The effects of ATP and α,β -methylene-ATP on cytosolic Ca^{2+} level and force in rat isolated aorta

¹Satoshi Kitajima, Hiroshi Ozaki & Hideaki Karaki

Department of Veterinary Pharmacology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Yayoi 1-1-1, Tokyo 113, Japan

1 The effects of a non-selective P_2 -receptor agonist ATP and a selective P_{2X} -receptor agonist α,β -methylene-ATP on intracellular free Ca^{2+} level ($[\text{Ca}^{2+}]_i$) and force were examined in rat isolated aorta without endothelium.

2 Both ATP (1–1000 μM) and α,β -methylene-ATP (0.1–100 μM) induced transient increase followed by small sustained increase in $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner. Compared with the force induced by a high concentration of KCl, the force induced by α,β -methylene-ATP was smaller and that induced by ATP was much smaller at a given $[\text{Ca}^{2+}]_i$.

3 An L-type Ca^{2+} channel blocker, verapamil (10 μM), completely inhibited the high K^+ -stimulated $[\text{Ca}^{2+}]_i$ and force. Verapamil partially inhibited the transient and sustained increases in $[\text{Ca}^{2+}]_i$ induced by 10 μM α,β -methylene-ATP and the sustained increase but not the transient increase induced by 1 mM ATP.

4 In the absence of extracellular Ca^{2+} (with 0.5 mM EGTA) 1 mM ATP caused transient increase in $[\text{Ca}^{2+}]_i$ while 10 μM α,β -methylene-ATP was ineffective

5 ATP, but not α,β -methylene-ATP, increased the tissue adenosine 3':5'-cyclic monophosphate (cyclic AMP) level.

6 These data suggest that ATP and α,β -methylene-ATP increase $[\text{Ca}^{2+}]_i$ by an activation of both L-type and non-L-type Ca^{2+} channels. In addition, ATP, but not α,β -methylene-ATP, increases $[\text{Ca}^{2+}]_i$ by a release of Ca^{2+} from an intracellular Ca^{2+} store. Possible reasons are discussed as to why the increase in $[\text{Ca}^{2+}]_i$ due to ATP and α,β -methylene-ATP resulted in only a small contraction.

Keywords: ATP; α,β -methylene-ATP; vascular smooth muscle; cytosolic Ca^{2+} level; contraction; Ca^{2+} sensitivity

Introduction

In smooth muscles, ATP is released from purinergic nerve endings as a transmitter or from cholinergic and adrenergic nerve endings as a cotransmitter and is involved in the physiological regulation of smooth muscles (for review, see Burnstock, 1990; Olsson & Pearson, 1990; El-Moatassim *et al.*, 1992). Although the responses mediated by ATP are usually inhibitory in oesophagus, stomach, small and large intestines, they are excitatory in vas deferens and urinary bladder (see, Gordon, 1986). In blood vessels, ATP induces either contraction or relaxation (Kennedy *et al.*, 1985; Kennedy & Burnstock, 1985; Ralevic *et al.*, 1991). The diversity of the effects of ATP on smooth muscle contractility may be due to the multiple receptor subtypes coupled to different signal transduction pathways.

Purinoreceptors are classified into P_1 and P_2 subtypes. P_2 receptors are further classified into P_{2X} and P_{2Y} subtypes (Burnstock & Kennedy, 1985). P_{2X} receptors are linked to non-selective cation channels whereas P_{2Y} receptors are linked to phospholipase C (Kennedy, 1990). ATP also activates a 'nucleotide' receptor in vascular smooth muscle (Chinellato *et al.*, 1992) which is also coupled to phospholipase C (O'Connor, 1992). In the phaeochromocytoma-derived PC12 cell line, ATP has been shown to elicit an inward current (Nakazawa *et al.*, 1990), Ca^{2+} influx and inositol 1,4,5-trisphosphate (IP_3) accumulation (Fasolato *et al.*, 1990). The existence of an ATP-activated Ca^{2+} -permeable cation channel has recently been reported in smooth muscles (Benham & Tsien, 1987; Honore *et al.*, 1989). Rembold *et al.* (1991) have demonstrated that ATP transiently increases $[\text{Ca}^{2+}]_i$ as measured by aequorin-luminescence, myosin light chain phosphorylation and force in swine carotid artery and suggested that ATP induces contraction by an increase in

transmembrane Ca^{2+} influx through activation of the P_{2X} receptor. On the other hand, in rat cultured aortic smooth muscle cells, ATP has been shown to mobilize intracellular Ca^{2+} by an increase in IP_3 (Tawada *et al.*, 1988). In canine gastric smooth muscle, ATP increases both Ca^{2+} release and protein kinase C activity (Ozaki *et al.*, 1992). In this muscle, products of phosphatidylinositol turnover inhibit spontaneous rhythmic contractions by reducing Ca^{2+} channel activity, possibly through the activation of Ca^{2+} -dependent K^+ channels (IP_3 -induced Ca^{2+} release from sarcoplasmic reticulum) and/or the direct inhibition of Ca^{2+} channels (phosphorylation by protein kinase C). These results suggest that different subtypes of purinoreceptors in smooth muscle are coupled to different signal transduction pathways.

In order to examine further the role of purinoreceptors in vascular smooth muscle, we observed the effects of ATP on $[\text{Ca}^{2+}]_i$, force, and Ca^{2+} sensitivity of contractile elements. For these purposes, we compared the effects of ATP with α,β -methylene-ATP, a poorly hydrolyzable and P_{2X} -specific analogue of ATP.

Methods

Male Wistar rats (250–300 g) were stunned and bled and the thoracic aorta was dissected. After removal of fat and connective tissues, the aorta was cut into helical strips approximately 2 mm in width and 8 mm in length. The 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, 1 μM car-

¹ Author for correspondence.

bachol, a releaser of endothelium-derived relaxing factor, did not change the contraction induced by $0.1 \mu\text{M}$ noradrenaline, suggesting that the endothelium had been removed. Normal PSS contained (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. A solution with elevated K^+ was made by replacing NaCl with equimolar KCl. A 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.

$[\text{Ca}^{2+}]_i$ 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 ($5 \mu\text{M}$) in the presence of 0.02% cremophor EL for 3 to 4 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 $[\text{Ca}^{2+}]_i$. Absolute $[\text{Ca}^{2+}]_i$ was not calculated because the dissociation constant of fura-2 for Ca^{2+} may change in smooth muscle cells

(Konishi *et al.*, 1988; Karaki, 1989). Ratios of fluorescence in the resting muscle and that in the depolarized muscle with elevated external K^+ (72.4 mM) were considered as 0 and 100%, respectively.

Tissue adenosine 3':5'-cyclic monophosphate (cyclic AMP) content was measured with a competitive radioimmunoassay (Abe & Karaki, 1988). Subsequent to an incubation, muscle strips were frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid solution. After centrifugation twice at 3,000 r.p.m., trichloroacetic acid in the supernatant was removed by washing with water-saturated ether, and the succinylated cyclic AMP was assayed by a competitive radioimmunoassay (Yamasa Shoyu, Tokyo, Japan). Tissue guanosine 3':5'-cyclic monophosphate (cyclic GMP) content was measured by competitive radioimmunoassay.

Chemicals used were verapamil hydrochloride, α,β -methylene-adenosine 5'-triphosphate (α,β -methylene-ATP) (Sigma Chemicals, St. Louis, U.S.A.), adenosine 5'-triphosphate (ATP) (Yamasa Shoyu, Tokyo, Japan), forskolin (Nihon Kayaku, Tokyo, Japan), acetoxymethyl esters of fura-2 (Dojindo Laboratories, Kumamoto, Japan) and cremophor EL (Nacalai Chemicals, Tokyo, Japan). The pH of ATP and α,β -methylene-ATP stock solutions was adjusted to 7.4 by addition of NaOH.

Figure 1 Typical recordings of the effects of ATP (a) and α,β -methylene-ATP (b) on $[\text{Ca}^{2+}]_i$ (upper trace) and contraction (lower trace) in rat isolated aorta loaded with a Ca^{2+} indicator, fura-2. ATP (1 mM) and α,β -methylene-ATP (10 μM) induced transient increase in $[\text{Ca}^{2+}]_i$ followed by sustained increase. As compared with the effects of high K^+ , ATP and α,β -methylene-ATP induced sustained increases in $[\text{Ca}^{2+}]_i$ with smaller contractions. Lower panels show concentration-dependent effects of ATP (c) and α,β -methylene-ATP (d) on $[\text{Ca}^{2+}]_i$ and force. The peak $[\text{Ca}^{2+}]_i$ (○) and force (○) and the values of $[\text{Ca}^{2+}]_i$ (●) and force (●) at 10 min are plotted against the concentrations. Each point represents mean $\pm$ s.e. mean of 4 experiments.

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

Results

Effects of ATP and α,β -methylene-ATP on $[\text{Ca}^{2+}]_i$ and force

Figure 1 shows the typical changes in $[\text{Ca}^{2+}]_i$ and force in response to 1 mM ATP and 10 μM α,β -methylene-ATP in rat isolated aorta. ATP (1 mM) induced a rapid increase in $[\text{Ca}^{2+}]_i$ ($162.8 \pm 7.0\%$, $n = 4$) followed by a small sustained increase which reached a plateau after about 10 min ($37.7 \pm 2.0\%$, $n = 4$). Although 1 mM ATP increased $[\text{Ca}^{2+}]_i$ to a level higher than that induced by high K^+ solution, it induced only a small transient force ($15.2 \pm 2.5\%$, $n = 4$). α,β -Methylene-ATP (10 μM) also induced a rapid increase in $[\text{Ca}^{2+}]_i$ ($138.4 \pm 4.2\%$, $n = 4$) followed by a small sustained increase which reached a plateau after about 10 min ($11.5 \pm 1.7\%$, $n = 4$). As with ATP, α,β -methylene-ATP induced a smaller force ($32.1 \pm 5.5\%$, $n = 4$) than high K^+ .

Figure 1c and d shows the effects of various concentrations of ATP (0.1–1000 μM) and α,β -methylene-ATP (0.01–100 μM) on $[\text{Ca}^{2+}]_i$ and force. Peak Ca^{2+} transient and force increased with the increase in the concentrations of ATP and α,β -methylene-ATP.

Effects of verapamil on $[\text{Ca}^{2+}]_i$ and force induced by ATP or α,β -methylene-ATP

The mechanism of the increases in $[\text{Ca}^{2+}]_i$ due to ATP and α,β -methylene-ATP was examined by use of the L-type Ca^{2+} channel blocker, verapamil (Figure 2a,b). Verapamil (10 μM) completely inhibited the high K^+ (72.4 mM)-induced increase in $[\text{Ca}^{2+}]_i$ and force ($n = 4$, data not shown). In the presence

of 10 μM verapamil, 1 mM ATP-induced peak $[\text{Ca}^{2+}]_i$ and force were not affected. Verapamil, on the other hand, inhibited the 10 μM α,β -methylene-ATP-induced peak $[\text{Ca}^{2+}]_i$ from 138.4 ± 4.2 to $79.3 \pm 4.1\%$ ($n = 4$, $P < 0.01$) and force from 32.1 ± 5.5 to $7.4 \pm 1.6\%$ ($n = 4$, $P < 0.01$). Verapamil also inhibited the sustained component of $[\text{Ca}^{2+}]_i$ in the presence of α,β -methylene-ATP more strongly (from 11.5 ± 1.7 to $3.8 \pm 0.3\%$, $n = 4$, at 10 min) than those in the presence of ATP (from 37.7 ± 2.0 to $31.3 \pm 1.4\%$, $n = 4$, at 10 min), although the sustained component of force was not affected by verapamil.

Effects of ATP and α,β -methylene-ATP on $[\text{Ca}^{2+}]_i$ and force in Ca^{2+} -free solution

Muscle strips were first treated with high K^+ (72.4 mM) for 5 min to load Ca^{2+} in storage sites (Karaki *et al.*, 1979). After the Ca^{2+} loading, external Ca^{2+} was removed which decreased $[\text{Ca}^{2+}]_i$ below the resting level. As shown in Figure 3, ATP (1 mM) induced a transient increase in $[\text{Ca}^{2+}]_i$ ($116.2 \pm 22.1\%$, $n = 4$) and force ($7.0 \pm 1.8\%$, $n = 4$) in the absence of external Ca^{2+} . Sequential addition of α,β -methylene-ATP (10 μM) did not change $[\text{Ca}^{2+}]_i$ or force.

In the absence of external Ca^{2+} , 10 μM α,β -methylene-ATP induced only a small increase in $[\text{Ca}^{2+}]_i$ ($6.7 \pm 3.5\%$, $n = 4$) with no detectable force. Sequential addition of ATP (1 mM) induced a transient increase in $[\text{Ca}^{2+}]_i$ ($122.1 \pm 14.2\%$, $n = 4$) with a small increase in force ($9.8 \pm 3.1\%$, $n = 4$).

Effects of ATP after the desensitization of the $\text{P}_{2\text{X}}$ receptor

It has been shown that α,β -methylene-ATP has the ability to desensitize $\text{P}_{2\text{X}}$ purinoceptors (Burnstock & Kennedy, 1985). As demonstrated in Figure 4, the increase in $[\text{Ca}^{2+}]_i$ and force induced by α,β -methylene-ATP (10 μM) became smaller

Figure 2 Effects of verapamil (●) on the increases in $[\text{Ca}^{2+}]_i$ and force induced by ATP (○) (a) and α,β -methylene-ATP (○) (b). After treatment of the muscle with verapamil (10 μM) for 7 min, ATP (1 mM) or α,β -methylene-ATP (10 μM) was added. Each point represents mean $\pm$ s.e.mean of 4 experiments. Significantly different from respective control with * $P < 0.05$ and ** $P < 0.01$.

Figure 3 Effects of ATP (a) and α,β -methylene-ATP (b) on $[Ca^{2+}]_i$ (upper trace) and contraction (lower trace) in the absence of external Ca^{2+} . After treatment of tissue with high K^+ (72.4 mM) for 5 min, external Ca^{2+} was removed (with 0.5 mM EGTA) for 2 min. ATP (1 mM) and α,β -methylene-ATP (10 μ M) were then added. ATP transiently increased $[Ca^{2+}]_i$ although α,β -methylene-ATP showed little effect. In the presence of ATP or α,β -methylene-ATP, α,β -methylene-ATP or ATP, respectively, was added sequentially. ATP transiently increased $[Ca^{2+}]_i$ even after treatment of the tissue with α,β -methylene-ATP.

Figure 4 Effects of ATP on $[Ca^{2+}]_i$ and contraction after repeated application of α,β -methylene-ATP (10 μ M) for 5 min at 5 min intervals. During the third application of α,β -methylene-ATP, ATP (1 mM) was added which produced an additional increase in $[Ca^{2+}]_i$ and contraction.

during repeated applications. The peak $[Ca^{2+}]_i$ and the peak force due to the third application of α,β -methylene-ATP were only $51.9 \pm 4.9\%$ ($n = 4$) and $57.4 \pm 14.8\%$ ($n = 4$), respectively, of those due to the first applications of α,β -methylene-ATP. Addition of 1 mM ATP during the third application of α,β -methylene-ATP induced additional increase in $[Ca^{2+}]_i$ to $78.4 \pm 6.4\%$ and force to $177.6 \pm 50.0\%$ ($n = 4$) of those obtained without α,β -methylene-ATP pretreatment.

Effects of ATP and α,β -methylene-ATP on cyclic AMP and cyclic GMP levels

Figure 5 summarizes the effects of ATP (1 mM) and α,β -methylene-ATP (10 μ M) on cyclic AMP content. ATP (1 mM) increased cyclic AMP content (to 183.4% in 1 min and 145.3% in 5 min). In contrast, α,β -methylene-ATP (10 μ M) did not increase cyclic AMP content. Treatment of the muscle for 5 min with an activator of adenylate cyclase, forskolin (1 μ M), increased cyclic AMP content to 352.8%.

Figure 5 Effects of ATP and α,β -methylene-ATP on cyclic AMP contents. Muscle strips were treated with ATP (1 mM), α,β -methylene-ATP (10 μ M) or forskolin (FK, 1 μ M) for 1 or 5 min. Significantly different from the control with * $P < 0.05$ and ** $P < 0.01$, respectively.

Cyclic GMP content of resting tissue was 139.2 ± 0.7 pmol g⁻¹ tissue ($n = 5$). ATP (1 mM) did not change the cyclic GMP content (131.1 ± 13.1 pmol g⁻¹ tissue 1 min⁻¹, $n = 5$ and 161.4 ± 6.4 pmol g⁻¹ tissue 5 min⁻¹, $n = 5$). α,β -methylene-ATP also did not change the cyclic GMP content (139.2 ± 12.6 pmol g⁻¹ tissue 1 min⁻¹, $n = 5$ and 127.8 ± 8.1 pmol g⁻¹ tissue 5 min⁻¹, $n = 5$).

Discussion

The results of the present study demonstrated that ATP elicited a large and transient increase followed by a small and sustained increase in $[Ca^{2+}]_i$. Verapamil did not inhibit the ATP-induced initial transient increase in $[Ca^{2+}]_i$ but it partially (by approximately 15%) inhibited the sustained increase. It has been demonstrated that, in rat aorta, noradrenaline, prostaglandin F_{2 α} and endothelin-1 induced sustained increases in $[Ca^{2+}]_i$ and verapamil almost complete-

ly inhibited these increases ($>80\%$; Hori *et al.*, 1992). These results suggest that the mechanism by which ATP increases $[\text{Ca}^{2+}]_i$ is different from that activated by other receptor agonists. It has been shown that ATP opens non-selective cation channels which are permeable to Ca^{2+} and not sensitive to Ca^{2+} channel blockers (Benham & Tsien, 1987; Honore *et al.*, 1989). Thus, it is possible that the ATP-induced increase in $[\text{Ca}^{2+}]_i$ may be partly mediated by the opening of this type of channel. In the absence of external Ca^{2+} , ATP induced a rapid and transient increase in $[\text{Ca}^{2+}]_i$. These results suggest that ATP releases Ca^{2+} from intracellular storage sites and this may partly be responsible for the initial phase of the ATP-induced response. This ATP-induced Ca^{2+} release might be induced by the increase in IP_3 , as has been reported in cultured vascular smooth muscle cells (Tawada *et al.*, 1988). Although we did not identify the type of non- P_{2X} receptor in the present experiments, either the P_{2Y} purinoceptor or nucleotide receptor may be mediating the Ca^{2+} release due to ATP (see Introduction). However, the P_{2Y} purinoceptors seem to be less likely because this type of receptor is generally found on the vascular endothelium (Burnstock & Kennedy, 1985) which was removed in the present study.

α,β -Methylene-ATP also induced an initial transient increase in $[\text{Ca}^{2+}]_i$ followed by a sustained increase. In contrast to the effects of ATP, verapamil partially inhibited $[\text{Ca}^{2+}]_i$ at the initial phase. Another difference was that α,β -methylene-ATP did not induce a transient increase in $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free solution. These results suggest that α,β -methylene-ATP does not release Ca^{2+} from storage sites and that it activates a non-selective cation channel. It was also found that repeated application of α,β -methylene-ATP induced partial desensitization which is one of the characteristics of P_{2X} purinoceptors (Burnstock & Kennedy, 1985). These results suggest that α,β -methylene-ATP activates mainly P_{2X} receptors, opens non-selective cation channels, and increases influx of Ca^{2+} (and possibly other cations) through this type of channel that in turn depolarizes membrane to activate voltage-dependent Ca^{2+} channels. The transient increase in $[\text{Ca}^{2+}]_i$ may partly be due to the rapid desensitization of P_{2X} receptors. In the muscle strips pretreated with α,β -methylene-

ATP, $[\text{Ca}^{2+}]_i$ and force stimulated by α,β -methylene-ATP decreased to 51.9% and 57.4%, respectively. In these desensitized muscle strips, ATP induced an additional increase in $[\text{Ca}^{2+}]_i$ and force even in the presence of α,β -methylene-ATP. The increase in $[\text{Ca}^{2+}]_i$ was 78.4% of that in the absence of pretreatment with α,β -methylene-ATP. These results support the suggestion that ATP activates not only P_{2X} purinoceptors but also other type of receptors.

Although, ATP and α,β -methylene-ATP induced large increases in $[\text{Ca}^{2+}]_i$, these stimulants induced only small contractions. Since it has been shown that ATP is metabolized to adenosine possibly by smooth muscle ecto-nucleotidase (Rembold *et al.*, 1991); that adenosine activates P_1 receptors to increase cyclic AMP (Burnstock & Kennedy, 1985), and that cyclic AMP decreases Ca^{2+} sensitivity of smooth muscle contractile elements (Karaki, 1989), we measured cyclic nucleotide contents. Results indicated that ATP but not α,β -methylene-ATP increased cyclic AMP content without changing cyclic GMP content. These results suggest that the dissociation between $[\text{Ca}^{2+}]_i$ and force stimulated by ATP may be partly explained by the cyclic AMP-induced Ca^{2+} desensitization of contractile elements. However, this is unlikely to be an important mechanism in dissociation since α,β -methylene-ATP also caused similar dissociation without increasing cyclic AMP content. An alternative possibility has been suggested by Himpens *et al.* (1992) in cultured smooth muscle cells; ATP may increase Ca^{2+} in the nucleus. Further studies are necessary to determine whether fura-2 detects not only the cytosolic free Ca^{2+} level but also the Ca^{2+} level in the nucleus.

In conclusion, ATP and α,β -methylene-ATP increase $[\text{Ca}^{2+}]_i$ by an activation of L-type (verapamil-sensitive) and non-L-type (verapamil-insensitive) Ca^{2+} channels possibly through activation of P_{2X} purinoceptors whereas ATP, but not α,β -methylene-ATP, has an additional effect, releasing Ca^{2+} from storage sites.

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