{2+} response is due to a Ca^{2+} activated Cl^- efflux.

The order of the efficacy of the nucleotides found here, as judged from the peak responses, was essentially similar to that which has been given for the $P2Y_2$ receptor (North and Barnard, 1997). UTP ≈ ATP > ATPγS. Nevertheless, significant differences were noted (see below). The initial response with low extracellular Ca^{2+} was very similar to the response when normal extracellular Ca^{2+} was used, indicating that the amplitude of the $[Ca^{2+}]_i$ peak is a good measure of the release from intracellular pools. This is consistent with the interpretation that the main receptor studied is the G protein coupled receptor described previously which also couples IP_3 generation to release from intracellular pools. It should be noted, however, that also platelet derived growth factor, homodimer of B-chain, (PDGF-BB) stimulation recruits $[Ca^{2+}]_i$ in mesangial cells by a transient release from intracellular pools (Wallmon et al., 1993), but this pathway involves tyrosine kinase activation and is partially independent of phosphatidyl inositide hydrolysis. Like PDGF-BB, ATP also has mitogenic effects on mesangial cells (Schultze-Lohoff et al., 1992). Therefore, it can not be excluded that ATP also raises $[Ca^{2+}]_i$ through pathways which are separate from the IP_3 mediated release from intracellular pools (Pfeilschifter, 1990; Pavenstädt et al., 1993).

It is interesting to note that for the mesangial cell, in contrast to what has been described for the general func-

tional response of the P2Y receptors, the amplitude of the $[Ca^{2+}]_i$ peak is similar (Fig. 2a) for all agonists used here. This observation contrasts with the reported sequence for the $P2Y_2$ receptor (North and Barnard, 1997) where 2MeS-ATP and ADP are reported to be without effect. This observation does not imply that the mesangial cells respond equally to all agonists. It suggests, however, that the intracellular recruitment has 'all-or-none' characteristics and from Fig. 3b it is clear that the response to ADP and 2MeS-ATP is significantly less than that to UTP and ATP. Nevertheless, it is interesting that the mesangial cells respond to ADP and 2MeS-ATP with a $[Ca^{2+}]_i$ peak which is characteristic of IP_3 mediated intracellular release. The magnitude and order of the responses do not fit with any of the known P2Y receptors. The data presented here support the hypothesis (Huber-Lang et al., 1997) that besides $P2Y_2$ receptors there are additional receptors on mesangial cells involved in the electrophysiological response to purine nucleotides. Nevertheless, the results presented here do not exclude that there may be only one type of purinoceptor on mesangial cells and with a characteristic sequence not yet reported.

It should be noted that there are a number of complicating factors which are inherent in all methods used in establishing the sequence order for purinergic agonists (North and Barnard, 1997). The complicating factors include enzymatic conversions at the level of the plasma membrane, contamination and spontaneous conversion between the diphosphate and triphosphate forms, autocrine stimulation and release of ATP from degraded cells. Also, the potency and previously presumed specificities for antagonists have been questioned. Nevertheless, the present methodology involves a steady flow of bath saline over a monolayer of cells attached to a glass support. The cellular environment is continuously washed free of products of cellular activity. The membrane-bound enzymatic conversions of the ATP analogues are therefore not likely to be fast enough to give a sufficient concentration of alternate phosphorylated forms. Second, the contamination levels of ADP in ATP may be significant but this is not of primary concern here. ATP is unstable and spontaneously converts to ADP. In contrast, however, in the present case it is the response to ADP which was unexpected and it is not likely that the contamination and possible conversion of ADP to ATP is quantitatively sufficient to explain the relatively large effects of ADP seen here. Third, the effect of 2MeS-ATP seen here can not be explained by the degradation to 2MeS-ADP since the latter is not expected to be active at $P2Y_2$ receptors.

Autocrine effects from stretch activation (as has been reported for fibroblasts, phylogenetically similar to mesangial cells; Wang et al., 1996) cannot explain the effects of ADP and 2MeS-ATP since a continuous flow was used here. Similarly, with the methods used here, the release of ATP to the medium, from cells that die, is too low to correlate with the effects of agonist activities.

It should be noted that in a photometric study where the total fluorescence from a number of cells is measured, the response is markedly different from that of a single cell. With a lumped response, neither the 'all-or-none' character of the $[Ca^{2+}]_i$ peak nor the percentage of cells responding is observable. Also, since the peaks are fast and occur with different delays the peaks are likely to be out of phase whereas the slow declining phases are likely to overlap. The photometric response will therefore generally accentuate the slow phase and it is possible to erroneously conclude that there is a significant slow phase. An apparent slow phase can result also from the out of phase overlap of peak responses. We frequently observed such time-lags in the responses in clusters of cells and found that superposing the responses introduced apparent plateaus in the response. It should be pointed out however, that this type of effect could not account for a marked plateau of the type observed here (i.e., in single cell measurements) and in earlier studies (Pavenstädt et al., 1993; Huber-Lang et al., 1997) where marked distinct plateaus were observed. Our data support the general conclusions of the earlier studies as well as the hypothesis of additional nucleotide receptors on mesangial cells. We have found that the presumed additional receptor type on mesangial cells also recruit $[Ca^{2+}]_i$. It is not yet clear, however, to what degree these additional receptors mediate a rise in $[Ca^{2+}]_i$ by recruitment from intracellular stores or, directly or indirectly, through activation of ion channels.

The ATP response in low bath Ca^{2+} gave an insignificantly smaller amplitude of $[Ca^{2+}]_i$ peak but showed a significantly lower average $[Ca_i^{2+}]$ increase in the minute following the start of the peak. This finding accentuates our conclusion that there is a significant contribution from the influx of extracellular Ca^{2+}. The pathway of extracellular influx was not characterised and may occur via non-specific cation channels (e.g., the so called ICRAC current subsequent to intracellular release; Hoth and Penner, 1992) or by modulation of Ca^{2+} exchangers. It may be noted that flufenamic acid, a non-specific cation channel blocking agent, was shown not to diminish the ATP induced depolarisation of mesangial cells (Huber-Lang et al., 1997).

The results also demonstrate that, for a slow physiological response to ATP, such as is expected in the glomerulus, conclusions on the involvement of $[Ca^{2+}]_i$ may be incorrect if it is not known whether the physiological response is associated with the peak or plateau phase (or both) of the $[Ca^{2+}]_i$ increase. It is also not adequate to evaluate only the amplitude of the $[Ca^{2+}]_i$ peak in single cells. The present findings demonstrate the need to complement the measurements of physiological responses with parallel measurements of $[Ca^{2+}]_i$ in individual cells as well as intact glomeruli.

In summary, we have examined the $[Ca^{2+}]_i$ response of first passage cultures of rat mesangial cells and found that the response corresponds essentially with that expected for a $P2Y_2$ receptor. Our results demonstrate that there is a

difference in the number of responding cells and average $[Ca^{2+}]_i$ increase over 1 min. We found evidence supporting the hypothesis that mesangial cells express receptors that, in addition to the $P2Y_2$ receptor, recruit $[Ca^{2+}]_i$. We found evidence for a significant influx of Ca^{2+} and that the purinergic agents may differ more in their activation of this influx than in the intracellular Ca^{2+} release.

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