Independent Pathways Regulate the Cytosolic $[Ca^{2+}]$ Initial Transient and Subsequent Oscillations in Individual Cultured Arterial Smooth Muscle Cells Responding to Extracellular ATP

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Stimulation with extracellular ATP causes a rapid initial transient rise followed by asynchronous periodic oscillations in cytosolic calcium ion activity ($[Ca^{2+}]_i$) in individual aortic smooth muscle cells in either HEPES-buffered or HCO_3^- -buffered saline. The dose at which one-half of the cells display an initial rise in cytosolic calcium is $0.11 \mu M$ ATP in the presence of external Ca^{2+} and $0.88 \mu M$ ATP in the absence of external Ca^{2+} ; the corresponding value for oscillations in the presence of external Ca^{2+} is $2.6 \mu M$ ATP. While the initial transient displays rapid desensitization, the oscillations persist for >30 min in the continuous presence of ATP. The presence of the agonist ATP is also absolutely required for the maintenance of the oscillations, presumably to provide continuous activation of P_2 purinoceptors. The average frequency of oscillation is $\sim 0.9 \text{ min}^{-1}$. The frequency depends only slightly on the concentration of ATP, and oscillations do not collapse into a prolonged elevated $[Ca^{2+}]_i$ at high concentrations of ATP. Both Ca^{2+} influx and release from internal stores participate in the initial transient. Oscillations are not produced in the absence of external Ca^{2+} but are initiated upon the addition of external Ca^{2+} in the continued presence of ATP. Oscillations in progress are abolished by the removal of extracellular Ca^{2+} with one additional peak occurring after the Ca^{2+} removal. These data suggest that extracellular Ca^{2+} influx is required for the maintenance of the posttransient oscillations, presumably to provide the Ca^{2+} necessary for refilling intracellular Ca^{2+} pools that are the source of the oscillating $[Ca^{2+}]_i$. The Ca^{2+} influx is not regulated by voltage-gated Ca^{2+} channels. The data in this report are consistent with the view that the initial transient has contributions from two receptor-mediated pathways, and the oscillations are controlled either by a mechanism separate from the ones that control the initial transient or by steps whose control diverges before the point of desensitization.

INTRODUCTION

The regulation of intracellular Ca^{2+} ion activity ($[Ca^{2+}]_i$)¹ is crucial for the regulation of the contractile state of

vascular smooth muscle cells (Aksoy *et al.*, 1983; Somlyo and Himpens, 1989). It is commonly accepted that the effect of Ca^{2+} is mediated by calmodulin-dependent

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† Abbreviations used: BBS, HCO_3^- -buffered saline; BSA, bovine serum albumin; $[Ca^{2+}]_i$, free cytosolic calcium ion activity; CCD, charge-coupled device; DMEM, Dulbecco's modified Eagle's me-

dium; EC_{50} , concentration at which half the cells respond; EGTA, [ethylenebis-(oxyethylenetriol)]tetraacetic acid; fura-2/AM, fura-2 acetoxymethyl ester; HBS, HEPES-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; IP_3 , inositol 1,4,5-trisphosphate; PBS, phosphate-buffered saline.

phosphorylation by myosin light chain kinase (Miller-Hance *et al.*, 1988; Taylor and Stull, 1988; Kamm *et al.*, 1989). Along with many other hormones and neurotransmitters, ATP, a purinergic agonist, induces receptor-mediated Ca^{2+} mobilization in many cell types, including smooth muscle from several species (Benham and Tsien, 1987; Tawada *et al.*, 1987; Benham, 1989; Rembold *et al.*, 1991). During crisis or signaling events such as platelet thrombus formation or neurotransmission, ATP (and/or ADP) is released into the extracellular space and probably reaches smooth muscle cells in high micromolar concentrations (Pearson *et al.*, 1983; Slakey, 1985; Gordon, 1986; White, 1988). Although ATP and ADP exert effects via P_2 purinoceptors, which are distinguished from the adenosine-activated P_1 purinoceptors (Burnstock, 1978), ATP is rapidly dephosphorylated to ADP, AMP, and eventually to adenosine by cell-surface ectonucleotidases (Pearson, 1985; Gordon, 1986; Gordon *et al.*, 1989; Burnstock, 1990). ATP and its metabolites can elicit a wide variety of physiologic effects including secretion, ion fluxes, phospholipid metabolism, growth and differentiation in both transformed and untransformed cells (Burnstock, 1990). ATP acts as a potent relaxant in some smooth muscle cell beds (intestine and rabbit portal vein) and a potent contractor in others (urinary bladder, vas deferens, and most vascular smooth muscles) (Burnstock and Kennedy, 1985).

Tawada *et al.* (1987) showed that populations of suspended rat smooth muscle cells gave a rapid transient rise followed by sustained elevated $[\text{Ca}^{2+}]_i$ in response to ATP. Isolated smooth muscle cells from rabbit ear artery were shown to express ATP-activated Ca^{2+} -permeable plasma membrane channels that mediate a Ca^{2+} influx in response to ATP (Benham and Tsien, 1987; Benham, 1989). We have previously reported that 50 μM extracellular ATP induces a robust initial transient rise in $[\text{Ca}^{2+}]_i$ followed by semiperiodic fluctuations from a baseline level in individual porcine aortic smooth muscle cells (Linderman *et al.*, 1990). The initial rise occurs rapidly and nearly synchronously within the temporal resolution of the experiment (5 s). The post-transient oscillations are asynchronous and vary in magnitude and frequency from cell to cell.

It has been postulated that the ATP-induced changes in $[\text{Ca}^{2+}]_i$ in vascular smooth muscle cells are regulated primarily through uptake or release of Ca^{2+} from the sarcoplasmic reticulum (SR) via ryanodine- and caffeine-sensitive and inositol 1,4,5-trisphosphate- (IP_3) gated Ca^{2+} channels (Somlyo and Himpens, 1989; Berridge, 1990). IP_3 -coupled $[\text{Ca}^{2+}]_i$ mobilization in response to ATP has been shown in other cell types (Charest *et al.*, 1985; Dubyak, 1986; Haggblad and Heilbronn, 1988; Berrie *et al.*, 1989; Gonzalez *et al.*, 1989). IP_3 mediates release of Ca^{2+} from smooth muscle sarcoplasmic reticulum (Ehrlich and Watras, 1988). However, the results from rabbit ear artery smooth muscle cells described by

Benham (1989) suggest that influx of extracellular Ca^{2+} also plays a role in the generation of the initial cytosolic calcium transient rise.

Oscillatory fluctuations in $[\text{Ca}^{2+}]_i$ have been observed in a variety of cell types responding to a variety of agonists (Berridge, 1990). Recently, Rembold and Murphy demonstrated that $[\text{Ca}^{2+}]_i$ and tension oscillate in phase in isolated strips of swine carotid artery smooth muscle (Rembold and Murphy, 1990). This finding supports the notion that $[\text{Ca}^{2+}]_i$ is the major factor in control of excitation-contraction coupling in smooth muscle. Thus, a detailed understanding of the control of cytoplasmic $[\text{Ca}^{2+}]$ is an important endpoint for this study. We have used digital fluorescence imaging of individual fura-2 loaded porcine aortic smooth muscle cells to characterize and quantitate the changes in $[\text{Ca}^{2+}]_i$ in response to the signaling agent ATP. We find several features in this system that are distinct from those in other cells.

MATERIALS AND METHODS

Cell Culture

Vascular smooth muscle cells were isolated from swine aorta with minor changes in the procedure described previously (Goldman *et al.*, 1983; Linderman *et al.*, 1990). Pieces of medial tissue (1 mm) were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 0.3% collagenase at 37°C overnight. The digest was then centrifuged (3000 rpm for 2 min) and the pellet first resuspended into a growth medium consisting of DMEM supplemented with 200 U/ml penicillin, 0.2 mg/ml streptomycin, and 10% fetal calf serum and then plated in a T-25 flask. This primary cell culture was allowed to grow to confluency (7–10 d) in serum-containing growth medium at 37°C in a 5% CO_2 /95% air atmosphere. Serial subcultures (1:4) were prepared by trypsinization with 0.05% trypsin/versene solution. Experiments were performed on cells between passages 3 and 8. Approximately $1\text{--}2 \times 10^5$ cells were seeded onto #1 glass coverslips in 35-cm² culture dishes and maintained in serum-containing growth medium overnight. Each day thereafter, the cells were fed with DMEM supplemented with 5.0 $\mu\text{g}/\text{ml}$ transferrin, 6.0 $\mu\text{g}/\text{ml}$ insulin, and 0.04 mg/ml ascorbic acid (defined medium) (Libby and O'Brien, 1983). Experiments were performed ~96 h after final plating onto coverslips.

Fura-2/AM Loading

Cells were loaded with 2 μM fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes, Eugene, OR) plus 0.02% pluronic F-127 in defined medium with 0.3% dimethylsulfoxide for 30 min at 37°C in a 5% CO_2 /95% air atmosphere. The coverslips were quickly rinsed several times with HEPES-buffered saline (HBS; 20 mM HEPES, pH 7.4, 125 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , 1 g/l D-glucose) or HCO_3^- -buffered saline (BBS; 44 mM NaHCO_3 , pH 7.4, 81 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , 1 g/l D-glucose) and mounted in the flow chamber for immediate use as previously described (Linderman *et al.*, 1990).

Agonist Addition

The volume of the cell chamber is 0.8 ml. Approximately 2.5 ml of agonist in HBS or BBS at 37°C was introduced into the flow chamber reservoir as previously described (Linderman *et al.*, 1990). Solution exchange was complete in ~2 s (Cheyette and Gross, 1991). ATP and nifedipine were purchased from Sigma Chemical (St. Louis, MO).

Calcium-free HBS had 100 μ M [ethylenebis-(oxyethylenetriamino)]tetraacetic acid (EGTA) in place of the $CaCl_2$ and ~ 5.0 ml were required to completely remove the calcium when the chamber previously contained standard HBS. Low calcium HBS had 90 μ M $CaCl_2$ plus 95 μ M EGTA in place of the 1.8 mM $CaCl_2$. High K^+ HBS contained a total of 130 mM KCl and no NaCl.

Fura-2 Imaging

Fura-2 fluorescence was imaged in a Zeiss IM-35 inverted microscope (Carl Zeiss, Thornwood, NY) equipped with a 75 W Hg-Xe arc lamp (Hamamatsu, Bridgewater, NJ) and a pair of monochromators (model H20, Instruments SA, Metuchen, NJ) for fluorescence epi-illumination as described previously (Linderman *et al.*, 1990). Images of the cells were detected by a charge-coupled device (CCD) instrumentation camera (Photometrics, Tucson, AZ) under control of a Masscomp 5550 computer (Concurrent Computer, Tinton Falls, NJ). One-half of the microscope field of view was physically masked by placing a partial field stop in a focal plane conjugate to the image plane. An image of the visible half of the field was collected by illumination at 365 nm. After the shutter closed, the collected image was electronically shifted from the unmasked half of the charge-coupled device (CCD) chip to the masked half in under 1 ms. A second image of the same half of the field was then collected by illumination at 334 nm. A 399-nm long-pass barrier filter and a 395-nm dichroic mirror completed the fluorescence system. Typical exposure times were 25 and 250 ms for 365 and 334 nm, respectively. All such images were binned 4×4 whereby photoelectrons of 4 pixels in the x direction and 4 pixels in the y direction were combined to form one large pixel. Images in a time lapse sequence were taken every 3 or 5 s. From these images plus a blank-field background image, ratio images (R) were generated, where $R = 100 \times F_{334}/F_{365}$ and F_{334} (F_{365}) is the fluorescence at 334 nm (365 nm) excitation. Computations of ratio images and subsequent processing were carried out with floating point precision. Because fura-2 within cells is difficult to calibrate with accuracy and because we have previously noted that the F_{334}/F_{365} ratio is linearly related to pCa, we report here pCa levels converted directly from F_{334}/F_{365} ratios. Because we previously showed that prestimulus $[Ca^{2+}]_i$ in these cells is near 100 nM (Linderman *et al.*, 1990), we have set each pCa prestimulus level to a value of 7. The amplitudes of transients and oscillations so calibrated agree with previous findings (Linderman *et al.*, 1990).

α -Isoactin Staining

After some fura-2 imaging experiments and while the cell chamber was maintained in position on the microscope, the cells were fixed and permeabilized with 1% formaldehyde and 0.5% Triton X-100 in HBS, respectively, for 5 min each. The cells were then washed extensively with 1% BSA in HBS and incubated for 30 min with mouse monoclonal antibodies (1:400) to smooth muscle cell-specific α -isoactin (Sigma Chemical). Excess primary antibody was removed by rinsing with 1% BSA in HBS and the cells were then incubated for 30 min with fluorescein conjugated Fab' fragments of goat anti-mouse IgG (1:50) (Organon Teknica, West Chester, PA) in HBS. Excess secondary antibody was washed away and the fluorescein-labeled isoactin was detected by excitation at 436 nm with a 510-nm dichroic mirror and a 530-nm long-pass barrier filter for emission.

Criteria for Counting Initial Transients and Oscillations

For dose-response measurements, an initial response was counted if the cell displayed a $[Ca^{2+}]_i$ spike $> 20\%$ above basal level within 1 min of the ATP stimulus. Oscillations were counted as present 1) if they were found > 1 min after ATP stimulus, 2) if at least two oscillation spikes were seen, and 3) if the spikes were elevated $> 20\%$ above basal at their peaks.

Fourier Transform and Least-Squares Analysis

F_{334}/F_{365} ratios are converted to pCa and then to $[Ca^{2+}]_i$. These data are interpolated at equal time intervals and then analyzed for frequency content by fast Fourier transform analysis with the program MatLab (The Math Works, S. Natick, MA) and also with the program Igor (WaveMetrics, Lake Oswego, OR). Nonlinear least-squares fitting was accomplished with the program Igor. After conversion of F_{334}/F_{365} ratio data to $[Ca^{2+}]_i$, a single exponential was fit to the decaying phase of the initial transient in Figures 5 and 6A.

RESULTS

Fura-2 Imaging, α -Isoactin Staining and Cytosolic $[Ca^{2+}]_i$ Changes on Stimulation with 50 μ M ATP

Figure 1A shows a representative group of cells loaded with fura-2, and Figure 1C shows the cytosolic $[Ca^{2+}]_i$ changes upon stimulation of the individually marked cells with 50 μ M ATP. All the cells produced initial cytosolic $[Ca^{2+}]_i$ transient rises that appeared to be synchronous (within the 5 s time resolution of this experiment). Cells 2, 3, 4, and 6 displayed posttransient oscillations all of different frequencies soon after the cytosolic $[Ca^{2+}]_i$ returned to near baseline level, whereas cell 5 showed only one additional spike within the time frame of this experiment. Cell 1 displayed no posttransient oscillations.

The average frequency of the oscillations of cells 2, 3, 4, and 6 in this particular experiment was 0.54 min^{-1} with a range from 0.79 to 0.45 min^{-1} . The oscillations persisted for the 20-min duration of the experiment. In other experiments, oscillations induced in response to 50 μ M ATP persisted at constant frequency for as long as 30 min. We examined over 300 cells stimulated with 50 μ M ATP; 97% of all cells (357/367) gave primary transients and 92% of the cells that were imaged for enough time to oscillate (319/347) oscillated.

To ascertain the purity of our smooth muscle cell cultures, after the above experiment shown in Figure 1, A and C, we fixed and stained the same cells with antibodies against smooth muscle-specific α -isoactin. Five of the six cells in the field of view bound the antibody and showed typical patterns of F-actin stress fibers (Figure 1B). We display this particular experiment to show an example of a cell that did not stain for α -isoactin (Figure 1B; cell 6). We have similarly examined 88 cells from 11 separate experiments; 85 of those cells (97%) contained smooth muscle specific α -isoactin. Thus, the cell to cell variability and asynchronicity in $[Ca^{2+}]_i$ oscillations observed in these smooth muscle cell cultures is not due to gross contamination by other cell types. We also note here one possibly dead cell that stained for α -isoactin but did not load with fura-2 (Figure 1B; bottom left). This cell, as with any cell that did not load with fura-2/AM, was not counted in the total number of cells not responding to ATP stimulus.

Figure 1. Primary transients and oscillations of $[Ca^{2+}]_i$ in response to 50 μ M ATP and corresponding smooth muscle cell-specific α -isoactin determination. A representative coverslip of fura-2 loaded smooth muscle cells (A) was stimulated with 50 μ M ATP to induce initial transients and subsequent oscillations in cytosolic $[Ca^{2+}]_i$. Results from different cells are offset vertically for clarity (C). After fura-2 imaging, the cells were stained for smooth muscle specific α -isoactin (B). Bar = 50 μ m.

HEPES and Bicarbonate Buffer Systems Do Not Affect the $[Ca^{2+}]_i$ Changes in Response to ATP

Agonist-stimulated cytosolic $[Ca^{2+}]_i$ changes measured with the indicator fura-2 in various cell types, including rat thoracic aortic smooth muscle cells stimulated with angiotensin II, have been shown to be dependent on the buffer system used (Ganz *et al.*, 1990). Although angiotensin II stimulation produced a rapid increase in $[Ca^{2+}]_i$ that was independent of the buffer system employed, a posttransient elevation of $[Ca^{2+}]_i$ persisted in HEPES buffer, but not in CO_2/HCO_3^- buffer in populations of rat thoracic aortic smooth muscle cells. This result suggests that HEPES buffer may induce these cells to support posttransient $[Ca^{2+}]_i$ oscillations that are reflected in the apparent elevation of $[Ca^{2+}]_i$ in a cell population. We examined the effect of these different buffers on the $[Ca^{2+}]_i$ dynamics observed in ATP-stimulated porcine aortic smooth muscle cells. In response to ATP in HCO_3^- -buffered saline, the cells produced initial

transients and oscillations comparable to the $[Ca^{2+}]_i$ traces in HEPES-buffered saline (Figure 1C). Interchanging buffers during the experiment did not affect the magnitude or frequency of the oscillations. Gas exchange in the cell chamber was minimal, and a stable pH was maintained for the short duration of the experiment. These results indicate that in these smooth muscle cells, oscillations are not an artifact due to HEPES buffering. We have chosen to use HEPES-buffered saline in the remainder of the experiments to eliminate any possibility of pH drift during the longer experiments.

Initial Transients and Oscillations of $[Ca^{2+}]_i$ in Response to Low Doses of ATP

To determine the sensitivity of the ATP-mediated response, we examined the $[Ca^{2+}]_i$ transients and oscillations expressed in response to lower doses of ATP. ATP (500 nM) produced an initial rise in cytosolic $[Ca^{2+}]_i$

Figure 1. (Continued)

in all five cells shown in Figure 2A, and four of those cells showed subsequent asynchronous oscillations. One cell was able to support only one small $[Ca^{2+}]_i$ transient subsequent to the initial one within the time frame of

the experiment (Figure 2A). In a different set of five cells, 250 nM ATP also produced primary transients in all cells (Figure 2B). However, oscillations were apparent in only three of the five cells and were not as robust as

Figure 2. Initial transients and oscillations of $[Ca^{2+}]_i$ in response to low doses of ATP. Three representative coverslips were stimulated at time 0 with ATP doses of 500 nM (A), 250 nM (B), and 50 nM (C). Five cells from each experiment are shown here. There were a total of 5 cells imaged on coverslip (A), 5 cells on coverslip (B), and 10 cells on coverslip (C).

the ones seen in cells responding to 500 nM ATP. At 50 nM ATP, only two of the five cells presented in Figure 2C produced initial transients, whereas one cell gave a $[Ca^{2+}]_i$ spike that was delayed by ~ 100 s after ATP stimulus. The two immediate initial transients were both followed by one smaller $[Ca^{2+}]_i$ transient, but no continuous oscillations were observed. It is clear that the two characteristic $[Ca^{2+}]_i$ responses (initial transient and oscillations) observed at a saturating dose of ATP were produced by cells in a dose-dependent manner.

Dependence of Cellular Response on ATP Concentration

It is conventional to measure sensitivity to agonists by examining the magnitude of response evoked by various agonist doses. The magnitude of response observed in a population of cells will have contributions from the time course, and magnitude of response of individual cells and from the number of cells responding. Rather

than determining the magnitude of response, we examined the dose dependence of the number of individual cells responding. Figure 3, A and B, shows the fraction of cells responding to ATP with initial transients in the presence or absence of extracellular Ca^{2+} . Figure 3C shows the fraction of cells displaying oscillations in cytosolic $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} as a function of ATP concentration. In assuming the functional form for the fraction of cells responding at a particular dose, we have assumed that the interaction between ATP and its receptor is monovalent, that it is in the steady state within the time that a particular cell responds, and that the probability of response of a cell is proportional to the fraction of purinoceptors activated. The solid lines in Figure 3, A–C, represents weighted least-squares fits of the data to the function

$$\text{fraction of cells responding} = 1/(1 + EC_{50}/[ATP])$$

where EC_{50} is the concentration of ATP at which one-half of the cells respond. The best-fit EC_{50} for producing the initial spike in the presence of extracellular Ca^{2+} is 0.11 ± 0.04 (SD) μM (Figure 3A) and in the absence of extracellular Ca^{2+} is 0.88 ± 0.34 μM (Figure 3B). Oscillations are produced with an EC_{50} of 2.6 ± 1.4 μM (Fig-

Figure 3. Dependence of cellular response on agonist concentration. Fraction of cells displaying a primary transient rise in the presence (A) and absence (B) of external calcium; the fraction of cells displaying oscillations in $[Ca^{2+}]_i$ (C); and frequency of the oscillations in response to varying doses of extracellular ATP (D). In A, total of 685 cells observed; in B, total of 281 cells observed; in C, total of 528 cells observed; in D, total of 161 cells observed. Doses were grouped at half-log intervals to reduce statistical fluctuations in the data in A and C. Error bars in A–C show uncertainty associated with counting n events; uncertainty = $\pm n^{-1/2}$. Error bars in D show the SD associated with each data point.

Figure 4. Fourier transform analysis of distinct and noisy oscillations. Two representative cells from the same experiment showing different oscillating cytosolic $[Ca^{2+}]_i$ patterns (A and B) and their respective fast Fourier transform spectra (C and D).

ure 3C). The error bars represent the expected relative uncertainty in counting n cells at each ATP dose level and equal $\pm n^{-1/2}$. Thus for small numbers of cells, the relative uncertainty is large.

Approximately 10% of cells examined showed one or more spikes before stimulation. This phenomenon is reminiscent of the spiking or oscillatory $[Ca^{2+}]_i$ transients in other cell types (Holl *et al.*, 1988; Ratan *et al.*, 1988; Weissberg *et al.*, 1989). In counting cells responding with oscillations to ATP (Figure 3C), we considered only cells that did not show spontaneous spikes in $[Ca^{2+}]_i$ before ATP stimulation.

The range of frequencies of oscillations observed as a function of agonist concentration is shown in Figure 3D. Although there is a small tendency for the frequency to be greater at high doses of ATP than it is at threshold doses, we have observed oscillations with frequencies as slow as 0.36 min^{-1} and as fast as 2.9 min^{-1} ($n = 100$) to the same dose of $50 \mu\text{M}$ ATP. Discrete oscillations are maintained even at very high agonist concentrations. Dramatic variations in the amplitude of the oscillations within a single cell or to varying doses of ATP were rare. Variation in oscillation frequency in a single cell was occasionally observed, with some cells showing a decrease and some showing an increase in frequency with time.

In response to $50 \mu\text{M}$ ATP, some cells oscillate at a clearly visible frequency whereas others exhibit repeated

transient spikes in calcium without an obvious regular frequency. To examine the possibility that the calcium transients in the latter cells were the sum of periodic and nonperiodic transients, we analyzed the data for frequency content by Fourier transform. For a typical cell exhibiting clear oscillations, the Fourier analysis identified, as expected, a strong fundamental oscillation frequency (Figure 4, A and C). Higher harmonics of the fundamental frequency are observed and likely are due to the nonsinusoidal form of the $[Ca^{2+}]_i$ transients in the oscillations. For a second cell from the same experiment but with much less apparent regularity in the response, Fourier analysis identified a characteristic frequency at 1.4 min^{-1} (Figure 4, B and D). The data from 18 cells whose response to $50 \mu\text{M}$ ATP was like that shown in Figure 4B were examined by Fourier analysis, and in every case a dominant frequency within the range shown in Figure 3D was uncovered. Thus, apparently "noisy" cells also have $[Ca^{2+}]_i$ oscillations of regular frequency.

Initial Transients Are Desensitized in the Presence of ATP

The initial transient $[Ca^{2+}]_i$ rise was produced rapidly upon $50 \mu\text{M}$ ATP addition and began to decay within 5–10 s (Figure 5). We examined whether the desensi-

Figure 5. Desensitization of the primary transient. A representative cell from one experiment in which 8 cells were stimulated with $50 \mu\text{M}$ ATP in HBS. Fifteen seconds later, cells were washed with HBS alone. Subsequent readdition of $50 \mu\text{M}$ ATP was 15 s after the HBS wash. Inset: higher time resolution during the initial transient with the washout period highlighted in bold. $\blacktriangle$, data points after ATP readdition, shifted to the left by 15 s to show that the declining phase of the response is nearly continuous with the slope established before the wash occurred. A least-squares fit to a single exponential function for the declining phase is superimposed on the shifted data.

tization of the initial transient was agonist dependent. An HBS wash to remove extracellular ATP, 15 s after an initial 50 μ M ATP stimulation, resulted in an immediate drop in $[Ca^{2+}]_i$. Readdition of 50 μ M ATP 15 s after the wash restored $[Ca^{2+}]_i$ to near, but slightly below, the level before ligand removal. Eight other cells in this experiment responded similarly. The inset in Figure 5 shows that the decline of the $[Ca^{2+}]_i$ signal from its peak level is reestablished when ATP is restored to the medium. Data points after the restoration of ATP are also plotted as filled triangles, offset 15 s to the left in the inset. They form an apparently smooth, continuous decline from the portion of the curve before ATP washout. The time course of the decay of the initial transient for this concatenated data was fit to a single exponential function ($[Ca^{2+}]_i = Ca_{\text{basal}} + Ca_{\text{peak}}e^{-kt}$ where Ca_{basal} is the prestimulus $[Ca^{2+}]_i$, Ca_{peak} is the amplitude of the peak of the transient, and k is the first order decay constant). The single exponential decay fit the data well with $k = 0.020 \pm 0.002 \text{ s}^{-1}$ as is shown by the best-fit curve in the inset. These data indicate that desensitization of the initial $[Ca^{2+}]_i$ transient stops in the absence of agonist. If the declining phase of the initial transient is due to the depletion of intracellular stores, then the store refilling process must be slow compared with the release. Because the restoration of a full magnitude initial response subsequent to a prior ATP-stimulated response in the continuous presence of extracellular Ca^{2+} requires a full 5 min, if store depletion determines desensitization, then it must require 5 min for complete refilling. This time course is unreasonably long (see below). Therefore, it is more likely that the desensitization is controlled at some level other than depletion of the internal Ca^{2+} stores.

In the Absence of External Ca^{2+} the Initial Transient, But Not Oscillations, Occurs in Response to 50 μ M ATP; Addition of Extracellular Ca^{2+} Initiates the Oscillations

Tawada *et al.* (1987) observed an elevated $[Ca^{2+}]_i$ level in the late phase of the initial $[Ca^{2+}]_i$ transient in the presence of external calcium in rat aortic smooth muscle cell suspensions. They postulated that this elevation is due to accelerated Ca^{2+} influx in the presence of external Ca^{2+} . We investigated whether Ca^{2+} influx contributes to either the initial transient or the subsequent oscillations. Smooth muscle cells were first stimulated with 50 μ M ATP in the presence of extracellular Ca^{2+} (Figure 6A). The extracellular ATP was removed and the cells were allowed to resensitize for 5 min. The cells were then stimulated with 50 μ M ATP in the absence of external Ca^{2+} . The transient produced in the absence of extracellular Ca^{2+} was clearly of lesser duration than that produced in the presence of extracellular Ca^{2+} (inset, Figure 6A). The reverse order of additions gave

Figure 6. Absence of external Ca^{2+} prevents oscillations but not the primary transient in response to 50 μ M ATP; readdition of extracellular Ca^{2+} initiates the oscillations. A representative cell from one experiment in which cells were stimulated with 50 μ M ATP followed by the removal of extracellular ATP (A). After sufficient time (5 min) had elapsed for resensitization, the cells were restimulated with 50 μ M ATP in the absence of external Ca^{2+} , resulting in another $[Ca^{2+}]_i$ transient. Inset: higher time resolution during the first transient (●) with the second transient (O) overlaid and matched to the time of ATP addition, revealing the prolonged late phase of the transient in the presence of external Ca^{2+} . Least-squares fits to the decaying phases of the two transients are overlaid (—). Similar behavior was observed in nine other cells in this experiment. Six individual cells from two separate experiments were stimulated with 50 μ M ATP ($t = 75 \text{ s}$) in the absence of extracellular Ca^{2+} (removed at $t = 50 \text{ s}$) to produce the initial spike in $[Ca^{2+}]_i$ (B). Readdition of 1.8 mM Ca^{2+} in the continued presence of external ATP ($t = 550 \text{ s}$) immediately initiated $[Ca^{2+}]_i$ oscillations. From three separate coverslips with a total of 26 cells, 6 cells gave one additional posttransient spike in the absence of external Ca^{2+} and 19 cells produced oscillations after Ca^{2+} addition.

identical results. As in Figure 5, least-squares fits to a single exponential decay function are superimposed on the traces in the inset. The best-fit exponential decay constants are $0.012 \pm 0.008 \text{ s}^{-1}$ in the presence of 1.8

mM extracellular Ca^{2+} and $0.038 \pm 0.003 \text{ s}^{-1}$ in its absence. As a control, this experiment was repeated but in the continuous presence of extracellular Ca^{2+} . The magnitude and time course of the two transients were identical. Although cells treated with $50 \mu\text{M}$ ATP in the absence of external Ca^{2+} were able to support the abbreviated initial transient, they were not able to support oscillations (Figure 6B). We observed only an isolated $[Ca^{2+}]_i$ spike in one cell following the primary transient, indicative of that cell's ability to support some postinitial $[Ca^{2+}]_i$ rise, likely due to release from partially depleted internal stores (Figure 6B). However, readdition of extracellular calcium in the continued presence of ATP immediately activated the oscillations (Figure 6B). On restoration of extracellular Ca^{2+} , oscillations initiated synchronously in most cells and, after two to three cycles, became asynchronous.

Extracellular ATP and Extracellular Ca^{2+} Are Required to Maintain the Oscillations

We examined the requirement for extracellular ATP and extracellular Ca^{2+} to maintain established oscillations. Four separate coverslips of cells (Figure 7, A–D) were stimulated with $50 \mu\text{M}$ ATP at $t = 0 \text{ s}$ in buffer containing 1.8 mM Ca^{2+} to establish the primary transients and oscillations. A subsequent wash with fresh ATP in the same buffer did not affect the magnitude or frequency of the oscillations (Figure 7A); however, we observed a $[Ca^{2+}]_i$ transient concomitant with the buffer change that was sometimes slightly greater in magnitude than the oscillations but never as large as that of the initial primary transient. We have seen this response in many other sets of cells stimulated with ATP, but never in cells washed with buffer containing no agonist. The wash may remove an extracellular inhibitory factor produced by the cells in the presence of ATP that acts to suppress the rise in cytosolic $[Ca^{2+}]_i$ (see DISCUSSION). On removal of ATP, oscillations were immediately abolished and $[Ca^{2+}]_i$ levels returned to baseline (Figure 7B).

In the continued presence of ATP, reduction of extracellular $[Ca^{2+}]$ from 1.8 mM to $17 \mu\text{M}$ caused a reduction in the frequency of $[Ca^{2+}]_i$ oscillations, similar to the results from phenylephrine-stimulated hepatocytes (Kawanishi *et al.*, 1989). In the continued presence of ATP, the removal of extracellular Ca^{2+} (Ca^{2+} -free HBS + $100 \mu\text{M}$ EGTA) abolished the oscillations but with a brief delay (Figure 7D). In previous reports describing results in other cell types, removal of extracellular Ca^{2+} decreased the frequency of oscillations but did not abolish them (McMillian *et al.*, 1987; Kawanishi *et al.*, 1989; Berridge, 1990). The data shown in Figure 7D show that, in ATP-stimulated porcine smooth muscle cells, although intracellular pools contain enough Ca^{2+} for one spike, continued oscillations are dependent

Figure 7. Oscillations of $[Ca^{2+}]_i$ in response to $50 \mu\text{M}$ ATP are abolished on the removal of extracellular ATP and/or extracellular Ca^{2+} . (A–D) representative cells were first stimulated with $50 \mu\text{M}$ ATP at 0 s to initiate primary transients and oscillations. For clearer resolution, only the oscillations are shown here with the plots offset vertically for clarity. ATP solution was replaced with fresh ATP at $t = 505 \text{ s}$ (A); ATP was replaced with HBS at $t = 490 \text{ s}$ (B); ATP was replaced with ATP in HBS containing $17 \mu\text{M}$ Ca^{2+} at $t = 555 \text{ s}$ (C); ATP was replaced with ATP in Ca^{2+} -free HBS + $100 \mu\text{M}$ EGTA at $t = 505 \text{ s}$ (D).

on extracellular Ca^{2+} , presumably to refill the internal Ca^{2+} stores.

In other experiments we found that the cells, after responding to ATP in the absence of Ca^{2+} , are still capable of eliciting a second initial transient 5 min after resensitization in the absence of ATP and in the continued absence of external Ca^{2+} . When stimulated with a third dose of ATP after a further 5 min resensitization in the absence of ATP and in the continued absence of extracellular Ca^{2+} , only a few cells displayed small transients whereas other cells gave no response. These data demonstrate that the Ca^{2+} pool released during the initial transient is not fully depleted by the first initial transient but that it is depleted after three transients.

Oscillations Are Not Mediated by L-Type Voltage-Gated Ca^{2+} Channels

Nifedipine, an L-type voltage-gated Ca^{2+} channel blocker, has been shown to inhibit voltage-gated Ca^{2+} channels in rabbit ear artery smooth muscle cells (Benham and Tsien, 1987). Benham and Tsien showed, however, that $1 \mu\text{M}$ ATP had no effect on the voltage-gated Ca^{2+} channels of either T or L type in the same cells (Benham, 1989; Benham and Tsien, 1987). The $[Ca^{2+}]_i$ oscillations established by ATP stimulus of porcine aortic smooth muscle cells are not affected by the presence of $5 \mu\text{M}$ nifedipine (Figure 8), suggesting that

Figure 8. Oscillations are not regulated through voltage-gated Ca^{2+} channels. Cells were stimulated with $50 \mu\text{M}$ ATP at $t = 50$ s. In the continual presence of ATP, $5 \mu\text{M}$ nifedipine was added at $t = 460$ s (---). Two cells are shown; similar behavior was observed in five cells examined in two different experiments.

voltage-gated calcium channels are not involved in the regulation of the $[\text{Ca}^{2+}]_i$ oscillations. In separate experiments, isotonic K^+ solution (130 mM) similarly did not affect the oscillations. Therefore, the oscillations do not appear to be regulated by changes in membrane potential or voltage-gated Ca^{2+} channels. This result also supports the notion that the ATP-stimulated $[\text{Ca}^{2+}]_i$ oscillations arise from IP_3 stimulated Ca^{2+} release rather than transmembrane Ca^{2+} flux, other than the flux required to refill the intracellular Ca^{2+} stores.

DISCUSSION

Individual aortic smooth muscle cells respond in a dose-dependent manner to ATP with an initial transient rise followed by periodic oscillations in cytosolic $[\text{Ca}^{2+}]_i$ that are asynchronous from cell to cell. Careful quantitative study of the initial $[\text{Ca}^{2+}]_i$ transient and oscillations in individual cells in the presence and absence of extracellular Ca^{2+} permits us to describe the minimum number of discrete responses to ATP needed to explain the complexity of the behavior. The data in this report are consistent with the view that the initial transient has contributions from two receptor-mediated pathways, and the oscillations are controlled either by a mechanism separate from the ones that control the initial transient or by steps whose control diverges before the point of desensitization.

There are two different sensitivities to ATP for the initial $[\text{Ca}^{2+}]_i$ transient as shown by the different EC_{50} s to produce the spike ($0.11 \pm 0.04 \mu\text{M}$ ATP in the pres-

ence of extracellular Ca^{2+} and $0.88 \pm 0.34 \mu\text{M}$ ATP in the absence of extracellular Ca^{2+}) (Figure 3, A and B). Tawada *et al.* (1987) observed a similar EC_{50} of $0.1 \mu\text{M}$ ATP for $[\text{Ca}^{2+}]_i$ responses in rat aortic smooth muscle cell suspensions in the presence of external Ca^{2+} . Tawada *et al.* also observed that IP_3 production was stimulated by ATP. In rabbit ear artery smooth muscle cells, low amplitude transmembrane cation currents were found with $0.01 \mu\text{M}$ ATP stimulus whereas maximal response was achieved at $1 \mu\text{M}$ ATP (Benham and Tsien, 1987). These authors did not observe any contribution of stored Ca^{2+} to the initial spike in $[\text{Ca}^{2+}]_i$. These data in aggregate suggest that in pig aortic smooth muscle cells, the initial transient is composed of Ca^{2+} influx via ATP-gated Ca^{2+} channels as well as Ca^{2+} release from internal Ca^{2+} stores. The mechanism that controls the influx of external Ca^{2+} is about 10 times more sensitive to extracellular ATP than the one that controls internal store release.

Further evidence that the ATP-induced initial transient has contributions from two distinct mechanisms lies in the shape of the initial $[\text{Ca}^{2+}]_i$ response. In the absence of external Ca^{2+} , the initial transient has a shorter time course (Figure 6A). This effect is not due to depletion of internal stores because a cell that responded to ATP in the absence of extracellular Ca^{2+} , when allowed to resensitize to ATP in the continued absence of external Ca^{2+} , was capable of eliciting another transient to a second dose of ATP. Thus, the shortened time course of the transient in the absence of external Ca^{2+} is due to lack of direct Ca^{2+} influx, not store emptying.

Subsequent to the initial transient in response to ATP, $[\text{Ca}^{2+}]_i$ oscillations are generated. Several features we observed suggest that the mechanism that controls the oscillations diverges significantly from the ones that control the initial transient. First, the EC_{50} for the initial transient in the presence of external Ca^{2+} is about 20-fold lower than that for the oscillations (Figure 3, A and C). Second, the whole initial transient response rapidly desensitizes while oscillations can persist >30 min in the continued presence of extracellular ATP (Figure 1C). Finally, the relationship between the internal pools that supply the initial transient and the oscillations is not simple. In the absence of external Ca^{2+} , an initial transient is still produced but oscillations are not (Figure 6B). This is not due to the complete emptying of the intracellular pools because the cells are still capable of eliciting another initial transient after resensitization in the absence of ATP and in the continued absence of external Ca^{2+} . This indicates that there are two Ca^{2+} pools that contribute to the initial transient, one of which also provides Ca^{2+} for the oscillations. In the absence of external Ca^{2+} , the ATP-induced initial transient depletes the pool that generates the oscillations while not completely emptying the other pool. The second pool

is responsible for the second release of Ca^{2+} after resensitization in the absence of extracellular Ca^{2+} . When stimulated with a third dose of ATP still in the continued absence of extracellular Ca^{2+} , only a few cells displayed small transients whereas other cells gave no response, indicative of depletion of the second pool. The absolute dependence of both the initial transient and the oscillations on the presence of extracellular ATP indicates that continued purinergic receptor occupancy is required for the maintenance of either response. The rapid abolition of either $[Ca^{2+}]_i$ response on removal of ATP also indicates that ATP rapidly dissociates from its receptor(s).

One model that fits these data involves at least two independent ATP-sensitive receptors on the cell surface that transduce $[Ca^{2+}]_i$ signals in swine aortic smooth muscle cells. This model is supported by the dose sensitivity differences between the initial transient (0.11 μ M) and the oscillations (2.6 μ M). In this model, the higher affinity receptor would desensitize over times of the order of 1 min, whereas the lower affinity receptor would not desensitize. One might postulate that the higher affinity receptor activates Ca^{2+} channels in the plasma membrane, whereas the lower affinity receptor activates release of Ca^{2+} from intracellular stores sensitive to IP_3 . It is not necessary for the two receptors to be different polypeptides; they might be forms of the same molecule that differ in posttranslational modification (glycosylation or phosphorylation) or in association with other regulatory molecules.

A second model involves a time- and agonist-dependent change in state of a single receptor type. In this model, the initial transient is produced by the receptor in its high-affinity state. Concomitant with the transduction of the initial $[Ca^{2+}]_i$ transient, the receptor undergoes a change to its low-affinity state that transduces the oscillatory phase of the $[Ca^{2+}]_i$ response. The conversion of the high- to low-affinity state must be nearly complete because we find that the initial transient totally desensitizes. If two receptor types of differing ligand affinities are present on the cell surface and if the two receptor types can interconvert freely (i.e., they are at thermodynamic equilibrium), then only one macroscopic ligand binding affinity will be found. Thus, in this model, the high-affinity receptor class cannot be in equilibrium with the low-affinity class during the initial transient phase of the response. This would seem to be at odds with the data of Figure 3 in which the EC_{50} of the initial transient in the absence of extracellular Ca^{2+} is not significantly different from that of the oscillations.

It is thought that most agonist-stimulated $[Ca^{2+}]_i$ oscillations are mediated by a rise in intracellular $[IP_3]$ that mobilizes stored intracellular calcium (Berridge, 1990). Although the $[Ca^{2+}]_i$ oscillations seen in ATP-stimulated porcine aortic smooth muscle cells are similar in many respects to those seen in other cell types responding to

various agonists, most other oscillatory $[Ca^{2+}]_i$ responses saturate, with high frequency oscillations collapsing to a persistent elevation of $[Ca^{2+}]_i$ at high agonist dose. Rat parotid acinar cells responding to acetylcholine (Gray, 1988), as well as porcine aortic smooth muscle cells responding to extracellular ATP (Figure 3D), show very little dependence of oscillation frequency on agonist dose level. Thus, most models of $[Ca^{2+}]_i$ oscillations (Meyer and Stryer, 1988; Goldbeter *et al.*, 1990; Parker and Ivorra, 1990; Cuthbertson and Chay, 1991) that predict agonist dependence of the frequency, do not accurately model the ATP- or acetylcholine-mediated responses in these cell types. This fact coupled with the observation of occasional spontaneous $[Ca^{2+}]_i$ oscillations in these and other cells implies that more than one mechanism of regulation of $[Ca^{2+}]_i$ oscillations is likely to be found.

A potential complication of extracellular ATP-mediated signaling in porcine aortic smooth muscle cells is the presence of ectonucleotidase activity capable of hydrolyzing $ATP \rightarrow ADP \rightarrow AMP \rightarrow$ adenosine (Gordon *et al.*, 1989). Although ATP is in sufficient excess in the bulk of the solution in the microscope cell chamber so that it is not significantly depleted by such ectonucleotidase activity over the typical time course of an experiment, the concentration of ATP and its metabolites may be altered from bulk solution values in the vicinity of the cell surface. The fact that ATP-mediated $[Ca^{2+}]_i$ oscillations persist undiminished for tens of minutes, even for low doses of ATP, indicates that cell-surface depletion of ATP does not play a dominant role in the control of the oscillations. Our observation that a chamber wash with fresh stimulant solution produces a $[Ca^{2+}]_i$ transient (Figure 7, A–D, and 8) only if ATP is present before, during, and after the wash suggests that an ATP metabolite, or a factor secreted by the cells, may play an inhibitory role in the regulation of $[Ca^{2+}]_i$ in these cells.

The finding that the fraction of cells responding to ATP is dependent on the dose, together with variation in the shape of oscillatory spikes, emphasizes that individual cells may vary strikingly from one another quantitatively, although all express the same fundamental response. Variation is not due to cells being at different points in the cell cycle; all cells have been on defined medium, in which they are quiescent (Libby and O'Brien, 1983; Xiong *et al.*, 1991), for 3 d before an experiment. The cell to cell variability of the $[Ca^{2+}]_i$ responses to extracellular ATP is also not due to gross contamination by other cell types. Contributing factors to the difference in response from cell to cell may include differing receptor numbers, variable ectonucleotidase activity or proximity to receptor (clearing the locally available pools of ATP), and cell-to-cell variation in extent of expression of the mediator of any step in the signal transduction pathway.

Because $[Ca^{2+}]_i$ is the primary regulator of contraction in smooth muscle (Rembold and Weaver, 1990), it is clear that differential regulation of two $[Ca^{2+}]_i$ responses, a transient initial response and prolonged oscillations, could differentially regulate the contractile state of individual muscle cells within a tissue. Rembold and Murphy's (1990) demonstration that oscillations of $[Ca^{2+}]_i$ track oscillations of force in porcine carotid medial tissue indicates that the ATP-mediated $[Ca^{2+}]_i$ oscillations that we have described may play an important role in the regulation of vascular tone in arterial smooth muscle.

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