British Journal of Pharmacology (1996) 118, 543-548

Dissociation of P_2 purinoceptor-mediated increase in intracellular Ca^{2+} level from myosin light chain phosphorylation and contraction in rat aorta

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1 The effects of P_2 agonists, adenosine-5'-triphosphate (ATP), α,β-methylene-adenosine-5'-triphosphate (α,β-me-ATP) and adenosine-5-O-(3-thiotriphosphate) (ATPγS), on the intracellular free Ca^{2+} level ($[Ca^{2+}]_i$), myosin light chain (MLC) phosphorylation and force of contraction were examined in vascular smooth muscle of rat aorta.

2 ATP (0.1 μM - 1 mM), α,β-me-ATP (0.1 - 100 μM) and ATPγS (1 - 100 μM) induced transient increases followed by sustained increase in $[Ca^{2+}]_i$. The effects of these agonists were concentration-dependent. Compared with the effects of a high concentration of KCl (17.5 - 72.4 mM), the contractions induced by these P_2 purinoceptor agonists were smaller at a given $[Ca^{2+}]_i$.

3 In the absence of extracellular Ca^{2+} (with 0.5 mM EGTA), ATPγS (10 μM) induced large transient increase in $[Ca^{2+}]_i$ with only small contraction in Ca^{2+}-free solution. In contrast, α,β-me-ATP (10 μM) induced only a very small increase in $[Ca^{2+}]_i$ and contraction.

4 ATP (1 mM), α,β-me-ATP (10 μM) and ATPγS (10 μM), added during stimulation with 0.1 μM noradrenaline, induced additional and transient increases in $[Ca^{2+}]_i$ which were also not associated with contraction.

5 High K^+ (72.4 mM) increased MLC phosphorylation with a similar time course to that of the increase in $[Ca^{2+}]_i$ (peak phosphorylation was 56% when $[Ca^{2+}]_i$ increased to 100%). In contrast, the time course of the increase in MLC phosphorylation due to ATP (1 mM) did not coincide with that of the large increases in $[Ca^{2+}]_i$; MLC phosphorylation increased to only 31% when $[Ca^{2+}]_i$ increased to 163%. The MLC phosphorylation due to α,β-me-ATP (10 μM) and ATPγS (10 μM), measured at peak $[Ca^{2+}]_i$, were only 19% and 14%, respectively, irrespective of a large increase in $[Ca^{2+}]_i$ (138% and 188%, respectively).

6 The absence of a clear relationship between P_2-purinoceptor-mediated increase in $[Ca^{2+}]_i$ (either by Ca^{2+} influx or Ca^{2+} release) and MLC phosphorylation or force generation appears to imply that elevation in $[Ca^{2+}]_i$ does not contribute to these responses.

Keywords: ATP; P_2 purinoceptors; cytosolic Ca^{2+} level; force; vascular smooth muscle

Introduction

Extracellular ATP mediates various biological processes, including platelet aggregation, neurotransmission, cardiac function, smooth muscle contraction and vascular tone (Gordon, 1986; Olsson & Pearson, 1990; El-Moatassim *et al.*, 1992; Burnstock, 1993; Dubyak & El-Moatassim, 1993). In blood vessels, ATP induces either contraction or relaxation (Kennedy & Burnstock, 1985; Kennedy *et al.*, 1985). The diversity of the effects of ATP on smooth muscle contractility may be due to the multiple receptor subtypes of the vascular bed (smooth muscle and endothelium) which may be coupled to different signal transduction pathways.

Purinoceptors are classified into P_1 and P_2 subtypes. The P_2 purinoceptors in blood vessels are further classified into P_{2X}, P_{2Y} and P_{2U} subtypes (Burnstock & Kennedy, 1985; O'Connor, 1992; Abbracchio *et al.*, 1993; Dubyak & El-Moatassim, 1993). The P_{2X} purinoceptor is linked to non-selective, Ca^{2+}-permeable cation channels (Benham & Tsien, 1987), whereas the P_{2Y} and P_{2U} purinoceptors are linked to phospholipase C and Ca^{2+} release (Abbracchio *et al.*, 1993). Activation of the P_{2Y} and/or P_{2U} purinoceptors in endothelial cells induces an endothelium-dependent vasorelaxation, whereas activation of the P_{2X} purinoceptors in vascular smooth muscle induces a vasoconstriction of vascular smooth muscle (O'Connor, 1992).

In rat aortic smooth muscle, on the other hand, ATP has been shown to mobilize intracellular Ca^{2+} by an increase in inositol 1,4,5-trisphosphate (IP_3) (Tawada *et al.*, 1988), suggesting that ATP activates not only the P_{2X} purinoceptor but also the other receptor subtype(s) which is linked to phosphatidyl inositide breakdown. Recently, we have demonstrated that ATP increases intracellular free Ca^{2+} level ($[Ca^{2+}]_i$) not only by Ca^{2+} influx but also by Ca^{2+} release from intracellular stores in rat aortic smooth muscle (Kitajima *et al.*, 1993), and that Ca^{2+} release from intracellular stores is mediated by the P_{2U} purinoceptor whereas Ca^{2+} influx is mediated by both the P_{2X} and P_{2U} purinoceptors by comparing the order of potency of several P_2 agonists (Kitajima *et al.*, 1994). Unexpectedly, however, the ATP-induced increase in $[Ca^{2+}]_i$, which is larger than the high K^+-induced increase, results in only a small contraction (Kitajima *et al.*, 1993). In order to examine further the dissociation between the increases in $[Ca^{2+}]_i$ and force, we examined the effects of ATP, α,β-me-ATP and ATPγS on $[Ca^{2+}]_i$, myosin light chain (MLC) phosphorylation and contractile force in rat aortic smooth muscle.

Methods

Preparations and solutions

Male Wistar rats (250 - 300 g) were stunned and bled and the thoracic aorta was dissected. After removal of fat and con-

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nective tissues, the aorta was cut into helical strips approximately 2 mm in width and 8 mm in length. Endothelium was removed by gently rubbing the intimal surface with a finger moistened with physiological salt solution (PSS). This procedure changed neither the magnitude of high K^+-induced contraction nor the threshold concentration of KCl required to induce contraction, suggesting that the smooth muscle layer was not damaged. In such tissue, a releaser of endothelium-derived relaxing factor (1 μM carbachol) did not change the contraction induced by 0.1 μM noradrenaline, suggesting that the endothelium had been removed completely. Normal PSS contained (in mM): NaCl, 136.9, KCl 5.4; $CaCl_2$, 1.5; $MgCl_2$ 1.0; $NaHCO_3$ 20.0; glucose 5.5 and EDTA, 0.01. The solution with elevated K^+ was made by replacing NaCl with equimolar KCl. Ca^{2+}-free solution was made by removing $CaCl_2$ and adding 0.5 mM EGTA. These solutions were maintained at 37°C and aerated with 95% O_2 and 5% CO_2. Muscle force was recorded isometrically with a force displacement transducer.

Measurement of $[Ca^{2+}]_i$ and muscle force

$[Ca^{2+}]_i$ was measured according to the method described by Ozaki *et al.* (1987) and Sato *et al.* (1988) using fura-2 (Grynkiewicz *et al.*, 1985). Muscle strips were exposed to the acetoxymethyl ester of fura-2 (5 μM) in the presence of 0.02% cremophor EL for 4 to 5 h at room temperature. The muscle strip was then transferred to the muscle bath integrated in the fluorimeter (CAF-100) and illuminated alternately (48 Hz) with two excitation wave lengths (340 nm and 380 nm). Fluorescence at 500 nm was measured, and the ratio of the fluorescence induced by these two wavelengths was calculated and used as an indicator of $[Ca^{2+}]_i$. Absolute $[Ca^{2+}]_i$ was not calculated because the dissociation constant of fura-2 for Ca^{2+} may change in smooth muscle cells (Konishi *et al.*, 1988). Ratios of fluorescence in the resting muscle and that in the depolarized muscle with high K^+ (72.4 mM), added just before the experiment, were considered as 0 and 100%, respectively. High K^+ (72.4 mM)-induced contraction, measured at 5 min, was considered as a reference response (100%).

Measurement of MLC phosphorylation

The extent of MLC phosphorylation was measured according to the method of Word *et al.* (1991). Strips of rat aorta were quickly frozen in liquid nitrogen and then homogenized with 10% trichloroacetic acid and 10 mM DTT. The homogenate was centrifuged at 10,000 *g* for 1 min and the pellet was washed with diethyl ether and then suspended in urea-glycerol buffer for electrophoretic analysis of light chain phosphorylation by imunoblotting. Polyacrylamide gels (10%) containing glycerol (40%, vol/vol) were pre-electrophoresed for 30 min at 300 V at room temperature. Reservoir buffer contained Tris-base (20 mM) and glycine (23 mM), pH 6.8; thioglycolate 2.3 mM and DTT 2.3 mM) were also included in the upper reservoir. Samples (5–10 mg protein) were electrophoresed for 300 V for 90 min at room temperature. Protein was transferred to nitrocellulose for 60 min at 2 mA cm^{-2} at room temperature. Nonphosphorylated and phosphorylated forms of MLC were localized on nitrocellulose paper with antibody against bovine tracheal MLC, peroxidase-conjugated goat anti-rabbit IgG, and 4-chloro 1-naphthol as substrate for the peroxidase. Relative amounts of nonphosphorylated and phosphorylated MLC were quantified by densitometry of imunostained nitrocellulose blots. The level of MLC phosphorylation is shown by the ratio of phosphorylated to total (phosphorylated and non-phosphorylated) MLC multiplied by 100.

Chemicals

Chemicals used were adenosine-5'-triphosphate (ATP) (Yamasa Shoyu, Tokyo, Japan), α,β-methylene-adenosine-5'-triphosphate (α,β-me-ATP), carbamylcholine chloride (carbachol) (Sigma Chemicals, St. Louis, U.S.A.), adenosine-5-O-(3-thiotriphosphate) (ATPγS) (Boehringer Mannheim Yamanouchi, Tokyo, Japan), noradrenaline bitartrate (Wako Pure Chemicals, Osaka, Japan), phentolamine mesylate (Nihon Ciba-Geigy, Osaka, Japan), acetoxymethyl esters of fura-2 (Dojindo Laboratories, Kumamoto, Japan) and cremophor EL (Nacarai Chemicals, Tokyo, Japan). The pH of ATP, α,β-me-ATP and ATPγS stock solutions were adjusted to 7.4 by adding NaOH.

Statistics

The numerical data were expressed as mean ± s.e. mean. Differences were evaluated by Student's *t* test, and the *P* value less than 0.05 was considered to be statistically significant.

Results

Correlation between $[Ca^{2+}]_i$, MLC phosphorylation and force

Figure 1a–c shows typical results on the changes in $[Ca^{2+}]_i$ and force in response to 1 mM ATP, 10 μM α,β-me-ATP and 10 μM ATPγS in rat isolated aorta. ATP (1 mM), α,β-me-ATP (10 μM) and ATPγS (10 μM) induced a rapid and large increase in $[Ca^{2+}]_i$ (162.8 ± 7.0%, 138.4 ± 4.2% and 188.0 ± 27.9% of high K^+-induced response, respectively; $n=4$ each) followed by a small sustained increase which reached a plateau after about 10 min. These agonists induced only smaller contractions (15.2 ± 2.5%, 32.1 ± 5.5% and 10.5 ± 2.5% of high K^+-induced response, respectively; $n=4$ each).

ATP (10 μM–1 mM), α,β-me-ATP (0.01–100 μM) and ATPγS (1–100 μM) induced a concentration-dependent increases in $[Ca^{2+}]_i$ and force. As summarized in Figure 1d, these P_2 agonists induced smaller contraction than high K^+ (17.5, 27.7 and 72.4 mM) for a given increase in $[Ca^{2+}]_i$.

Western blotting of the homogenate after immunoprecipitation shows that high K^+ (72.4 mM) induced a substantial increase in MLC phosphorylation while ATP (1 mM) induced only a slight increase in MLC phosphorylation when measured at peak $[Ca^{2+}]_i$ (Figure 2). Figure 3 shows the time courses of the increases in $[Ca^{2+}]_i$ and MLC phosphorylation due to high K^+ (72.4 mM) and ATP (1 mM). High K^+ induced a rapid increase in $[Ca^{2+}]_i$, reaching the maximum at approximately 5 s, which was then slightly decreased reaching a steady state level. The level of MLC phosphorylation in control tissue was 9.3 ± 1.3% ($n=6$). High K^+ rapidly increased the MLC phosphorylation to 55.6 ± 5.8% within 10 s and maintained this level up to 60 s. ATP (1 mM) also rapidly increased $[Ca^{2+}]_i$ which reached a maximum (162.8 ± 7.0, $n=4$) at 3–12 s. ATP induced a small sustained increased in $[Ca^{2+}]_i$ (37.7 ± 2.0, $n=4$) after 60 s. In response to the rapid increase in $[Ca^{2+}]_i$, MLC phosphorylation increased to 30.5 ± 2.5% ($n=6$) at 6 s and maintained this level up to 60 s.

Figure 4 summarizes the effects of high K^+ (72.4 mM), ATP (1 mM), α,β-me-ATP (10 μM) and ATPγS (10 μM) on $[Ca^{2+}]_i$, myosin light chain (MLC) phosphorylation (measured at peak $[Ca^{2+}]_i$) and contractile force. Treatment of the tissue with 72.4 mM K^+ increased $[Ca^{2+}]_i$ to 100% ($n=4$), MLC phosphorylation to 55.6 ± 5.8% ($n=4$) and force to 100% ($n=4$). ATP (1 mM), α,β-me-ATP (10 μM) and ATPγS (10 μM) also increased $[Ca^{2+}]_i$ to 162.8 ± 7.0% ($n=4$), 138.4 ± 4.2% ($n=4$) and 188.0 ± 27.9% ($n=4$), respectively. Unlike high K^+ solution, ATP, α,β-me-ATP and ATPγS induced only small, if any, increase in MLC phosphorylation to 30.5 ± 2.5% ($n=4$; $P<0.01$), 18.8 ± 0.9% ($n=4$; $P<0.01$) and 13.7 ± 2.3% ($n=4$; not significantly different from the resting level), respectively. These P_2 receptor agonists also induced only small contractions as has been described.

Responses in Ca^{2+}-free solution

As shown in Figure 5, muscle strips were first treated with high K^+ (72.4 mM) for 5 min to load storage sites with Ca^{2+}

(Karaki *et al.*, 1979). After the Ca^{2+} loading, external Ca^{2+} was removed which resulted in decrease in $[Ca^{2+}]_i$ below the resting level. After the removal of Ca^{2+} for 2 min, α,β-me-ATP and ATPγS were added. In the absence of external Ca^{2+}, 10 μM α,β-me-ATP induced only a small increase in $[Ca^{2+}]_i$ (6.7±3.5%, $n=4$) with no detectable force (Figure 5a). In contrast, 10 μM ATPγS induced a large transient increase in $[Ca^{2+}]_i$ (122.4±19.8%) and a small contraction (7.6±2.2%, $n=4$) (Figure 5b). ATP (1 mM) also induced a large transient increase in $[Ca^{2+}]_i$ (137.6±15.6%) and small force (6.2±2.1%, $n=4$) in Ca^{2+}-free solution as has been reported by Kitajima *et al.* (1993).

Effects on noradrenaline-induced $[Ca^{2+}]_i$ and contraction

As shown in Figure 6a, noradrenaline (0.1 μM) induced a rapid increase in $[Ca^{2+}]_i$ followed by a decrease to a new steady level and induced a sustained contraction. ATP (1 mM), added during the sustained increases in $[Ca^{2+}]_i$ and contraction, induced a transient increase in $[Ca^{2+}]_i$ by 106.3±11.6% ($n=4$) with no detectable phasic increase in force. Although $[Ca^{2+}]_i$ returned to the original level, muscle tension was decreased to 83.4±4.1% ($n=4$). Similarly, addition of α,β-me-ATP (10 μM) and ATPγS (10 μM), induced additional increases in $[Ca^{2+}]_i$ (86.1±20.6%, $n=4$, and 86.5±9.2%, $n=4$, respectively) with no detectable change in force (Figure 6b and c). However, these P_2 agonists did not change the noradrenaline-induced contraction.

Discussion

In the endothelium-free rat aorta, a P_{2X} selective agonist, α,β-me-ATP, and ATPγS induced a large and transient increase in $[Ca^{2+}]_i$ with only a small contraction. It has been shown that the increases in $[Ca^{2+}]_i$ due to α,β-me-ATP and ATPγS are largely attributable to Ca^{2+} influx and Ca^{2+} release, respectively, in the rat aorta (Kitajima *et al.*, 1994). These results indicate that neither Ca^{2+} influx nor Ca^{2+} release stimulated by P_2 purinoceptors is effectively coupled to smooth muscle contraction.

It is generally accepted that the increase in $[Ca^{2+}]_i$ activates MLC kinase which phosphorylates MLC to induce smooth muscle contraction (Kamm & Stull, 1985). Measurement of MLC phosphorylation showed that ATP, α,β-me-ATP and ATPγS induced only a small increase in the amount of phosphorylated MLC, suggesting that increase in $[Ca^{2+}]_i$ due to activation of P_2 purinoceptors is not effectively utilized to phosphorylate MLC.

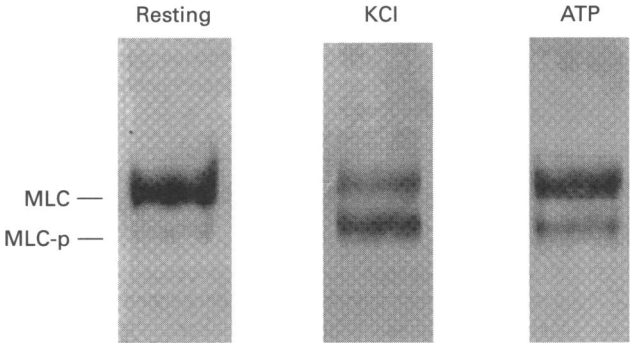

Figure 2 Representative result of Western blotting of non-phosphorylated and phosphorylated MLC after stimulation of tissue with high K^+ (72.4 mM) and ATP (1 mM) for 10 and 6 s, respectively. MLC, non-phosphorylated MLC; MLC-p, phosphorylated MLC.

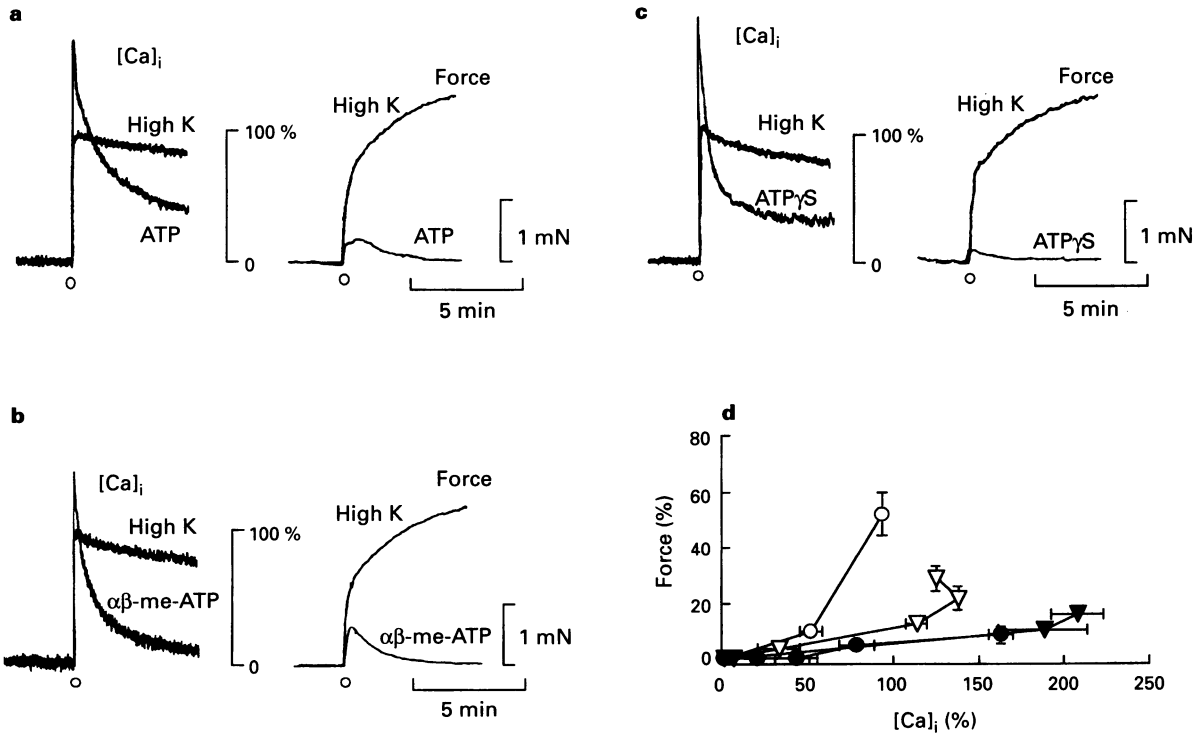

Figure 1 Effects of ATP (a), α,β-me-ATP (b) and ATPγS (c) on $[Ca^{2+}]_i$ (left) and contraction (right) in rat isolated aorta without endothelium. ATP (1 mM), α,β-me-ATP (10 μM) and ATPγS (10 μM) induced a transient increase in $[Ca^{2+}]_i$ followed by a sustained increase. High K^+ (72.4 mM)-induced responses, obtained in the same muscle strip before adding ATP, α,β-me-ATP or ATPγS, are overlaid for comparison. $[Ca^{2+}]_i$-force relationship for high K^+, α,β-me-ATP and ATPγS are also shown in (d). Contractile force induced by high K^+ (5.4, 17.5, 27.7, 72.4 mM) (○), ATP (0.1, 1, 10, 100 and 1000 μM) (●), α,β-me-ATP (0.01, 0.1, 1, 10, 100 μM) (▽) or ATPγS (1, 10, 100 μM) (▼) is plotted against peak $[Ca^{2+}]_i$ (approximately 12 s after the addition of drugs). Each point represents mean±s.e. mean of 4 experiments.

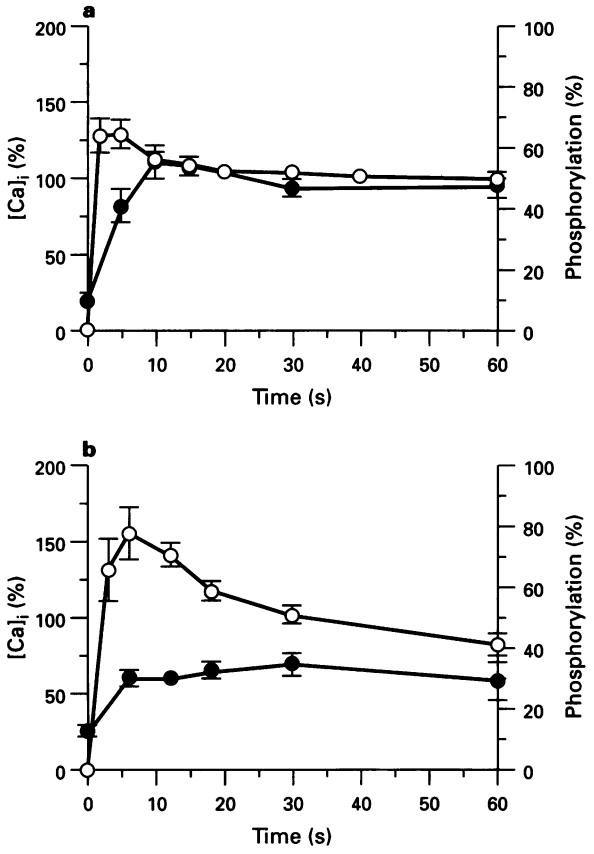

Figure 3 Time course of the increase in $[Ca^{2+}]_i$ (○) and MLC phosphorylation (●) induced by high K^+ (72.4 mM) (a) and ATP (1 mM) (b). Each points represents mean ± s.e. mean of 6 experiments.

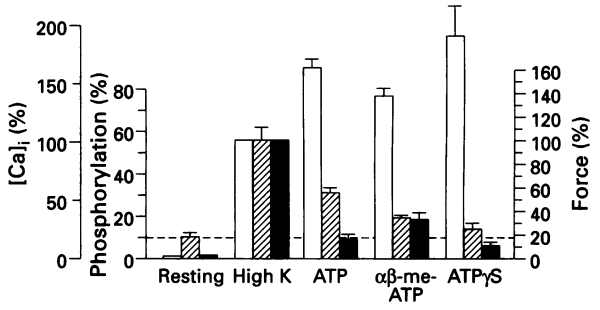

Figure 4 Changes in $[Ca^{2+}]_i$ (open columns), MLC phosphorylation (hatched columns) and force (solid columns) due to 72.4 mM high K^+, 1 mM ATP, 10 μM α,β-me-ATP and 10 μM ATPγS. MLC phosphorylation was measured at peak $[Ca^{2+}]_i$ (at 10 s for high K^+ and at 6 s for purines). Although all the stimulants induce significant increase in $[Ca^{2+}]_i$, only high K^+ induced a substantial increase in MLC phosphorylation. Values are expressed as mean ± s.e. mean of 4 experiments.

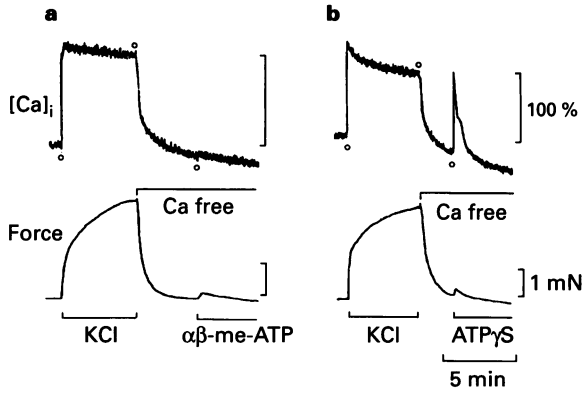

Figure 5 Effects of α,β-me-ATP (a) and ATPγS (b) on $[Ca^{2+}]_i$ (upper trace) and contraction (lower trace) in the absence of external Ca^{2+}. After the muscle was treated with high K^+ (72.4 mM) for 5 min, external Ca^{2+} was removed (with 0.5 mM EGTA) for 2 min and than α,β-me-ATP or ATPγS was added. ATPγS (10 μM) transiently increased $[Ca^{2+}]_i$ while α,β-me-ATP (10 μM) had no effect.

There are at least three possibilities for the dissociation of MLC phosphorylation from increased $[Ca^{2+}]_i$. The first possibility is that the P_2 purinoceptor agonists may decrease the Ca^{2+} sensitivity of contractile elements. In order to examine this possibility, we observed the effects of the P_2 agonists on the noradrenaline-induced contraction. α,β-me-ATP and ATPγS did not inhibit the noradrenaline-induced contraction. Therefore, the activation of P_2 receptors does not seem to inhibit the Ca^{2+} sensitivity of contractile elements. On the other hand, ATP partially (by 17%) inhibited the contraction with no significant change in $[Ca^{2+}]_i$. Khakh *et al.* (1995) suggested that the low potency of contractile effect of ATP is attributable to the rapid breakdown by ecto-nucleotidase in rat vas deferens. A breakdown product of ATP, adenosine, activates adenylate cyclase to produce cyclic ATP (Olsson & Pearson, 1990; Rembold *et al.*, 1991), which decreases the Ca^{2+} sensitivity of contractile elements (Karaki, 1989; Abe & Karaki, 1989; Ozaki *et al.*, 1992). Rembold *et al.* (1991) have also demonstrated in the swine carotid artery that ATP induced large and transient increases in $[Ca^{2+}]_i$ (as measured by aequorin-luminescence) with only a small transient elevation in force, and further suggested that the low Ca^{2+} sensitivity of MLC phosphorylation with ATP stimulation as compared with histamine. In the rat aorta, decrease in Ca^{2+} sensitivity of contractile elements in the presence of ATP has been confirmed by showing that adenosine (1 mM) inhibited the contraction induced by noradrenaline (0.1 μM) or high K^+ (72.4 mM) with only a small decrease in $[Ca^{2+}]_i$ (unpublished observation). However, this mechanism may not be responsible for the dissociation between $[Ca^{2+}]_i$ and force due to α,β-me-ATP because this compound is poorly hydrolysable. Similarly, this mechanism may not play a role in the dissociation between $[Ca^{2+}]_i$ and force due to ATPγS because the inhibitory effect of adenosine is observed at concentrations above 100 μM which is much higher than the concentration of ATPγS (10 μM) used in this study.

The second possibility is that purinoceptor agonists may increase $[Ca^{2+}]_i$ not only in the cytoplasm but also in the cellular space where contractile elements are absent, such as the sub-membrane space, mitochondria or nucleus. Himpens *et al.* (1992) have reported that ATP produces a greater increase of Ca^{2+} in the nucleus than in the cytoplasm in pig cultured aortic cells. Since the volume of the nucleus is 20–30% in vascular smooth muscle cells, the increase in fura-2 signals in the nucleus would tend to overestimate the cytoplasmic $[Ca^{2+}]_i$. Although the volume of the space may be significantly smaller than the nucleus, a possible high Ca^{2+} area associated with sub-membrane space (Chen & Van Breemen, 1993) cannot be ruled out for the mechanism of dissociation between $[Ca^{2+}]_i$ and force. Recently, the 'Ca^{2+} sparks' have been visibly detected near the surface membrane of vascular smooth muscle cell, which cause vasorelaxation through the activation of K^+ channels with little effect on spatially averaged $[Ca^{2+}]_i$ (Nelson *et al.*, 1995).

In the present study, the fluorescence of the Ca^{2+} indicator was measured from the outer surface of the tissue whilst contractility was measured from the whole tissue. Thus, it is possible that the dissociation between $[Ca^{2+}]_i$ and force is a

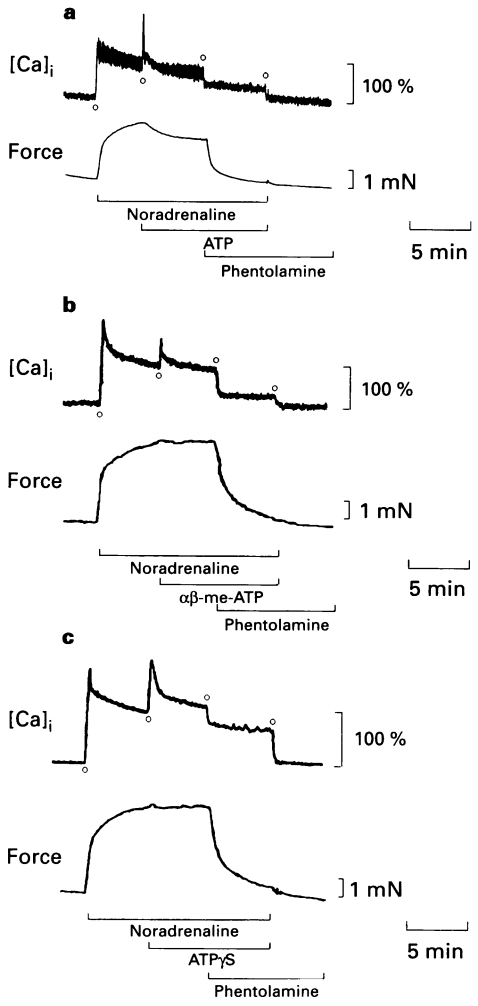

Figure 6 Effects of ATP (a), α,β-me-ATP (b) and ATPγS (c) on $[Ca^{2+}]_i$ (upper trace) and force (lower trace) in noradrenaline-stimulated aorta. After treatment with noradrenaline (0.1 μM) for 5 min, ATP (1 mM), α,β-me-ATP (10 μM) and ATPγS (10 μM) were added. After application of each P_2 agonist for 5–7 min, phentolamine (1 μM) was added to inhibit the effects of noradrenaline.

consequence of access/diffusion differences between P_2 agonists and K^+. Previously, however, we have shown that it is possible to measure $[Ca^{2+}]_i$ of endothelial cells in intact rat aorta either from the intimal or adventitial surface (Sato *et al.*, 1990). This result suggests that the Ca^{2+} signal does not originate only from the superficial muscle layer but also from the inner layers of the preparation. Since the preparation used in this study was without endothelium, both K^+ and P_2 agonists are able to diffuse from both surfaces. Furthermore, in response to the rapid increase in $[Ca^{2+}]_i$ due to the application of ATP, we first detected the increase MLC phosphorylation at 6 s and could not observe any delayed peak in the level of MLC phosphorylation. These results do not support the suggestion that the dissociation between $[Ca^{2+}]_i$ and MLC phosphorylation is due to the difference in the access/diffusion among the agonists used.

Ca^{2+} is the ubiquitous second messenger modifying various cell functions. Dorn *et al.* (1992) reported that prostaglandin $F_{2\alpha}$-induced increase in $[Ca^{2+}]_i$ correlates better with protein synthesis than with the contractile response in the rat aortic cells. Erlinge *et al.* (1993) showed that ATP-induced Ca^{2+} influx correlated well with ATP-induced thymidine incorporation. Malam-Souley *et al.* (1993) reported that *c-fos* and *c-myc* mRNA levels are increased by ATP at μM concentration ranges in the rat aortic cells. It is necessary to clarify which cellular Ca^{2+} compartment is modified by these agonist or how Ca^{2+} signals modulate these agonist-mediated cell functions.

In conclusion, the absence of a clear relationship between P_2-purinoceptor-mediated increase in $[Ca^{2+}]_i$ (either by Ca^{2+} influx or Ca^{2+} release) and MLC phosphorylation or force generation appears to imply that elevation in $[Ca^{2+}]_i$ does not contribute to these responses. Further studies are necessary to substantiate this finding.

This work was partly supported by Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture, Japan. We thank Dr James T. Stull (University of Texas Dallas, South-Western Medical Center) for supplying us with the antibody of MLC.

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(Received August 10, 1995
Revised January 26, 1996
Accepted February 9, 1996)