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Dipyridamole inhibits intracellular calcium transients in isolated rat arteriole smooth muscle cells*

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Summary. Dipyridamole, an inhibitor of adenosine uptake as well as a cGMP phosphodiesterase inhibitor, is commonly used in prophylactic therapy for patients with angina pectoris. However, the effects of dipyridamole on systemic blood vessels, especially on the peripheral vascular system, are not well understood. Therefore, the effect of dipyridamole on ATP-induced arteriole contraction was examined with special reference to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using real-time confocal microscopy. In cases of 0.1–10 μM range, dipyridamole induced only slight $[Ca^{2+}]_i$ decreases in smooth muscle cells of both testicular and cerebral arterioles. However, 100 μM dipyridamole induced substantial $[Ca^{2+}]_i$ decreases in the cells. In the presence of 10 μM dipyridamole, changes in ATP-induced $[Ca^{2+}]_i$ were found to be inhibited in smooth muscle cells of testicular arterioles but not in those of cerebral arterioles. In addition, α, β–methylene ATP-induced $[Ca^{2+}]_i$ increases in testicular arteriole smooth muscle cells were also partially inhibited in the presence of dipyridamole. When testicular arterioles were perfused with dipyridamole, no increases in nitric oxide levels were detected. High levels of K^+ induced a $[Ca^{2+}]_i$ increase in testicular arterioles that was also partially inhibited by dipyridamole. In the presence of substances that affect protein kinase A or G, ATP-induced $[Ca^{2+}]_i$ was not completely inhibited. These findings suggest that dipyridamole may act not only as an inhibitor of adenosine uptake and as a cGMP phosphodiesterase inhibitor, but also as a calcium channel blocker in arteriole smooth muscle cells.

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Introduction

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) play an essential role in stimulus-response coupling in a wide variety of tissues, and cytosolic Ca^{2+} homeostasis is critically regulated in various cells, including vascular smooth muscle cells. It is also well known that a variety of vasoactive agonists cause vascular smooth muscle cell contraction and/or stimulate cell growth by causing an increase in $[Ca^{2+}]_i$ (van Breemen and Saida, 1989; Somlyo and Somlyo, 1994).

Dipyridamole, which has been clinically used since the early 1960s as a coronary vasodilator, has undergone a renaissance as an antithrombotic drug (Diener *et al*., 1996). This chemical increases the plasma concentration of adenosine (an endogenous platelet inhibitor) by inhibiting adenosine uptake (Gresele *et al*., 1983, 1986) and attenuating adenosine catabolisms (Ferrandon *et al*., 1994). In addition, it has been reported to inhibit cyclic nucleotide degradation by phosphodiesterases (PDE), including the cGMP-specific phosphodiesterase type V (Ahn *et al*., 1989; Gillespie and Beavo, 1989; Sakuma *et al*., 1990).

However, the mechanism of action of dipyridamole remains an enigma because this drug may affect the systemic circulation. In some clinical instances, blood pressure falls after the administration of dipyridamole. This implies that dipyridamole may be concerned with inhibiting the contraction of arteriole smooth muscle cells. Thus, the aim of this study was to clarify precisely the effect of dipyridamole in the peripheral vascular system. We recently succeeded in showing that ATP participates in a variety of actions in arterioles of different tissues (Saino *et al*., 2002) in relation to the change in $[Ca^{2+}]_i$, and reported that smooth muscle cells in coronary arterioles, like testicular arterioles, respond to extracellular ATP *via* P2X purinoceptors (Matsuura *et al*., 2004). In the present study, we will show that the effect of dipyridamole with respect to ATP in arterioles is almost the same as that observed in the absence of extracellular Ca^{2+} although dipyridamole has little effect against arteriole smooth muscles. Based on these findings, we will propose the possibility that dipyridamole not only inhibits the uptake of adenosine or cGMP phosphodiesterase in arterioles, but also serves as an inhibitor of Ca^{2+} influx into arteriole smooth muscles.

Materials and Methods

Preparation of arterioles

Experiments were conducted in accordance with the guidelines of the ethics committee for animal treatment of Iwate Medical University. Adult male rats (Wistar, 8–12 weeks old, body weight 250–400g) were killed by carbon dioxide gas. They were then perfused via the left cardiac ventricle with Ringer's solution (147 mM NaCl, 4 mM KCl and 2.25 mM $CaCl_2$) at 25℃ at a hydrostatic pressure of approximately 1 m of H_2O. After washing out blood cells from vessels, the brain and testis were removed and placed in Hepes-buffered Ringer's solution (HR). The HR 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; all were adjusted to pH 7.4 with NaOH. Small arteries were isolated and digested with purified collagenase (100 U/m*l*; Elastin Products, Owensville, MO, USA) in HR for 2 h at room temperature (20–25℃). Connective tissues were then carefully removed. Ca^{2+}-deficient solutions were prepared by replacing $CaCl_2$ with EGTA (1 mM; Sigma).

Dye loading for $[Ca^{2+}]_i$ and intracellular NO concentration measurement

Spatiotemporal changes in $[Ca^{2+}]_i$ in small arteries were determined by ratiometry using Indo-1. DAF-2 was used to measure intracellular nitric oxide concentrations ($[NO]_i$). Dye loading was facilitated *via* the use of either acetoxymethyl esters (Indo-1/AM; Dojindo, Kumamoto) or diacethyl esters (DAF-2/DA; Daiichikagaku, Tokyo). To measure $[Ca^{2+}]_i$ levels, the specimens were transferred to HR that also contained 0.02% cremophor® EL (Nacalai Tesque, Kyoto) and 5 μM Indo-1/AM, followed by incubation for 12 h at 4℃. To measure $[NO]_i$ levels, the specimens were transferred to HR that contained 0.02% cremophor® EL and 10 μM DAF-2/DA followed by incubation for 1 h at room temperature. After incubation, they were placed on coverslips coated with Cell-Tak® (a nontoxic adhesive reagent; Collaborative Biomedical, Bedford, MA, USA) in modified Sykus-Moor chambers and then continuously perfused with HR that also contained selected stimulants.

A real-time confocal microscope (RCM/Ab, modified type of RCM-8000, Nikon, Tokyo) was used to measure changes in $[Ca^{2+}]_i$ and $[NO]_i$. Cells, loaded with either Indo-1 or DAF-2, were respectively exposed to an ultraviolet-beam (351 nm) or to a blue-beam (488 nm) for measurements of changes in $[Ca^{2+}]_i$ and $[NO]_i$. A triazole derivative of DAF-2 emits light at 515 nm on excitation at 488 nm; the intensity of this light is proportional to the amount of NO present (Kojima *et al*., 1998; Nakatsubo *et al*., 1998). 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. Images were immediately stored on a high-speed hard disk, and a ratio image from each pair was then computed: the fluorescence intensity of less than 440 nm ($F_{<440}$) to that greater than 440 nm ($F_{>440}$). A higher ratio ($F_{<440}/F_{>440}$) is indicative of a higher $[Ca^{2+}]_i$. The acquisition time per image frame was 1/30 sec using this system. Digital images in laser scanning microscopic imaging were composed of 512 × 480 pixels with a density resolution of 8 bits/pixel. Each pixel gave a spatial resolution of approximately 0.3 μm. Fluorescent intensity ivas displayed in pseudocolors of 256 grades with red representing a high $[Ca^{2+}]_i$ and purple and blue a low $[Ca^{2+}]_i$.

Stimulation by ATP and selected reagents

Specimens were then stimulated by replacing the standard HR with an HR solution containing the following

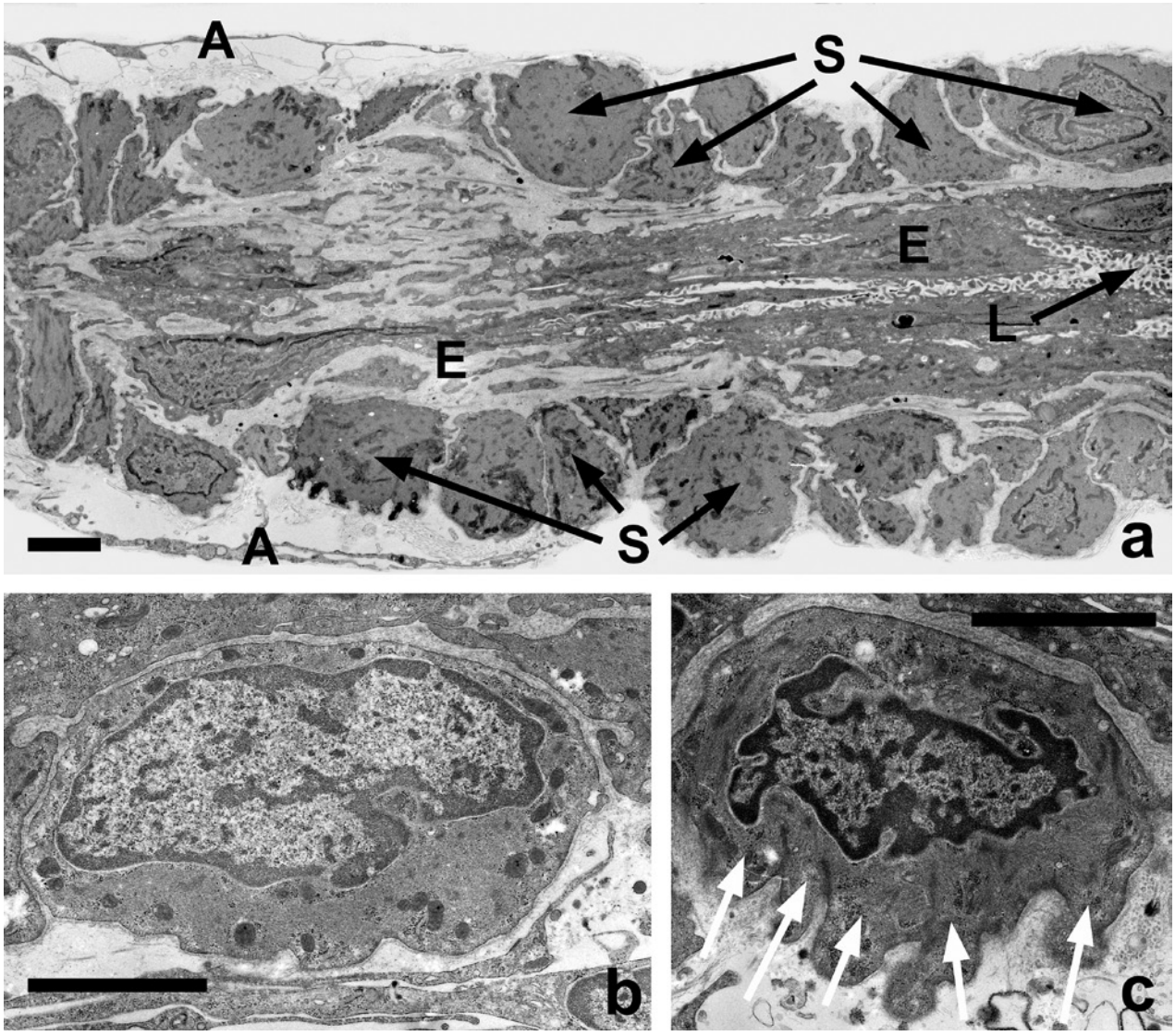

Fig. 1. Electron micrographs of a control and ATP-added rat testicular arteriole. **a:** longitudinal section of a non-stimulated control arteriole. A: fibroblasts of the adventitia, E: endothelia, L: lumen, S: smooth muscle cells. **b:** In control arterioles, the contour of smooth muscle cells is almost smooth. **c:** After the ATP stimulation, smooth muscle cells have shrunk (white arrows). Bars = 10 μm

agonists and/or antagonists: adenosine 5'-triphosphate (ATP 10 μM; Kohjin, Japan), adenosine (10 μM; Sigma), dibutyryl cGMP (50 μM; Sigma), dibutyryl cAMP (50 μM; Sigma), dypiridamole (10 μM; Sigma), forskolin (1 μM; Sigma), H8 (10 μM; calbiochem, La Jolla, CA, USA), indomethacin (10 μM; Sigma), isobutylmethylxanthine (IBMX 500 μM; Wako, Osaka), KT 5823 (1 μM; calbiochem), S-nitroso-N-acetylpenicillamine (SNAP 100 μM; calbiochem), and zaprinast (20 μM; Sigma).

Ultrastructure

To monitor ultrastructural changes in smooth muscles that could occur during the experiments, testicular arterioles were observed by electron microscopy. After measuring

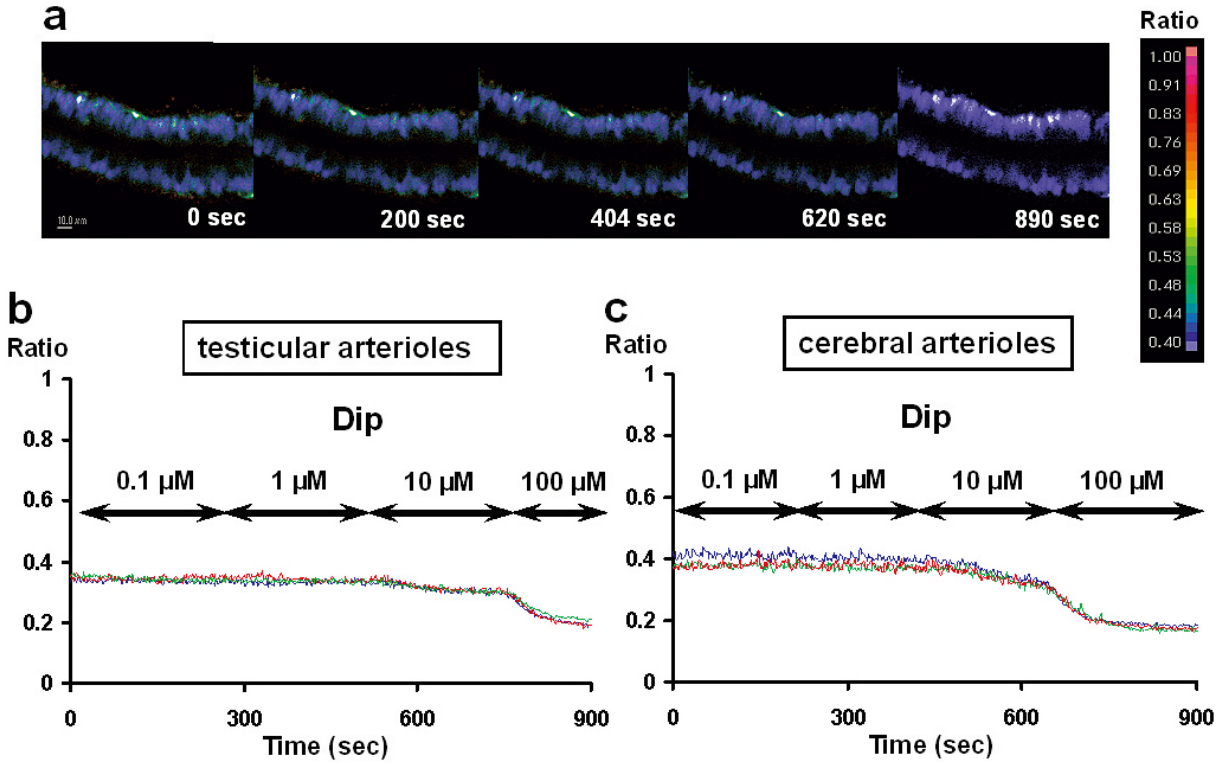

Fig. 2. Pseudocolor images of testicular arteriole smooth muscle cells showing $[Ca^{2+}]_i$ changes, measured by ratiometry of Indo-1 (**a:** 0, 200, 404, 620, 890 sec) and time courses for $[Ca^{2+}]_i$ dynamics induced by dipiridamole (Dip) in testicular and cerebral arteriole smooth muscle cells in a concentration-dependent manner (**b** and **c ;** blue, red, and green lines) at certain areas (about 1 μm^2). In the presence of extracellular Ca^{2+}, arterioles were stimulated with dipyridamol at room temperature. The smooth muscle cells showed no obvious contractions, dilation, or $[Ca^{2+}]_i$ increases(**a**). When arterioles were perfused with dipyridamole (0.1-10 μM), slight dipyridamole-induced $[Ca^{2+}]_i$ changes were observed in testicular and cerebral arterioles : i.e. an imperceptible $[Ca^{2+}]_i$ decrease (**b** and **c**). However, dipyridamole (100 μM) induced marked $[Ca^{2+}]_i$ decreases in either of the arteriole smooth muscle cells (**b** and **c**). Color scale bar: fluorescence ratio represents $[Ca^{2+}]_i$.

the $[Ca^{2+}]_i$ dynamics, the arterioles were fixed in 0.125% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered saline (PBS; 100 mM) for approximately 4 h 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 solutions 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 (approximately 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 by electron microscopy (H-7100; Hitachi Co, Hitachi).

Results

Ultratructures of testicular arterioles

The testicular arterioles studied here exhibited the normal structural integrity (Fig. 1a). They were surrounded by smooth muscle cells in a circular fashion with little ultrastructural damage (e.g. swollen mitochondria, vacuolation of sarco/endoplasmic reticulum) detected in the muscle cells. In control testicular arterioles, the contour of smooth muscle cells was usually smooth and the intercellular spaces were not enlarged (Fig. 1b).

However, after ATP perfusion, the contour of the smooth muscle cells appeared to be undulated due to the contraction of the cells (Fig. 1c). ATP-stimulated

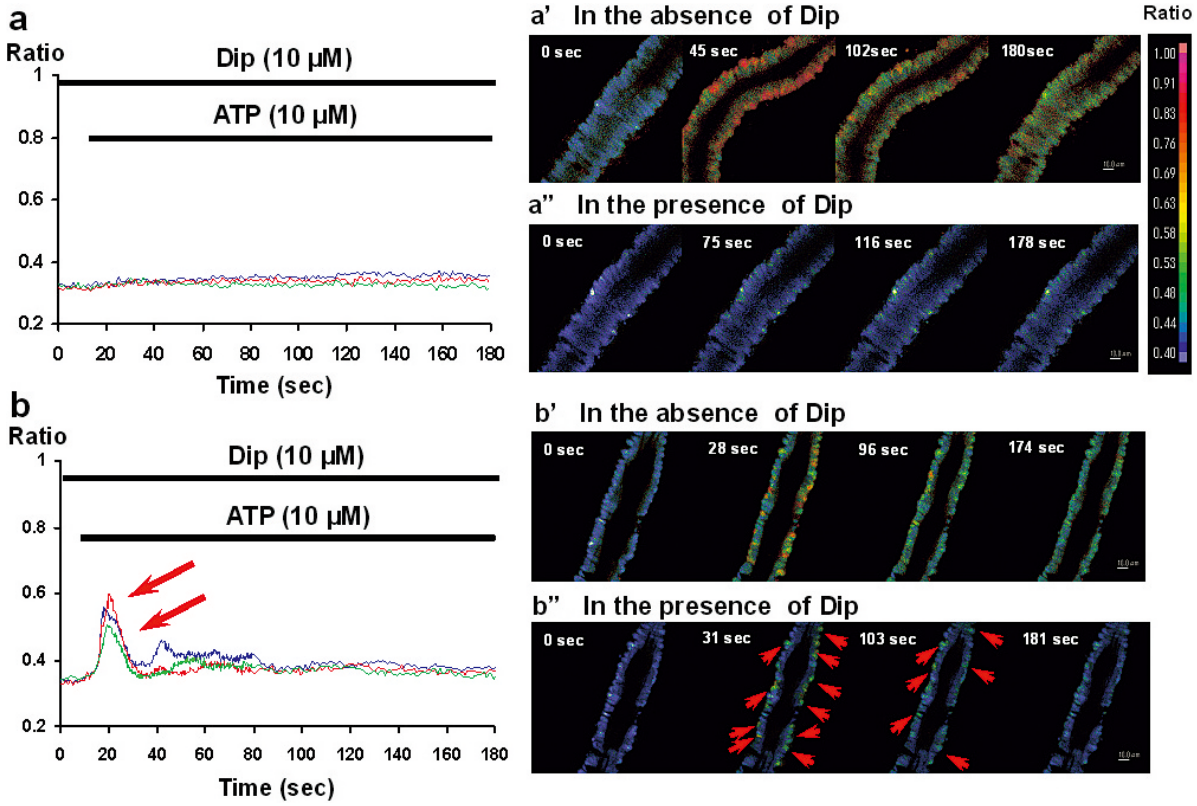

Fig. 3. Time courses for $[Ca^{2+}]_i$ dynamics induced by dipyridamole in testicular and cerebral arteriole smooth muscle cells (**a** and **b** ; blue, red, and green lines) at certain areas (about 1 μm^2); pseudocolor images show changes in $[Ca^{2+}]_i$, as measured by the ratiometry of Indo-1 (**a'**, **a"**, **b'** and **b"**). In the absence of dipyridamole (10 μM), ATP (10 μM)-induced $[Ca^{2+}]_i$ increases in smooth muscle cells (**a'**). However, dipyridamole completely inhibits ATP-induced $[Ca^{2+}]_i$ dynamics in testicular arteriole smooth muscle cells (**a** and **a"**). In the case of cerebral arterioles, ATP-induced $[Ca^{2+}]_i$ increases in smooth muscle cells (**b'**). In the presence of dipyridamole, stimulation by ATP led to an increased Ca^{2+} in smooth muscle cells (**b** and **b"**; red arrows).

cells appeared to be dark, compared with non-stimulated cells. No other structural differences between the non-stimulated and ATP-loaded specimens were observed. From the above findings, we concluded that the specimens were nearly intact.

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

Arteriole specimens were perfused with normal Hepes-buffered Ringer's solution for 5 min prior to stimulation by the selected reagents. Some injured cells in arterioles, which showed high $[Ca^{2+}]_i$ in resting conditions, were excluded from the subsequent analyses. In the present study, the effect of dipyridamole on testicular and cerebral arterioles was examined in a dose dependent manner. Dipyridamole (0.1-10 μM) induced little $[Ca^{2+}]_i$ change in the smooth muscle cells of both testicular and cerebral arterioles (Fig. 2a–c), and no dilatation of these arterioles was observed (Fig. 2a). However, dipyridamole (100 μM) greatly decreased $[Ca^{2+}]_i$ in the arteriole smooth muscle cells (Fig. 2a–c) although no obvious dilatation of the arterioles was detected (Fig. 2a). We therefore chose the concentration of dipyridamole (10 μM).

Because dipyridamole had little effect against arteriole smooth muscles, we examined the effect of dipyridamole in the presence of ATP. According to our previous studies, smooth muscle cells in testicular arterioles

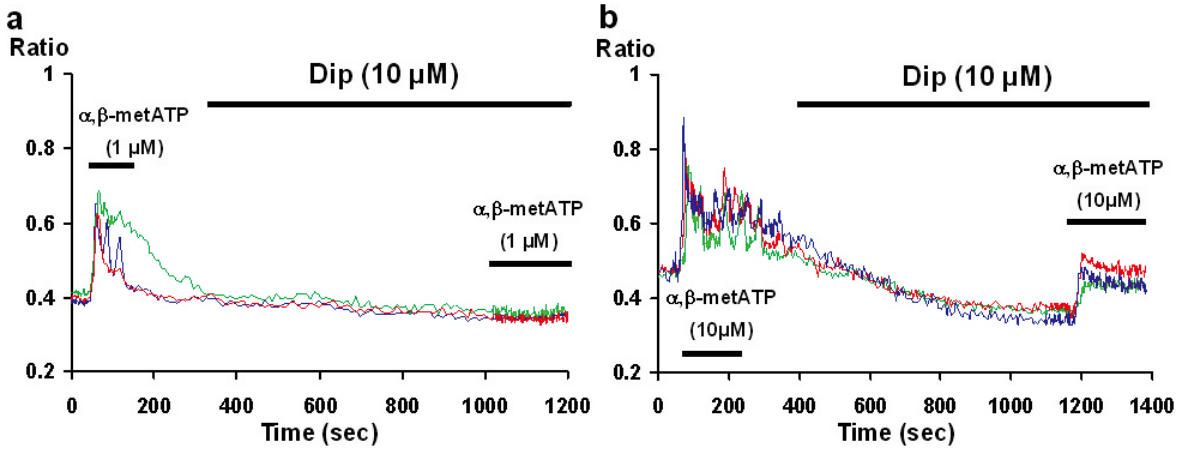

Fig. 4. Time courses for $[Ca^{2+}]_i$ dynamics induced by α, β–methylene ATP in testicular arteriole smooth muscle cells (**a** and **b;** blue, red, and green lines) in certain areas (about 1 μm^2). Arterioles were stimulated by treatment with α, β–methylene ATP (1 μM or 10 μM) at room temperature. α, β–methylene ATP-induced $[Ca^{2+}]_i$ increases in the smooth muscle cells were observed. After washing out the residual α, β–methylene ATP for 3 min, the specimens were pre-equilibrated with dipyridamole (10 μM) for 10 min before the addition of α, β–methylene ATP. In the presence of dipyridamole, α, β–methylene ATP(1 μM)-induced $[Ca^{2+}]_i$ increases in arteriole smooth muscle cells were completely inhibited (**a**). In cases of stimulation with 10 μM α, β–methylene ATP, α, β–methylene ATP-induced $[Ca^{2+}]_i$ increases in the cells were partially inhibited (**b**). Color scale bar: fluorescence ratio represents $[Ca^{2+}]_i$.

contain P2X (ligand-gated ion channels) receptors, and cerebral arteriole cells contain both P2X and P2Y (G-protein coupled types) receptors (Saino *et al*., 2002), indicating that the muscle cells in both types of arterioles respond to extracellular ATP via P2X purinoceptors. Actually, ATP (10 μM) induced $[Ca^{2+}]_i$ increases in the smooth muscle cells in the present experiment (Fig. 3a', b'). In the presence of dipyridamole, these ATP-induced $[Ca^{2+}]_i$ changes were inhibited in testicular arteriole smooth muscle cells (n=12)(Fig. 3a and compare a' with a''), but they not completely inhibited in cerebral arteriole cells (n=14)(Fig. 3b and compare b' with b''). The effect of dipyridamole in both arterioles was the same as that observed in the absence of extracellular Ca^{2+} (n=14)(data not shown). α,β-methylene ATP (a typical agonist of P2X purinoceptors, 1 μM) also induced $[Ca^{2+}]_i$ increases in testicular arteriole cells but were completely inhibited by dipyridamole (n=6)(Fig. 4a). In cases of stimulation with 10 μM α, β-methylene ATP, $[Ca^{2+}]_i$ increases in the cells were not completely inhibited by dipyridamole (n=8) (Fig. 4b).

Whether nitric oxide (NO) participates in this reaction was also investigated. Dipyridamole caused no increases in intracellular NO concentration in smooth muscle cells in testicular arterioles (n=12)(Fig.5a). S-nitroso-N-acetylpenicillamine(SNAP)(100 μM), a NO donor, also failed to inhibit ATP-induced $[Ca^{2+}]_i$ increases in testicular arterioles (n=12)(Fig. 5b). These results indicate that, under these conditions, NO is not involved in dipyridamole-mediated $[Ca^{2+}]_i$ reactions in arteriole smooth muscle cells.

Effect of dipyridamole on depolarization-induced $[Ca^{2+}]_i$ dynamics

To clarify whether smooth muscle cells are electrically excitable, we observed the $[Ca^{2+}]_i$ dynamics of membrane depolarization, as caused by a high potassium ion (high K^+) environment (56 mM). Depolarization by high K^+ induced a $[Ca^{2+}]_i$ increase in smooth muscle cells of testicular arterioles (n=10)(Fig. 6a, b, d). However, dipyridamole partially inhibited any high K^+-induced $[Ca^{2+}]_i$ increase (n=10) (Fig. 6c, d). In the absence of dipyridamole, a second introduction of high K^+ produced the same increase in calcium that had been observed in response to the first application (data not shown). Thus, the reduced responses observed during dipyridamole treatment were not due to receptor desensitization but, rather, to the effects of dipyridamole.

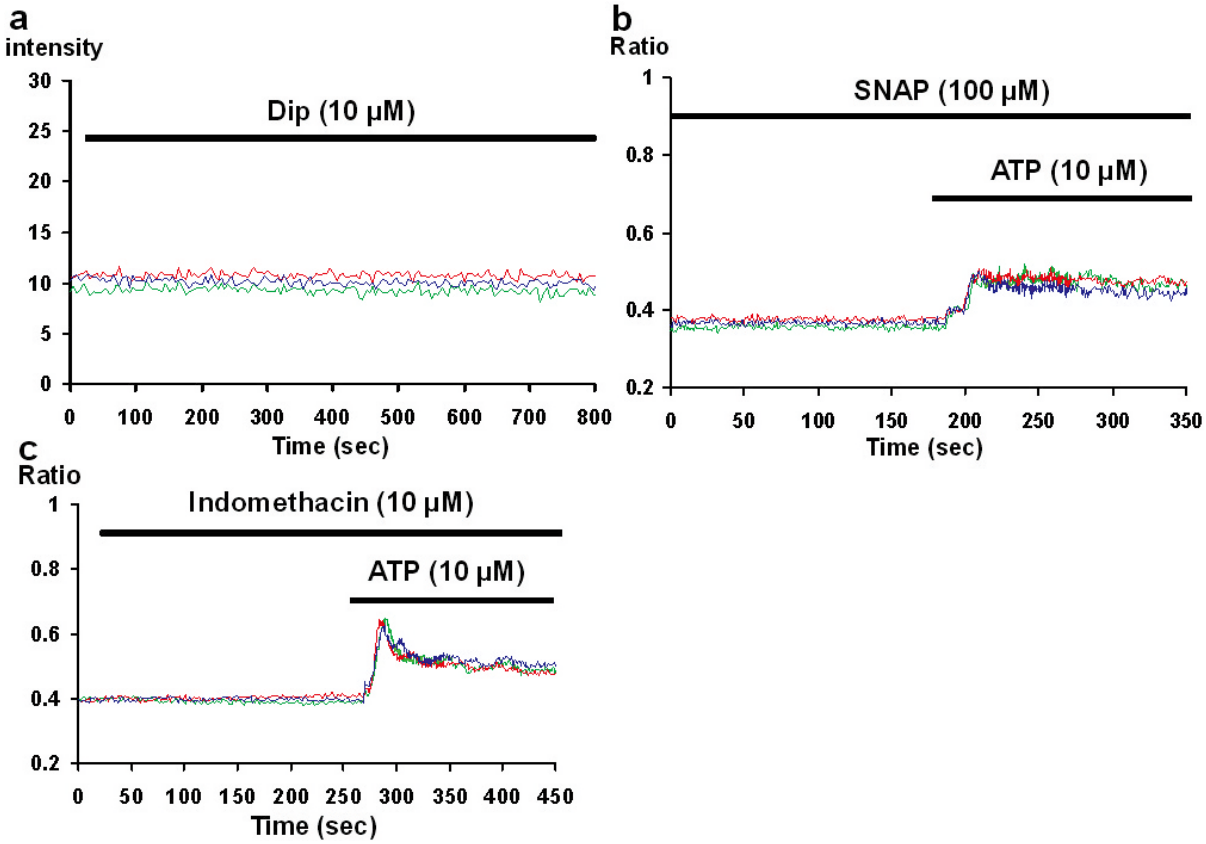

Fig. 5. Time courses for intracellular NO concentration induced by dipyridamole and $[Ca^{2+}]_i$ dynamics induced by ATP in the presence of drugs involved in NO and prostaglandins (**a**–**c**; blue, red, and green lines) in testicular arteriole smooth muscle cells at certain areas (about 1 μm^2). DAF-2/DA was used to measure NO concentrations. When arterioles were perfused with dipyridamole (10 μM), the issue of whether NO was produced in arteriole smooth muscle cells was determined. Dipyridamole failed to induce an increase in NO intensity in testicular arteriole smooth muscle cells (**a**). In the presence of S-nitroso-N-acetylpenicillamine (SNAP, 100 μM), the ATP (10 μM)-induced $[Ca^{2+}]_i$ increases were not completely inhibited in the arteriole smooth muscle cells (**b**). After treatment with indomethacin (10 μM), ATP caused $[Ca^{2+}]_i$ increases in the cells (**c**).

Effect of some reagents on ATP-induced $[Ca^{2+}]_i$ dynamics

We next attempted to clarify the mechanism of the intracellular action of dipyridamole. Because it is well-known that dipyridamole is an inhibitor of adenosine uptake and also acts as a cGMP phosphodiesterase inhibitor, we examined the effect of dipyridamole to adenosine and cGMP phosphodiesterase.

Adenosine (10 μM), a P1 purinoceptor agonist, failed to cause $[Ca^{2+}]_i$ increases in testicular arteriole smooth muscle cells (Fig. 7a). In the presence of adenosine, the first rapid phase of ATP-induced $[Ca^{2+}]_i$ increases was not inhibited in the cells, but the second plateau phase disappeared (Fig. 7a, blue arrowheads). This result shows that adenosine is partially involved in dipyridamole-mediated $[Ca^{2+}]_i$ reactions in these cells. While it is known that both isobutylmethylxanthine (IBMX; 500 μM) and forskolin (1 μM) are activators of protein kinase A, ATP-induced $[Ca^{2+}]_i$ increases in arteriole smooth muscle cells were not inhibited by pretreatment with these activators (n=12)(Fig. 7b, c). In the presence of dibutyryl cAMP (50 μM), a cell permeable cAMP analog, stimulation by ATP failed to inhibit an increase in Ca^{2+} in these cells (n=14) (Fig. 7d). These results show that activations of protein kinase A are not involved in dipyridamole-mediated

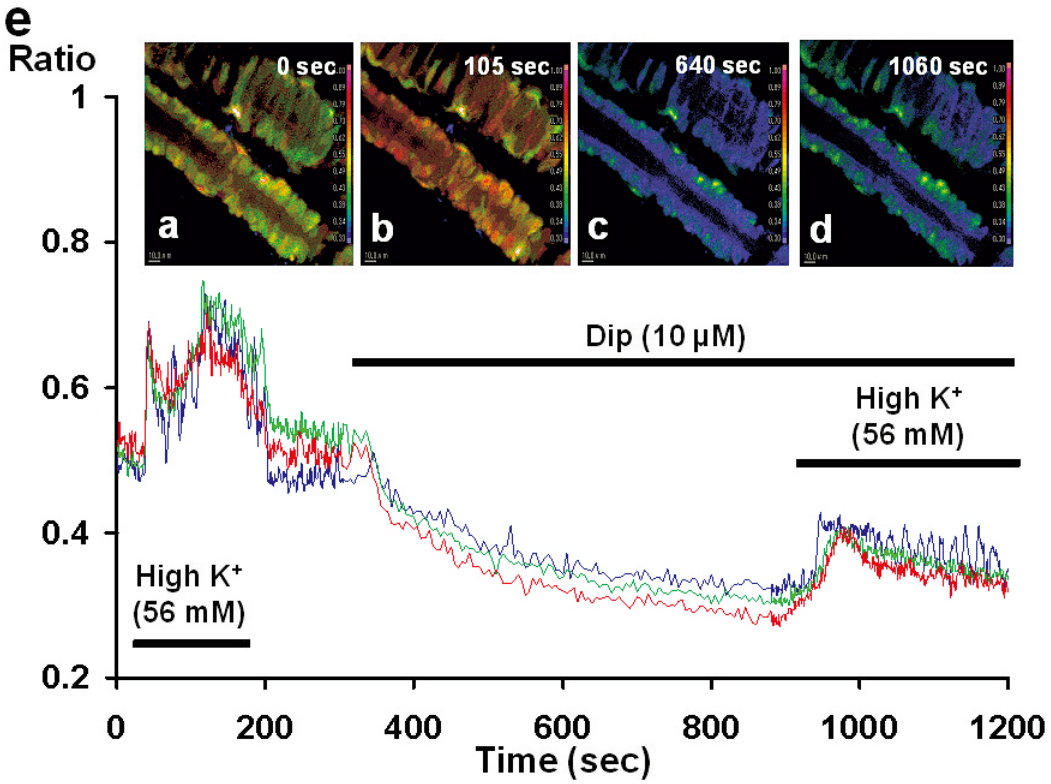

Fig. 6. Pseudocolor images of testicular arteriole smooth muscle cells show $[Ca^{2+}]_i$ changes, as measured by the ratiometry of Indo-1 (**a:** 0 sec, **b:** 105 sec, **c:** 640 sec, d: 1060 sec) and time courses for $[Ca^{2+}]_i$ dynamics induced by high K^+ in testicular arteriole smooth muscle cells (**e:** blue, red, and green lines) at certain areas (about 1 μm^2). High K^+ (56 mM)-induced a $[Ca^{2+}]_i$ increase in the cells (compare **a** with **b**). After washing out the residual high K^+ for 2.5 min (**c**), the specimens were pre-equilibrated dipyridamole (10 μM) for 10 min before the addition of high K^+. Dipyridamole partially inhibited any high K^+-induced $[Ca^{2+}]_i$ increase (**d** and **e**).

$[Ca^{2+}]_i$ reactions in arteriole smooth muscle cells.

To investigate the possible contribution of cGMP phosphodiesterase, the effect of zaprinast, an inhibitor of cGMP phosphodiesterase, was also examined, along with dipyridamole. Zaprinast (20 μM) failed to inhibit ATP-induced $[Ca^{2+}]_i$ increases (n=14)(Fig. 8a). ATP-induced $[Ca^{2+}]_i$ increases were not completely inhibited in the cells, either in the presence of dibutyryl cGMP (a cell permeable cGMP analog; 50 μM)(Fig. 8b) or in the presence of H8 (an inhibitor of protein kinase G; 10 μM) (Fig. 8c). Similarly, in the presence of KT 5823 (an inhibitor of protein kinase G; 1 μM), stimulation by ATP failed to inhibit an increase in Ca^{2+} in these cells (n=14) (data not shown). These findings confirm that cGMP pathways do not directly contribute to dipyridamole-mediated $[Ca^{2+}]_i$ reactions in testicular arterioles.

Taking all of these factors into consideration, the effect of dipyridamole in arteriole smooth muscle cells appears to be inhibited by an influx of extracellular Ca^{2+}.

Discussion

The $[Ca^{2+}]_i$ dynamics of dipyridamole using ATP and certain types of reagents were examined in smooth muscle cells of both testicular and cerebral arterioles. To our knowledge, there are only a few studies on the mechanism of action of dipyridamole in systemic blood vessels including arterioles (Ziegler *et al.*, 1995, 1998; Meyer *et al.*, 1996; Kruuse *et al.*, 2001)—showing that

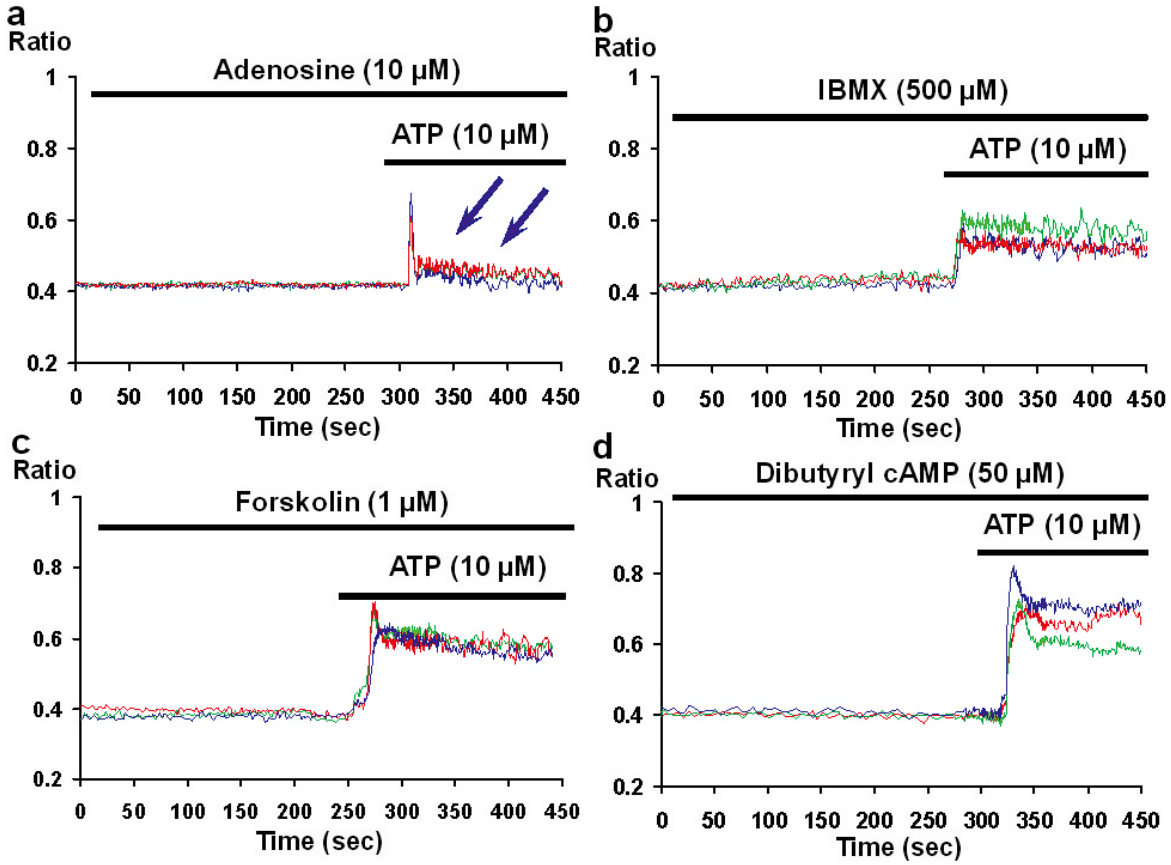

Fig. 7. Time courses for intracellular $[Ca^{2+}]_i$ dynamics induced by ATP in the presence of different drugs involved in P1 purinoceptors in testicular arteriole smooth muscle cells (**a**–**d**; blue, red, and green lines) in certain areas (about 1 μm^2). Adenosine (10 μM) had no effect on the $[Ca^{2+}]_i$ dynamics (**a**). In the presence of adenosine, stimulation by ATP (10 μM) did not completely inhibit the increase in Ca^{2+} in testicular arteriole smooth muscle cells (**a**). The first rapid phase of ATP-induced $[Ca^{2+}]_i$ increases was not inhibited in the cells. However, the second plateau phase disappeared (**a**, blue arrow heads). In the presence of activators of protein kinase A (such as IBMX (500 μM) or forskolin (1 μM)), ATP-induced $[Ca^{2+}]_i$ increases were not completely inhibited (**b** and **c**). When arterioles were perfused with dibutyryl cAMP (50 μM), the ATP-induced $[Ca^{2+}]_i$ increases were not completely inhibited (**d**).

dipyridamole induces the dilatation of arteries. However, the methods in these studies were only for observations of the percentage of relaxation of the pre-contraction in some arteries. Furthermore, they did not investigate $[Ca^{2+}]_i$ dynamics in arteries. This therefore is the first study to clearly demonstrate that dipyridamole abolished ATP-induced $[Ca^{2+}]_i$ dynamics in arterioles by real-time confocal microscopy. Also the effect of dipyridamole in arteriole smooth muscle cells appears to be inhibited by an influx of extracellular Ca^{2+}. In this study, we further showed that dipyridamole causes dilation through intracellular calcium mechanisms by multiple potential mechanisms.

Response of smooth muscle cells in testicular and cerebral arterioles with respect to dipyridamole and ATP

As described in the results, dipyridamole inhibits adenosine uptake (German *et al*., 1989; Ferrandon *et al*., 1994), and cGMP phosphodiesterase (Ahn *et al*., 1989; Gillespie and Beavo, 1989; Sakuma *et al*., 1990).

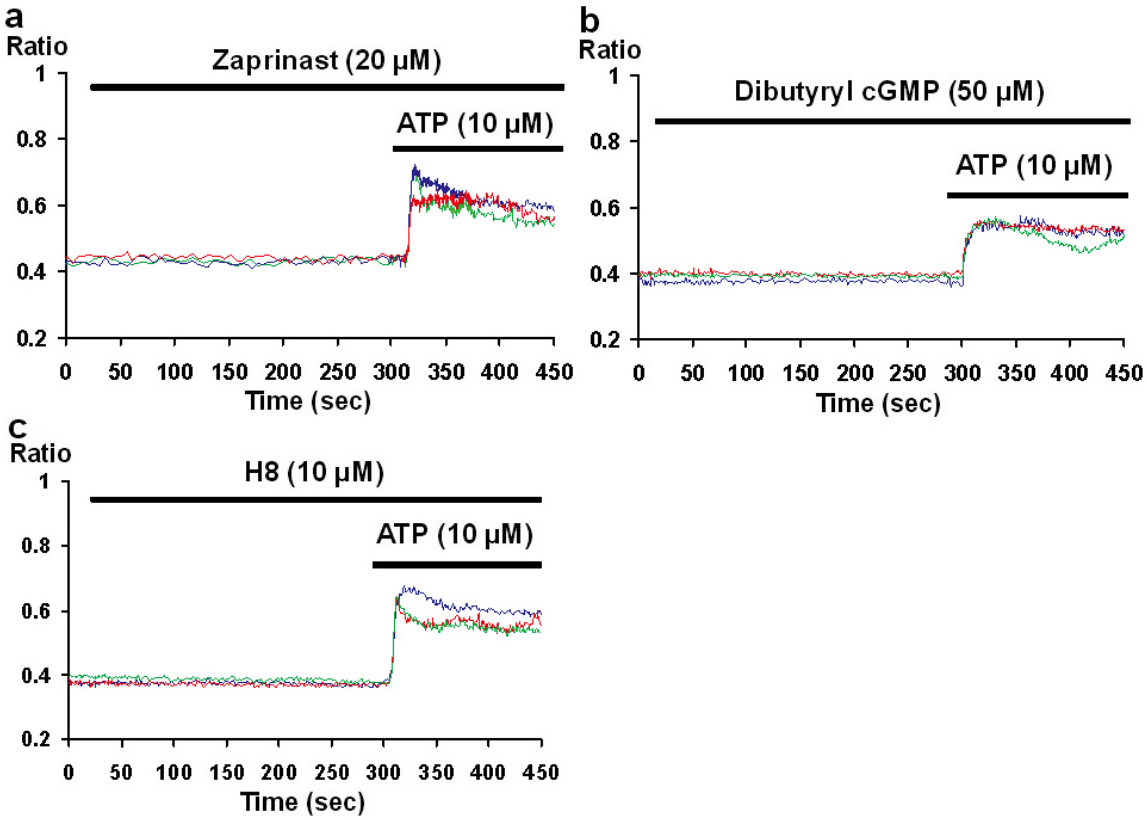

Fig. 8. Time courses for intracellular $[Ca^{2+}]_i$ dynamics induced by ATP(10 μM) in the presence of drugs involved in cGMP (**a–c;** blue, red, and green lines) in testicular arteriole smooth muscle cells in certain areas (about 1 μm^2). When zaprinast (20 μM) was used as a stimulus, a $[Ca^{2+}]_i$ increase was not observed (**a**). In the presence of zaprinast, ATP-induced $[Ca^{2+}]_i$ increases were not inhibited (**a**). When arterioles were perfused with dibutyryl cGMP (50 μM), ATP-induced $[Ca^{2+}]_i$ increases were not completely inhibited (**b**). In the presence of H8 (10 μM), the ATP-induced $[Ca^{2+}]_i$ increase was not completely inhibited (**c**).

Several studies have also shown the dilatory effects of dipyridamole as more or less selective phosphodiesterase inhibitors on intact cerebral arteries (Harris *et al*., 1989; Cosentino *et al*., 1992; Parfenova *et al*., 1993; Rosenblum *et al*., 1993; Willette *et al*., 1997). The present study demonstrated that dipyridamole induced only small changes in $[Ca^{2+}]_i$ in testicular or cerebral arteriole smooth muscle cells, and no dilation was observed in these arterioles. In testicular arterioles, the addition of dipyridamole abolished Ca^{2+} related responses to ATP. In contrast, in the presence of dipyridamole, the ATP-induced $[Ca^{2+}]_i$ changes in cerebral arterioles were not completely inhibited. The inhibition of the influx of Ca^{2+} abolished Ca^{2+} related responses to ATP in testicular arterioles. Taking our recent data into consideration (Saino *et al*., 2002), almost the same inhibitory patterns against an ATP-mediated $[Ca^{2+}]_i$ increase were observed between the addition of dipyridamole and the absence of extracellular Ca^{2+}.

The α,β–methylene ATP (1 μM)-induced $[Ca^{2+}]_i$ increases in testicular arteriole smooth muscle cells were completely inhibited by pretreatment with dipyridamole. However, in cases of stimulation with 10 μM α,β–methylene ATP, $[Ca^{2+}]_i$ increases in the cells were not completely inhibited. These findings suggest that dipyridamole is not a specific inhibitor of P2X purinoceptors but may block the influx of calcium from the extracellular spaces. In contrast with our findings, Naito *et al*. (2004) showed that dipyridamole had no effect on vasoconstrictor responses to ATP in canine splenic arteries. There might be differences in species, organs, or the sizes of arterial vessels because we used

"arterioles" in our experiments, and the findings suggest a strong effect of dipyridamole on rat testicular arterioles.

Response of smooth muscle cells in testicular arterioles with respect to nitric oxide

Nitric oxide (NO), which is generated in biological tissues, is an important regulator of a broad range of functions (Moncada *et al*., 1991). NO is a physiologically important vasodilator, and stimulates vasorelaxation in part, through the activation of guanylyl cyclase and the formation of cGMP (Schmidt *et al*., 1993; Lincoln *et al*., 1996, Hewitson *et al*., 2002). Sodium nitroprusside inhibited the activation of thromboxane synthase by thrombin in a dose-dependent manner, which was clearly enhanced by further incubation with dipyridamole (Aktas *et al*., 2003). Dipyridamole also has been reported to directly stimulate the release of endothelial prostacyclin (Neri Serneri *et al*., 1981; Mehta and Mehta, 1982; Costantini *et al*., 1990). However, we showed that no change in $[NO]_i$ was observed in the presence of dipyridamole. Furthermore, in the presence of S-nitroso-N-acetylpenicillamine (SNAP) or indomethacin, ATP-induced $[Ca^{2+}]_i$ responses were not completely inhibited. These findings are not consistent with the general view that dipyridamole activates an increase in NO synthesis and releases prostacyclin; instead, they show how the possibility that dipyridamole is indirectly involved in these reactions cannot be excluded.

Response of smooth muscle cells in testicular arterioles with respect to high K^+

In our study, dipyridamole partially inhibited a depolarization-induced $[Ca^{2+}]_i$ increase. This finding is consistent with the statement that dipyridamole might inhibit the sickling-induced fluxes of Na^+, K^+, and Ca^{2+} in sickled red blood cells (Joiner *et al*., 2001). However, the order of potency for the calcium entry blocking effect from greatest to weakest was verapamil to diltiazem to adenosine to lidoflazine and finally to dipyridamole (Nakagawa *et al*., 1986). It is difficult to conclude that dipyridamole is a simple Ca^{2+} channel blocker. Further studies are needed to clarify the relationship between a Ca^{2+} channel and dipyridamole in arteriole smooth muscle cells.

Response of smooth muscle cells in testicular arterioles with respect to adenosine

Because dipyridamole inhibits the uptake of adenosine by endothelial cells, its vasodilating activity has previously been attributed to an increase in circulating adenosine levels (Roos and Pfleger, 1972; German *et al*., 1989; Ferrandon *et al*., 1994). Adenosine significantly dilates the arterioles and produces a decrease in vessel wall calcium (Meininger *et al*., 1991). Adenosine also causes vasodilation through the stimulation of P1 receptors in vascular smooth muscle cells (Ralevic and Burnstock, 1998). In our previous report, we showed that adenosine induced no changes in $[Ca^{2+}]_i$ in testicular or cerebral arteriole smooth muscle cells (Saino *et al*., 2002). In the present study, we further demonstrated that, in the presence of adenosine, an ATP-induced $[Ca^{2+}]_i$ response was observed in arteriole smooth muscle cells—although this reaction was partially inhibited. Because adenosine A1 receptors mediate the inhibition of L-type Ca^{2+} channels (Di Perri *et al*., 1989; Rocher *et al*., 1999), the effects of dipyridamole on resistance vessels may be partly explained by the potentiation of adenosine mechanisms rather than the potentiation of NO or other cGMP-mediated actions (Gamboa *et al*., 2005).

Response of smooth muscle cells in testicular arterioles with respect to cGMP and cAMP

Dipyridamole and zaprinast are two widely recognized, potent phosphodiesterase type V inhibitors; both have been shown to exhibit a vasodilating activity (Rosenkrantz *et al*., 1972; Lugnier *et al*., 1986; Thomas *et al*., 1990; Braner *et al*., 1993; McMahon *et al*., 1993; Clarke *et al*., 1994; Ziegler *et al*., 1995, 1998; Meyer *et al*., 1996; Kruuse *et al*., 2001). In the present study, zaprinast failed to block the increase in ATP-induced $[Ca^{2+}]_i$ in testicular arterioles, indicating that dipyridamole and zaprinast affect vasodilation *via* different mechanisms.

In this study, little relation was evident between dipyridamole and cGMP or cAMP in testicular and cerebral arterioles although some previous authors have simultaneously measured the effects of phosphodiesterase inhibitors on cyclic nucleotide levels (Kim *et al*., 1992; Parfenova *et al*., 1993). Further experiments are necessary to clarify more completely the relationship between dipyridamole and cGMP or cAMP in arteriole smooth muscle cells.

Conclusions

The effect of dipyridamole with respect to ATP in the peripheral vascular system, especially testicular and cerebral arterioles, was examined using a Ca^{2+} imaging technique by real-time confocal microscopy. In this paper, we showed that dipyridamole exerts no direct

effects on arteriole smooth muscle cells. The findings here indicate that dipyridamole may act not only as an inhibitor of adenosine uptake or cGMP phosphodiesterase but also as a calcium channel blocker in arteriole smooth muscle cells.

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