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Response to ATP is accompanied by a Ca^{2+} influx *via* P2X purinoceptors in the coronary arterioles of golden hamsters*

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Summary. In the vascular wall, adenosine-5'-triphosphate (ATP) released along with noradrenaline from sympathetic nerve terminals is considered to play an important role in controlling intracellular calcium ion ($[Ca^{2+}]_i$) levels in arteries. The present study examined how vascular smooth muscle cells in coronary arterioles respond to ATP in relation to $[Ca^{2+}]_i$ dynamics. For this purpose, the dynamics of $[Ca^{2+}]_i$ in the coronary arterioles of golden hamsters was examined by real-time laser scanning confocal microscopy. This technique enabled the visualization of $[Ca^{2+}]_i$ changes in response to ATP in the intact coronary arterioles, the ultrastructure of which was well preserved. It was shown that an increase in $[Ca^{2+}]_i$ in the arteriole smooth muscle cells was elicited by ATP. While P1 purinoceptor agonists have no effect on this process, P2 purinoceptor agonists were found to induce a $[Ca^{2+}]_i$ increase in the smooth muscle cells. Suramin (an antagonist of P2X and P2Y receptors) completely inhibited ATP-induced $[Ca^{2+}]_i$ dynamics, but reactive blue 2 (a P2Y receptor antagonist) did not. Uridine-5'-triphosphate (a P2Y receptor agonist) had no effect on $[Ca^{2+}]_i$, but α,β - methylene ATP (a P2X receptor agonist) caused a strong increase in $[Ca^{2+}]_i$. We conclude that smooth muscle cells of the hamster coronary arterioles possess P2X, but not P1 or P2Y purinoceptors. The smooth muscle cells probably respond to extracellular ATP via P2X purinoceptors, resulting in the contraction of the coronary arterioles.

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Introduction

Adenosine 5'-triphosphate (ATP), a ubiquitous compound, can be found in extracellular spaces as a neurotransmitter or co-transmitter that is released from nerve endings (Burnstock, 1972; Gordon, 1986; von Kügelgen and Starke, 1991; Dubyak and El-Moatassim, 1993; Burnstock, 1995). ATP is also known to leak from injured or stimulated cells (Burnstock, 1996; McConalogue *et al.*, 1996). ATP is involved in specific extracellular signaling actions regulating a variety of cellular functions (e.g. development, proliferation, and differentiation) (Abbracchio and Burnstock, 1998).

Purinergic receptors are important when considering these signaling actions. They can be divided into two main categories: P1 purinoceptors (adenosine receptors), and P2 purinoceptors (ATP receptors) (Burnstock, 1978). P2 purinoceptors can be grouped into different two subfamilies: P2X and P2Y. Since P2X purinoceptors are ligand-gated ion channels, an increase in intracellular calcium ion ($[Ca^{2+}]_i$) mediated by P2X purinoceptors is caused by an influx of Ca^{2+}. P2Y purinoceptors are comprised of seven membrane-spanning receptors, which are coupled to G-proteins, resulting in the mobilization of $[Ca^{2+}]_i$ from internal Ca^{2+} stores (Dubyak, 1991; Abbracchio and Burnstock, 1994; Burnstock, 1996, 1997; Fredholm *et al.*, 1997; Kunapuli and Daniel, 1998).

It has been reported that isolated coronary arteries possess P2X and P2Y purinoceptors (Hopwood and Burnstock 1987; Corr and Burnstock 1994). These studies, however, only observed the phenomena of the extension and

contraction of coronary arteries using either rounded slices of heart specimens or isolated Langendorff perfused heart specimens. On the other hand, some recent researchers have reported that cultured smooth muscle cells from the coronary artery display pharmacological characteristics that are consistent with activities associated with P2Y purinoceptors (Strobaek *et al.*, 1996; Seiler, 1999; Weirich *et al.*, 2001). However, the above data were based on *in vitro* experiments, and our understanding of purinergic receptors of the arterioles *in situ* remains limited. Studies on the effect of extracellular ATP on subcellular $[Ca^{2+}]_i$ changes in the intact tissues are awaited to clarify the physiological role of purinergic receptors in the coronary arterioles.

Digital imaging analysis of $[Ca^{2+}]_i$ dynamics in intact living tissues may represent a useful approach for distinguishing different cellular responses to a transmitter. With the development of techniques for the digital imaging of $[Ca^{2+}]_i$, characteristic features such as Ca^{2+} oscillation (Berridge and Galione, 1988; Berridge, 1990; Jacob, 1990; Tsien and Tsien, 1990) and Ca^{2+} waves (Meyer and Stryer, 1991; Thomas *et al.*, 1992; Berridge, 1993; Pozzan *et al.*, 1994) have been reported in many cell types. Employing these techniques, previous studies have mainly used cultured or isolated cells as an experimental model (Bouchelouche, 1993). However, these isolated/cultured cells lose their natural conformation and structure, and the intracellular signaling of such cells may be altered (Nelson *et al.*, 1990). Consequently, the best approach appears to be a study of $[Ca^{2+}]_i$ dynamics in individual cells in intact tissue specimens using real-time confocal microscopy (Satoh *et al.*, 1997; Kimura *et al.*, 1999; Mori *et al.*, 2000; Shinohe and Saino, 2000; Kumagai and Saino, 2001; Saino *et al.*, 2002a, b; Kubo-Watanabe *et al.*, 2002, 2003; Saino and Satoh, 2004).

Thus, the aim of this study was to clarify the mechanism of ATP-induced $[Ca^{2+}]_i$ dynamics in coronary arteriole smooth muscle cells. To achieve this, we examined the $[Ca^{2+}]_i$ dynamics of semi-intact coronary arterioles from golden hamsters that retain their essential vascular structures. We recently succeeded in showing that ATP participates in a variety of actions in arterioles of different tissues (Saino *et al.*, 2002b). The present study especially attempts to specify the types of purinoceptors that are present in the smooth muscle cells of the coronary arterioles.

Materials and Methods

Preparation of arterioles

Experiments were conducted according to the guidelines of the ethics committee for animal treatment at Iwate Medical University. Adult male and female golden hamsters, *Mesocricetus auratus*, (10–12 weeks old, body weight 100–120 g) were used. The hamsters were killed by carbon dioxide gas followed by exsanguination. The heart was quickly removed and soaked in Hepes-buffered Ringer's (HR) solution. The HR solution contained 118 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.13 mM $MgCl_2$, 1 mM NaH_2PO_4, 5.5 mM D-glucose, MEM amino acids solution (Gibco, Grand Island, NY, USA), 0.2% bovine serum albumin (Sigma, St. Louis, MO, USA) and 10 mM Hepes; pH 7.4, adjusted with NaOH. Arterioles were isolated from the heart and digested with collagenase (100 U/m*l*; purified by HPLC; Elastin Products, Owensville, MO, USA) in the HR solution for 2h at room temperature (20–25℃). Connective tissues were then carefully removed. Ca^{2+}-deficient solutions were prepared by replacing $CaCl_2$ with EGTA (1.0mM; Sigma).

Intracellular Ca^{2+} imaging

In order to measure $[Ca^{2+}]_i$, coronary arterioles were placed on a glass cover slip, set in a perifusion chamber, and incubated with 10 μM Indo-1/AM (Indo-1/AM; Dojindo, Kumamoto) in the HR solution for 45 min at 15℃. Indo-1 (excited by ultraviolet light) is a ratiometric dye that is used in the quantitative determination of $[Ca^{2+}]_i$. The emission maximum of Indo-1 shifts from 475 nm in a Ca^{2+}-free medium to 400 nm when the dye solution is saturated with Ca^{2+}. The ratio of emission intensity shorter than 440 nm to that longer than 440 nm can be used to estimate $[Ca^{2+}]_i$; a higher ratio indicates a higher $[Ca^{2+}]_i$. Artifacts which include photobleaching and dye leakage can be eliminated by this ratiometry. We measured $[Ca^{2+}]_i$ in restricted areas (an approximately 0.5 μm^2 spot size).

A real-time confocal microscope (RCM/Ab; a modified version of a Nikon model RCM-8000, Tokyo) was used to measure $[Ca^{2+}]_i$ changes. Cells loaded with Indo-1 were exposed to an ultraviolet-beam (351 nm). An argon-ion laser was equipped with an inverted microscope (TE-300, Nikon), and the fluorescence emission was passed through a water-immersion objective lens (Nikon C Apo 40×, N.A. 1.15) to a pinhole diaphragm. The acquisition time per image frame was 1/30 sec using this system. Images were immediately stored on high-speed hard disks. The digital images in the laser scanning microscopic imaging were composed of 512×480 pixels with a density resolution of 8 bits/pixel. The fluorescent intensity was displayed as pseudocolors with 256 hues, with red representing a high $[Ca^{2+}]_i$ and purple and blue a low $[Ca^{2+}]_i$.

Stimulation by ATP and ATP-analogs

The $[Ca^{2+}]_i$ dynamics of arterioles was examined in a

perfusion chamber as quickly as possible after the dye-loading procedure. After perfusion with the standard HR solution for a few minutes at room temperature, intact arterioles were selected and examined under the microscope. Specimens were continuously perfused with the HR solution containing the following agonists and/or antagonists: adenosine (Sigma; 100 μM), adenosine 5'-triphosphate (ATP; Kohjin, Tokyo; 100 μM), adenosine 5'-diphosphate (ADP; Sigma; 100 μM), adenosine 5'-monophosphate (AMP; Sigma; 100 μM), α,β-methyleneadenosine 5'-triphosphate (α,β-methylene ATP; a typical agonist of $P2X_{1,3}$-purinoceptors; Sigma; 100 μM), uridine triphosphate (UTP; an agonist of $P2Y_{2,4,6}$-purinoceptors; Sigma; 100 μM), suramin (an antagonist of P2 purinoceptors; Research Biochemicals International, Natick, MA, USA; 90 μ M), reactive blue-2 (RB-2; an antagonist of P2Ypurinoceptors; Research Biochemicals International; 30 μM), thapsigargin (a microsomal Ca^{2+}ATPase inhibitor; Alomone Labs, Israel; 1 μM), pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; an antagonist of P2Xpurinoceptors; Sigma; 100 μM), $GdCl_3$ (a nonspecific cation channel blocker, Wako, Japan; 100 μM), and diltiazem (a L-type Ca^{2+} channel blocker; Sigma; 40 μM).

Ultrastructure

To monitor ultrastructural changes of the smooth muscles that could occur during the experiments, coronary arterioles were observed by electron microscopy. After measuring the $[Ca^{2+}]_i$ dynamics, the arterioles were fixed in 0.125% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered saline (PBS; 100 mM) for about 4 hrs at room temperature. Specimens were then postfixed in 1% osmium tetroxide (Merck, Germany) in PBS for 1.5 h at 4℃, dehydrated in a series of ethanol, and embedded in Epon 812 (TAAB, Berkshire, UK). Longitudinal sections were consecutively cut through the arterioles using an ultramicrotome (2088 Ultrotome; LKB, Bromma, Sweden). Semithin sections (about 1 μm thick) were stained with toluidine blue and observed by light microscopy. Ultrathin sections (about 0.07 μm thick) were doubly stained with uranyl acetate and lead citrate, and examined in an electron microscope (H-7100; Hitachi Co, Hitachi).

Results

The ultratructures of coronary arterioles

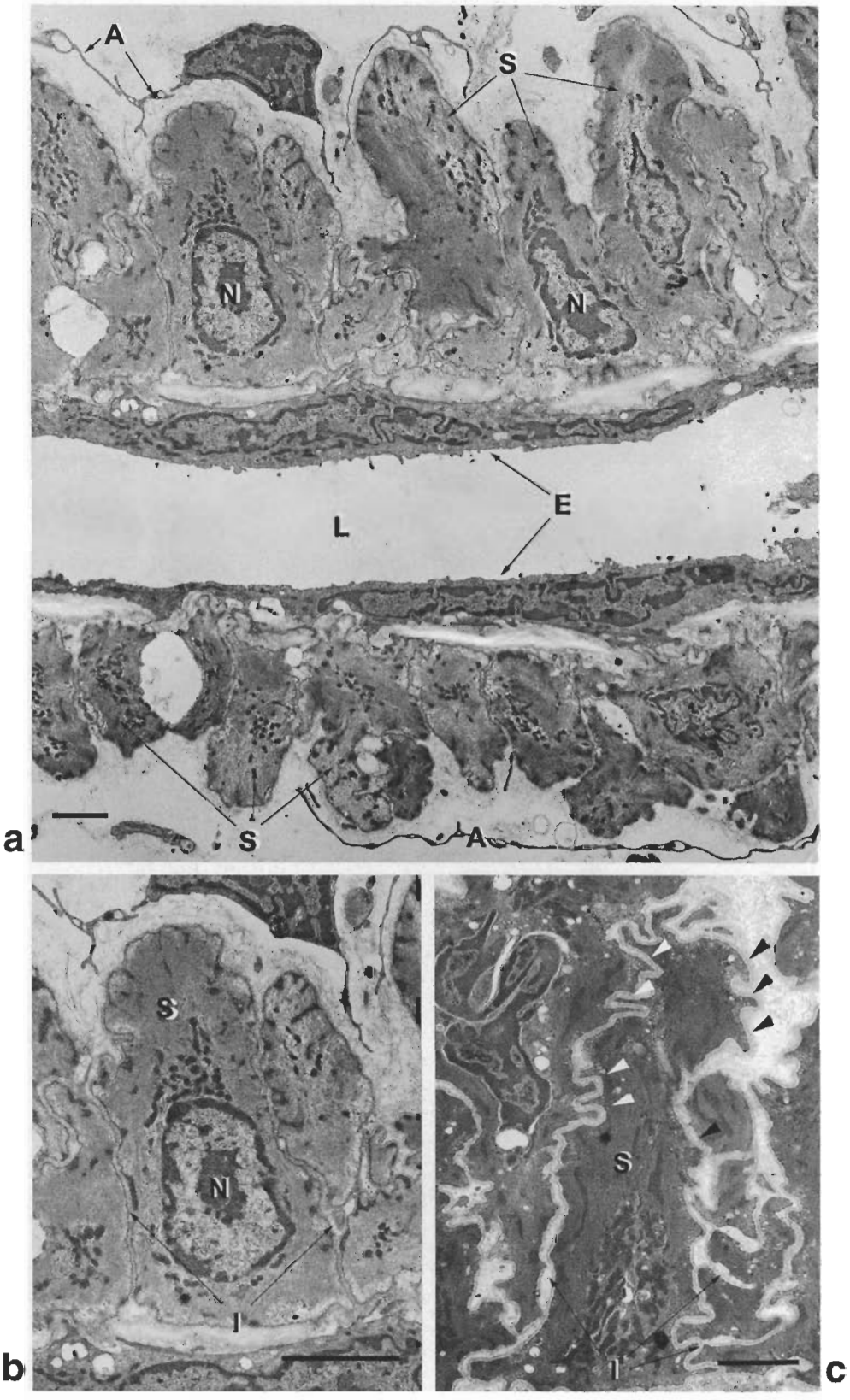
Electron microscopy revealed that the specimens prepared for the intracellular calcium imaging study maintained the typical structural integrity of coronary arterioles (Fig. 1a). They were surrounded by smooth muscle cells in a circular fashion, and no significant ultrastructual damage (e.g. swollen mitochondria, vacuolation of sarco/endoplasmic reticulum) was detected. In the coronary arterioles without the ATP perfusion, profiles of the smooth muscle cells were rather smooth and intercellular spaces were not enlarged, signs indicative of normal intercellular communication (Fig. 1b). However, after the ATP perfusion, the outline of the smooth muscle cells appeared undulated because of the contraction of the smooth muscle cells (Fig. 1c). The ATP-stimulated smooth muscle cells appeared to be dark compared with the non-stimulated smooth muscle cells. No other structural differences between the non-stimulated and ATP- loaded specimens were detected.

Effect of ATP on $[Ca^{2+}]_i$ dynamics

No spontaneous $[Ca^{2+}]_i$ changes were observed in the smooth muscle cells of the coronary arterioles. A small percentage of injured cells, which showed high $[Ca^{2+}]_i$ at resting conditions, were excluded from subsequent analyses. The exposure of the arteriole to extracellular ATP led to an increase in $[Ca^{2+}]_i$ of the smooth muscle cells (n=12) (Fig. 2a–h). In the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$), ATP failed to induce increases in $[Ca^{2+}]_i$ of these cells (n=14) (compare Fig. 3a and 3a' to 3b and 3b'). Diltiazem (40 μM), a L-type Ca^{2+} channel blocker, also inhibited the ATP-induced $[Ca^{2+}]_i$ increases (n=8) (Fig. 4a), and Gd^{3+} (100 μM), a nonspecific cation channel blocker, showed the same effect (data not shown). To investigate the possible contribution of internal Ca^{2+} stores, thapsigargin, an inhibitor of the Ca^{2+} pump of the sarco/endoplasmic reticulum, was used. The depletion of Ca^{2+} stores by thapsigargin (1 μM) failed to inhibit ATP-induced increases in $[Ca^{2+}]_i$ (n=9) (Fig. 4b), suggesting that an influx of Ca^{2+} from the extracellular spaces could be a major factor in the $[Ca^{2+}]$ changes in the coronary arteriole smooth muscle cells.

Effect of ATP analogs on $[Ca^{2+}]_i$ dynamics

P1 purinoceptors are more responsive to adenosine and AMP than ADP and ATP, and conversely, P2 purinoceptors are more responsive to ADP and ATP than adenosine and AMP (Burnstock, 1978). ADP (100 μM) and ATP (100 μM) induced an increase in $[Ca^{2+}]_i$ in the smooth muscle cells (n=10), whereas adenosine (100 μM) and AMP (100 μM) did not induce any $[Ca^{2+}]_i$ increases (n=9) (Fig. 5a–d). A typical agonist of P2X purinoceptors, α,β-methylene ATP (100 μM), induced a $[Ca^{2+}]_i$ increase (Fig. 6a). The ATP-induced $[Ca^{2+}]_i$ increase was significantly inhibited by pretreatment with an antagonist of P2X purinoceptors, PPADS (100 μM) (n=8) (Fig. 6b), while a typical



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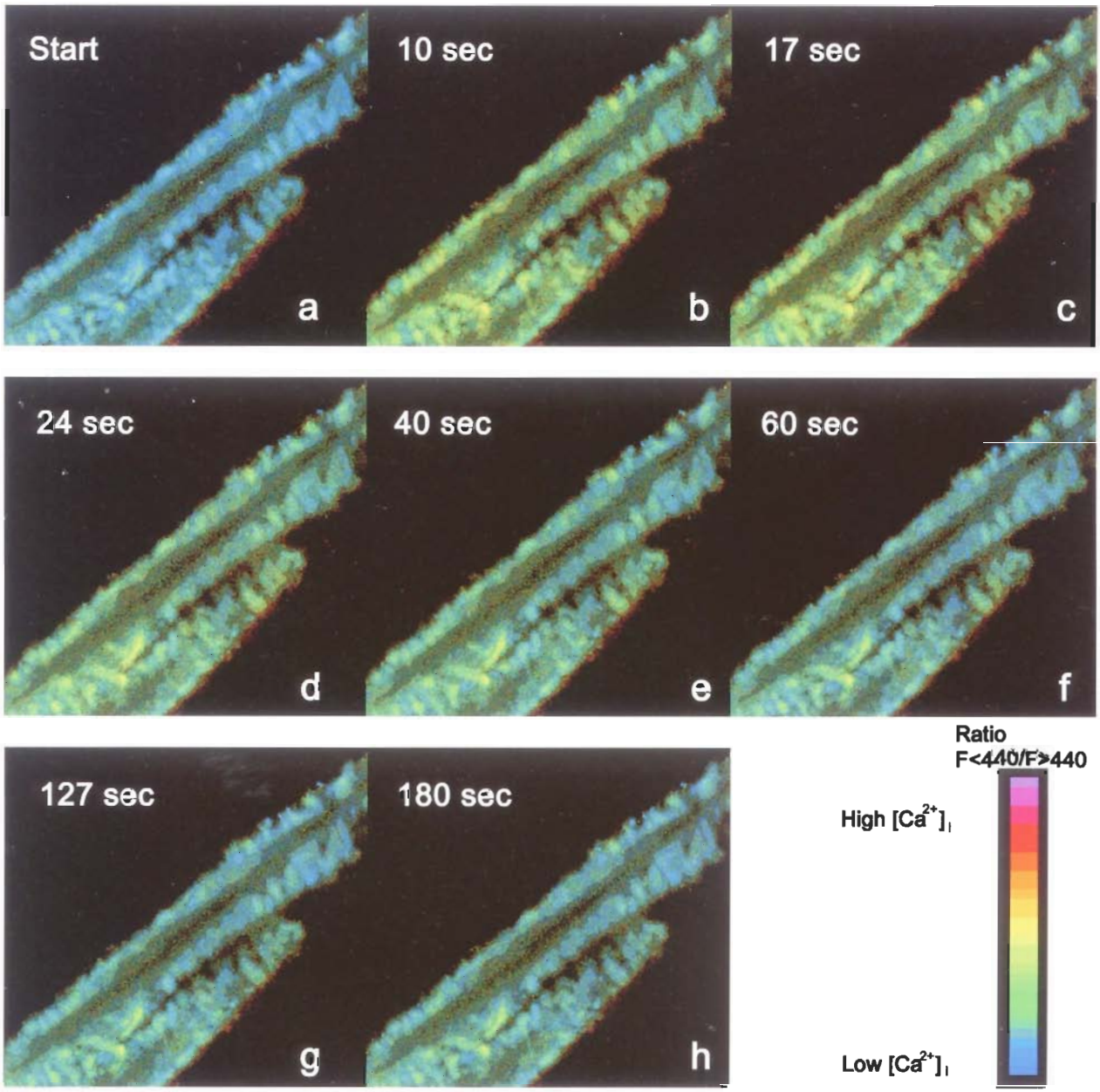

Fig. 2. Pseudocolor images of smooth muscle cells of the coronary arteriole showing $[Ca^{2+}]_i$ changes, which were measured by ratiometry using Indo-1 (**a–h**). In the presence of extracellular Ca^{2+} (1.25 mM), arterioles were stimulated with ATP at room temperature. After 10 sec of stimulation, the smooth muscle cells became contracted and $[Ca^{2+}]_i$ was increased in the cells (**b**). Thereafter, the $[Ca^{2+}]_i$ increase spread to adjacent cells (compare **a** with **b–h**). Color scale bar: Fluorescence ratio represents $[Ca^{2+}]_i$.

Fig. 1. Electron micrographs of a golden hamster coronary arteriole prepared for the intracellular calcium imaging. **a:** Longitudinal section of a non-stimulated control arteriole. **b:** In non-stimulated arterioles, the profiles of smooth muscle cells are almost smooth. **c:** After ATP stimulation, most of the smooth muscle cells are shrunken (black arrow heads) and have meandering intercellular spaces (white arrow heads). A: fibroblasts of adventitia, E: endothelia, I: intercellular spaces, L: lumen, N: nuclei, S: smooth muscle cells. Bars = 25 μm

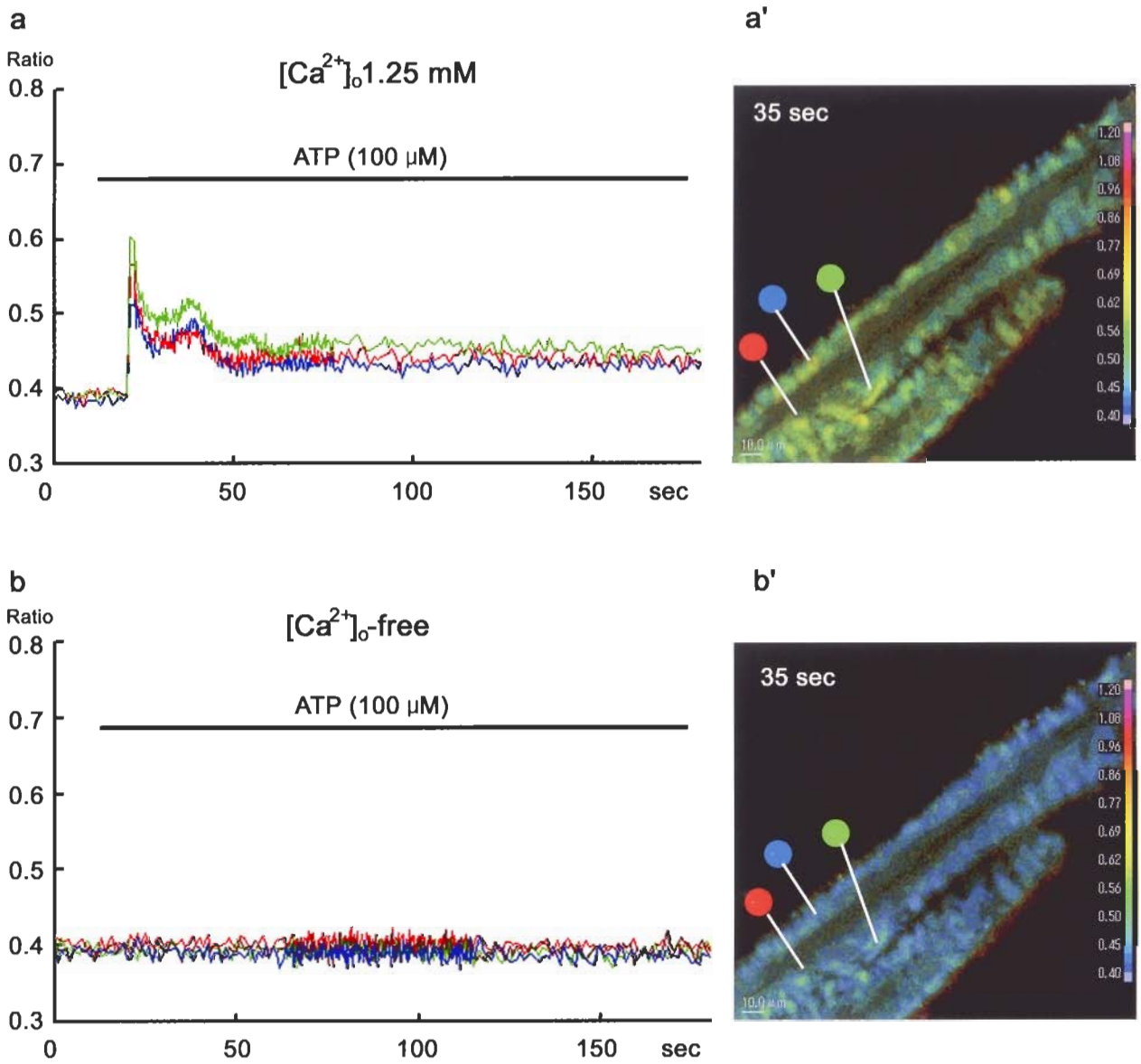

Fig. 3. Dependence of extracellular Ca^{2+} on ATP-induced $[Ca^{2+}]_i$ dynamics. Time courses of $[Ca^{2+}]_i$ dynamics induced by ATP in coronary arteriole smooth muscle cells (**a** and **b**; green, red, and blue lines) at certain areas (about 1 μm^2). Each green, red, and blue line represents $[Ca^{2+}]_i$ at the respective green, red, and blue boxes in the fluorescent images, respectively. ATP (100 μM)-induced $[Ca^{2+}]_i$ increases in the cells (**a** and **a'**). Under extracellular Ca^{2+}-free conditions ($[Ca^{2+}]_o$-free), stimulation by ATP led to no further Ca^{2+} increase in the cells (**b** and **b'**).

agonist of P2Y purinoceptors, UTP (100 μM), had no effect (Fig. 6c). ATP-induced $[Ca^{2+}]_i$ increases in the cells were prevented by pretreatment with an antagonist of P2 purinoceptors, suramin (90 μM) (n=8) (Fig. 7a). Reactive blue-2 (30 μM), an antagonist of P2Y-purinoceptors, failed to inhibit ATP-induced $[Ca^{2+}]_i$ increases (n=8) (Fig. 7b).

We thus concluded that smooth muscle cells of the hamster coronary arterioles possess P2X, but not P1 or P2Y purinoceptors.

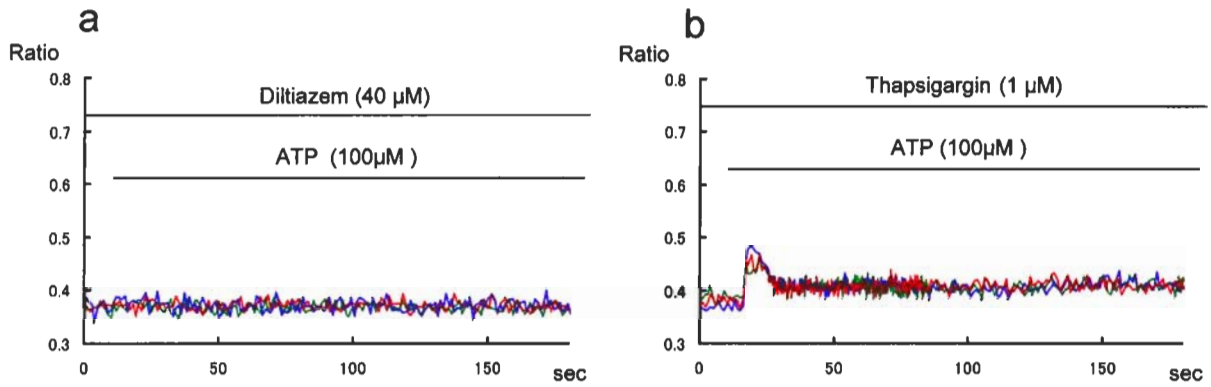

Fig. 4. Time courses for the ATP-induced $[Ca^{2+}]_i$ dynamics in coronary arteriole smooth muscle cells (**a**, **b**; green, red, and blue lines) at certain areas (about 1 μm^2). Diltiazem (40 μM) inhibits ATP (100 μM)-induced $[Ca^{2+}]_i$ dynamics in the cells (**a**). After depleting intracellular Ca^{2+} stores by treatment with thapsigargin (1 μM), ATP (100 μM) caused a $[Ca^{2+}]_i$ increase in the cells (**b**).

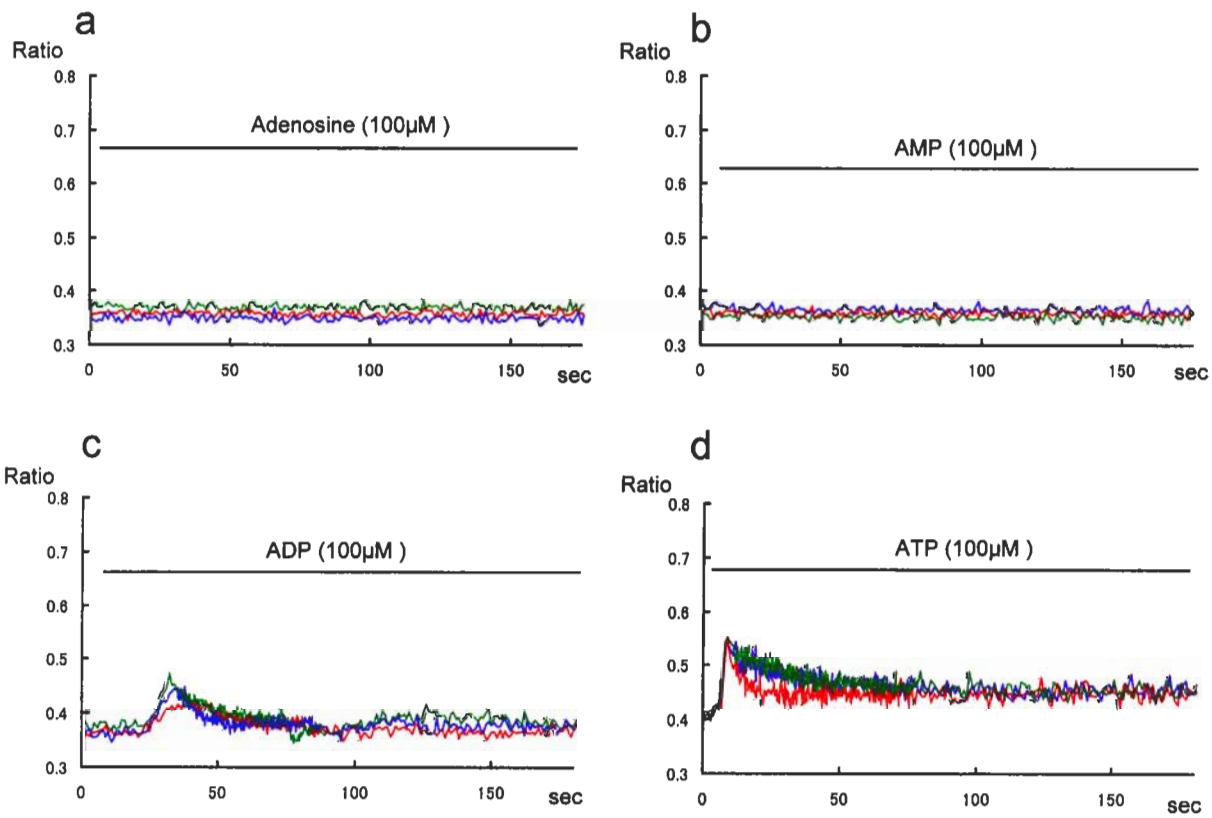

Fig. 5. Time courses for $[Ca^{2+}]_i$ dynamics induced by ATP and ATP-analogs in coronary arteriole smooth muscle cells (**a**–**d**; green, red, and blue lines) at certain areas (about 1 μm^2). Adenosine (100 μM) and AMP (100 μM) had no effect on $[Ca^{2+}]_i$ dynamics (**a** and **b**). ADP (100 μM) had an effect on $[Ca^{2+}]_i$ dynamics (**c**). ATP (100 μM) was more potent than ADP (**d**).

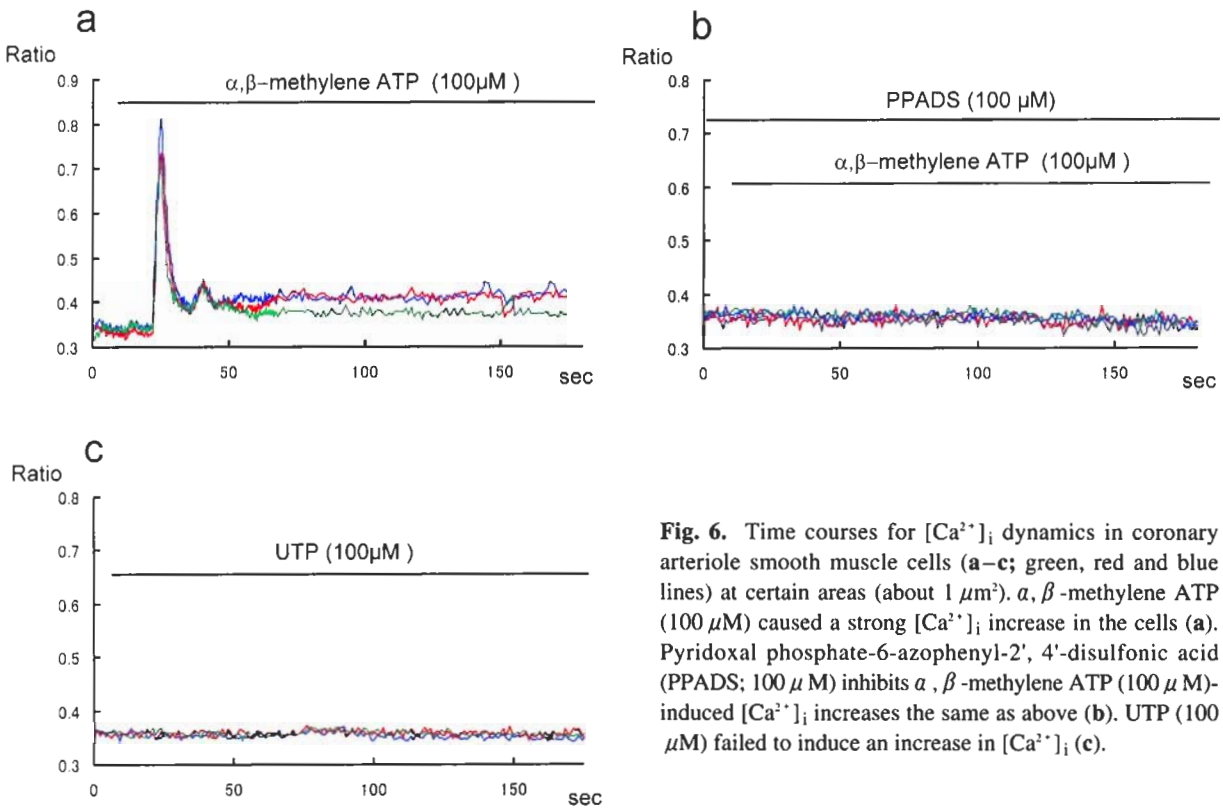

Fig. 6. Time courses for $[Ca^{2+}]_i$ dynamics in coronary arteriole smooth muscle cells (**a–c;** green, red and blue lines) at certain areas (about 1 μm^2). α, β-methylene ATP (100 μM) caused a strong $[Ca^{2+}]_i$ increase in the cells (**a**). Pyridoxal phosphate-6-azophenyl-2', 4'-disulfonic acid (PPADS; 100 μM) inhibits α, β-methylene ATP (100 μM)-induced $[Ca^{2+}]_i$ increases the same as above (**b**). UTP (100 μM) failed to induce an increase in $[Ca^{2+}]_i$ (**c**).

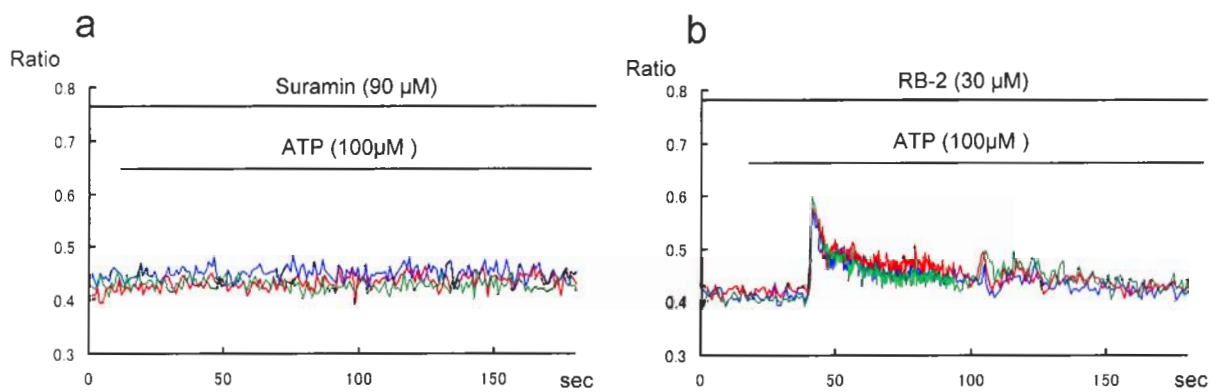

Fig. 7. Time courses for $[Ca^{2+}]_i$ dynamics induced by ATP in the presence of purinoceptor antagonists (**a** and **b;** green, red, and blue lines) at certain areas (about 1 μm^2). Suramin (90 μM) inhibited ATP (100 μM)-induced $[Ca^{2+}]_i$ increases (**a**). Reactive blue-2 (RB-2; 30 μM) did not inhibit ATP (100 μM)-induced $[Ca^{2+}]_i$ increases (**b**).

Discussion

The present study is the first to reveal the involvement of $[Ca^{2+}]_i$ dynamics with respect to ATP and its analogs in intact coronary arterioles. Ca^{2+} responses have been examined mainly in cultured or isolated vascular smooth muscle cells by various imaging techniques (Iino *et al.*, 1994; Yip and Marsh, 1996; Li *et al.*, 1997; Bolton and Gordienko, 1998; Jaggar *et al.*, 1998). In the course of the cultured process of vascular smooth muscle cells, cellular responses induced by a P2X receptor were lost, whereas $[Ca^{2+}]_i$ increases mediated by a P2Y receptor appeared (Pacaud *et al.*, 1995; Erlinge, 1998). In addition, functional coupling between the sarco/endoplasmic reticulum and mitochondria was found to be altered during the dedifferentiation and/or redifferentiation process which occurs in the rat aortic smooth muscle cells in culture (Vallot *et al.*, 2001). It is also possible that the smooth muscle cells in intact arterioles differ in characteristics from the cultured cells. In this context, it is crucial to analyse the intact smooth muscle cells by real-time confocal microscopy.

Heterogenous responses of smooth muscle cells in the coronary arterioles with respect to ATP

Based on the present results, we consider that the major pathway of the ATP-induced $[Ca^{2+}]_i$ increase is the influx of Ca^{2+} *via* ion channels in the smooth muscle cells of coronary arterioles. P2, but not P1 purinoceptors, can be involved in the ATP-mediated $[Ca^{2+}]_i$ increase. A potent agonist of P2X purinoceptors caused a $[Ca^{2+}]_i$ increase, but a P2Y purinoceptor agonist had no effect. P2X antagonists inhibited an ATP-induced $[Ca^{2+}]_i$ response. Therefore, the response of the coronary arteriole to ATP can be mediated by P2X, but not P2Y purinoceptors.

Several P2X receptors are reported to be present in vascular tissues, including the coronary artery, aorta, cerebral artery and basilar artery (von Kugelgen and Starke, 1990; Bo *et al.*, 1998a; Nori *et al.*, 1998); the main subtype is thought to be the $P2X_1$ purinoceptor (Vulchanova *et al.*, 1997; Bo *et al.*, 1998b; Hansen *et al.*, 1999), as judged from molecular biological data and electrophysiological results obtained in studies of P2X receptor-expressing cells and freshly isolated vascular cells. The present results are consistent with the view that vascular smooth muscle cells contain P2X purinoceptors.

Previous researchers reported that both P2X- and P2Y-purinoceptor subtypes are present in smooth muscle in isolated rabbit mesenteric and coronary arteries (Mathieson and Burnstock, 1985; Hopwood and Burnstock, 1987; Corr and Burnstock, 1994). On the other hand, we recently demonstrated that smooth muscle cells in the testicular arterioles contain solely the P2X purinoceptor, and those in the cerebral arterioles contain both P2X and P2Y purinoceptors (Saino *et al.*, 2002b). A series of our studies show that arteriole smooth muscle cells might have a great variety of receptor subtype which could not have been revealed using culture cells; it is possible that arteriole functions in different tissues are controlled in different ways.

The ATP-dependent $[Ca^{2+}]_i$ transient in primarily cultured smooth muscle cells from porcine coronary arteries is mediated via a UTP-activated P2Y purinoceptor subtype, which might be $P2Y_2$ purinoceptor (Seiler *et al.*, 1999; Weirich *et al.*, 2001). Obviously, there are many differences between smooth muscle cells of the intact arterioles and cultured vascular smooth muscle cells.

Endothelial cells responses to ATP

In the vascular wall, ATP is known to be released along with noradrenaline from sympathetic nerve terminals (von Kügelgen and Starke, 1991). In the present study, ATP was added to the perfused solution, which mimicks a situation in which ATP is released from nerve terminals around arterioles.

On the other hand, it can be argued that endothelial cells also play a role in $[Ca^{2+}]_i$ changes in arteriole smooth muscles. It was recently reported that the ATP-stimulated endothelium (via P2Y purinoceptors) produces nitric oxide, and then vasodilation occurs (Konduri *et al.*, 2004). In our preliminary experiments, we observed $[Ca^{2+}]_i$ changes in endothelial cells in arterioles, but no response to ATP was detected. Therefore, the participation of endothelial cells in the ATP-induced muscle contractions in the present study can be discounted in the present study.

Conclusion

The present study revealed that purinoceptors of coronary arteriole smooth muscle cells are P2X ion channel type receptors. Real-time confocal microscopy was found to be a useful tool for investigating structural and functional changes in living vascular smooth muscle cells. Clarification of their specificities by this type of study is essential for the development of therapies that are designed to improve the blood circulation in certain tissues/organs.

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