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Dual regulation of cerebrovascular tone by UTP: P_{2U} receptor-mediated contraction and endothelium-dependent relaxation

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1 The mechanisms of vascular tone regulation by extracellular uridine 5'-triphosphate (UTP) were investigated in bovine middle cerebral arterial strips. Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and force were simultaneously monitored by use of front-surface fluorometry of fura-2.

2 In the arterial strips without endothelium, UTP (0.1 μM - 1 mM) induced contraction in a concentration-dependent manner. However, when the endothelium was kept intact, cumulative application of UTP (0.1 - 100 μM) (and only at 1 mM) induced a modest phasic contraction in arterial strips. This endothelium-dependent reduction of the UTP-induced contraction was abolished by 100 μM N^{ω}-nitro-L-arginine (L-NOARG) but not by 10 μM indomethacin. In the presence of intact endothelium, UTP (30 μM) induced a transient relaxation of the strips precontracted with 30 nM U-46619 (a stable analogue of thromboxane A_2), which was completely inhibited by pretreatment with L-NOARG but not with indomethacin.

3 In the endothelium-denuded strips, the contractile response to UTP was abolished by desensitization to either ATPγS or ATP (P_{2U} receptor agonists), but not by desensitization to α,β-methylene-ATP (P_{2X} receptor agonist) or to 2-methylthio-ATP (P_{2Y} receptor agonist). Desensitization to UTP abolished the contractile response to ATP.

4 In the endothelium-denuded artery, a single dose application of UTP induced an initial transient, and subsequently lower but sustained increase in $[Ca^{2+}]_i$ and force. In the absence of extracellular Ca^{2+}, UTP induced only the initial transient increases in $[Ca^{2+}]_i$ and force, while the sustained increases in $[Ca^{2+}]_i$ and force were abolished. UTP (1 mM) had no effect on the basic $[Ca^{2+}]_i$- force relationship obtained on cumulative application of extracellular Ca^{2+} at steady state of 118 mM K^+-depolarization-induced contraction.

5 We conclude that in the presence of an intact endothelium, UTP-induced relaxation of pre-constricted middle cerebral artery is mainly mediated indirectly, by the production of an endothelium-derived relaxing factor, but at high doses of UTP, vascular smooth muscle contraction is mediated directly via activation of P_{2U} purinoceptor and $[Ca^{2+}]_i$ elevation without Ca^{2+}-sensitization of the contractile apparatus. UTP may thus exert a dual regulatory effect upon cerebrovascular tone, but in cases where the endothelium is impaired, it may also act as a significant vasoconstrictor.

Keywords: UTP; U-46619; cerebral vasospasm; smooth muscle; intracellular calcium; Ca^{2+}-sensitivity; endothelium; endothelium-derived relaxing factor

Introduction

Brain is abundant in UTP (Keppler *et al.*, 1970). Since UTP has a potent vasoconstrictor action (Urquilla, 1978), it may be one of the possible spasmogens in cerebral vasospasm after subarachnoid haemorrhage (Shirasawa *et al.*, 1983). On the other hand, it has been reported that UTP induces prostacyclin (PGI_2) production in cultured endothelial cells (Needham *et al.*, 1987; Motte *et al.*, 1993) and endothelium-dependent relaxation of arterial smooth muscle, which is not inhibited by haemoglobin, an inhibitor of endothelium-derived relaxing factor (EDRF) (Hardebo *et al.*, 1987). Although PGI_2 may contribute to the relaxation of vascular smooth muscle (Moncada, 1982), it remains to be determined whether or not PGI_2 production is significantly involved in the UTP-regulation of cerebrovascular tone.

UTP elicits $[Ca^{2+}]_i$ elevation as potently as ATP in rat aortic smooth muscle cells (Tawada *et al.*, 1987; Erlinge *et al.*, 1993), implicating the possible involvement of the P_{2U} purinoceptor (one of the subtypes of P_2 purinoceptors, which is characterized by the rank order of potency for ATP analogues; UTP = ATP > ATPγS > 2-methylthio-ATP, β,γ-methylene-ATP) (O'Connor *et al.*, 1991). On the other hand, it was also reported that the vascular smooth muscle has a pyrimidine receptor (von Kügelgen *et al.*, 1987, 1990; Juul *et al.*, 1992; Sajag *et al.*, 1992), which is activated by UTP and distinct from the P_2 purinoceptor (Benham, 1989; Ralevic & Burnstock, 1991). At present, it is not clear whether or not there is a receptor common to UTP and ATP, that is a P_{2U} purinoceptor, to regulate vascular smooth muscle tone. In addition, it is not clear what mechanisms are involved in the regulation of UTP-induced contraction, especially $[Ca^{2+}]_i$ dynamics.

Thus, the mechanism underlying the cerebrovascular tone regulation by UTP is not well understood. The purpose of the present study is to investigate, (1) the mechanism of UTP-induced endothelium-dependent relaxation, and (2) the mechanism of UTP-induced contraction of cerebral arterial smooth muscle. To our knowledge, this is the first report showing the P_{2U} receptor-mediated increases in $[Ca^{2+}]_i$ and force in vascular smooth muscle.

Methods

Tissue preparation and force measurement

Bovine middle cerebral arteries ($n = 21$ vessels) were obtained immediately after slaughter of the animal at a local abattoir

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and brought to the laboratory in ice-cold physiological saline solution (PSS), as described (Miyagi *et al.*, 1995a, b). Briefly, segments 1–3 cm from the bifurcation of the internal carotid artery were excised. The arachnoid membranes were carefully excised and strips (1 × 4 mm) cut with scissors were prepared. In the case of endothelium-denuded strips, the luminal surface of the strip was rubbed off gently with a cotton swab under a microscope. The complete denudation of the endothelium was confirmed by a lack of relaxation induced by 1 μM bradykinin. Strips were mounted vertically in a quartz organ bath, being connected to a force-transducer (TB-612T Nihon Koden, Tokyo, Japan). During a 1 h equilibration-period, the strips were stimulated with 118 mM K^+-PSS every 15 min, and the resting tension was adjusted to 200 mg.

Chemical denervation

Prior to the experiment, chemical denervation was performed with KH_2PO_4- and $NaHCO_3$-free PSS containing 20 μM glutathione and 1.2 μM 6-hydroxydopamine, as described by Aprigliano & Hermsmeyer (1976). In addition, all solutions used in this study contained 3 μM tetrodotoxin, 1 μM phentolamine and 1 μM guanethidine (Agnus *et al.*, 1988). These procedures for chemical denervation had no effect on the contractile responses to 118 mM K^+-depolarization (Miyagi *et al.*, 1995b).

$[Ca^{2+}]_i$ measurement

The changes in $[Ca^{2+}]_i$ of bovine middle cerebral arteries were assessed by fura-2 front-surface fluorometry, as described before (Miyagi *et al.*, 1995a, b). Briefly, vascular strips were loaded with fura-2 by incubating in Dulbecco's modified Eagle's medium containing 50 μM fura-2/AM (an acetoxymethyl ester form) and 5% foetal bovine serum for 4 h at 37°C, under aeration with 95% O_2 and 5% CO_2. The fura-2-loaded strips were washed with normal PSS to remove the dye in the extracellular space, and were then further incubated in normal PSS for 1 h. Fluorescence intensities of the fura-2-Ca^{2+} complex were simultaneously monitored with the force development, using a fluorometer specially designed for fura-2 front-surface fluorometry (CAM-OF3, Japan, Spectroscopic Co., Tokyo, Japan) (Ushio-Fukai *et al.*, 1993) and the ratio of the fluorescence intensities (500 nm emission) at 340 nm excitation to those at 380 nm excitation was monitored. Before starting each measurement, the responsiveness of each strip to 118 mM K^+-PSS was recorded, for the purposes of normalization. The force and fluorescence ratio were expressed as a percentage (% force and % ratio), assuming the values in normal (5.9 mM K^+) and 118 mM K^+-PSS, to be 0% and 100%, respectively. Because the calibration of the absolute levels of $[Ca^{2+}]_i$ at the end of experiments is liable to be uncertain, statistical analysis of $[Ca^{2+}]_i$ signals was performed using % ratio which is stable over 1 h of measurement (Miyagi *et al.*, 1995a, b).

Drugs and solutions

Normal PSS was composed as follows (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. Ca^{2+}-free version of PSS (Ca^{2+}-free PSS) contained 2 mM ethylene-glycol-*bis* (β-amino-ethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) instead of 1.25 mM $CaCl_2$. K^+ (118 mM)-PSS was identical to normal PSS, except for an equimolar substitution of KCl for NaCl. All solutions were aerated with a mixture of 5% CO_2 and 95% O_2 (pH 7.4; 37°C). EGTA and Fura-2/AM were purchased from DOJINDO (Kumamoto, Japan). Fura-2/AM was dissolved in dimethyl sulphoxide (DMSO) and diluted in medium just before loading the dye. The following drugs were used: UTP, ATP, α,β-methylene ATP, ATPγS, tetrodotoxin, prazosin, guanethidine (Sigma, St. Louis, U.S.A.), 2-methylthio-ATP (Research Biochemicals Incorporated, MA, U.S.A.), phentolamine (Chiba Geigy, Hyogo, Japan), N^{ω}-nitro-L-arginine (Aldrich Chemical, WI, U.S.A.), and indomethacin (WAKO Pure Chemical Industries, Osaka, Japan). U-46619 was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.) and dissolved in DMSO (1 mM) and stored at −20°C.

Data analysis

Values were expressed as mean ± s.e.mean. Comparisons among the four groups were made by one-way analysis of variance followed by Dunnett's test. Student's *t* test was used to determine statistical significance between two groups and an analysis of covariance was used to determine the non-overlapping (or shift) of the $[Ca^{2+}]_i$-force relationship.

Results

Force development by UTP in cerebral arterial smooth muscle with and without intact endothelium

To determine the characteristics of UTP-induced force development, the arterial strips were stimulated by cumulative application of UTP (0.1 μM–1 mM) (Figure 1). With intact endothelium, UTP at concentrations below 100 μM did not induce any contraction, but a transient slight decrease of the tone (Figure 1a,e). Only at 1 mM, did UTP cause a phasic contraction, the peak level of the force was 38 ± 6.6% of the response to 118 mM K^+-PSS. In contrast, in the absence of endothelium, UTP induced contraction in a concentration-dependent manner (Figure 1b,e). At a concentration higher than 10 μM, UTP apparently induced contraction; at 1 mM, UTP-induced contraction was 81 ± 13%. The concentration-response curves of UTP did not appear to reach maximum at 1 mM, and as a result, therefore the concentrations generating half maximal response (EC_{50}) could not be determined. Thus, the endothelium markedly attenuated the UTP-induced contraction of the arterial strips. To investigate the mechanism underlying the inhibitory effect of endothelium on UTP-induced contraction, strips were incubated with 100 μM N^{ω}-nitro-L-arginine (L-NOARG), which is known to inhibit nitric oxide synthesis (Moncada *et al.*, 1991), for 40 min before the initiation of the experiment. In the strips preincubated with 100 μM L-NOARG, UTP-induced contraction was first observed at 100 μM and reached 80 ± 1.7% of the level of 118 mM K^+-induced contraction at 1 mM, the extent of which was similar to that of endothelium-denuded strips (79 ± 10%) (Figure 1c,e). Preincubation with 10 μM indomethacin for 40 min had no effect on this inhibitory effect of endothelium at all concentrations of UTP examined ($P > 0.05$) (Figure 1d,e).

U-46619 (a stable analogue of thromboxane A_2 induced a sustained contraction (in the presence of endothelium, 112 ± 4.1%; in the absence of endothelium, 112 ± 4.8%) (Figure 2). When the strips were precontracted with 30 nM U-46619, in the presence of endothelium, 30 μM UTP induced a transient relaxation, in which the reduction of force reached the lowest level (32.3%) at 1 min and almost disappeared at 15 min (Figure 2a,e), whereas in the absence of endothelium 30 μM UTP neither increased nor decreased the force of precontracted strips (Figure 2b,e). Preincubation of the strips with endothelium with 100 μM L-NOARG for 40 min abolished the transient relaxation completely (Figure 2c,e), while preincubation with 10 μM indomethacin did not attenuate but rather enhanced the extent of UTP-induced relaxation ($P < 0.05$) (Figure 2d,e).

Subtype of P_2 purinoceptor mediating UTP-induced contraction of cerebral arterial smooth muscle

In this study, we investigated whether a pyrimidine receptor distinct from a P_2 receptor or a subtype (P_{2U}) of P_2 receptor is involved in the direct contractile effect of UTP on cerebral arterial smooth muscle. As shown in Figure 3a,b,d, the desensitization of P_{2X} receptor by incubating arterial strips with

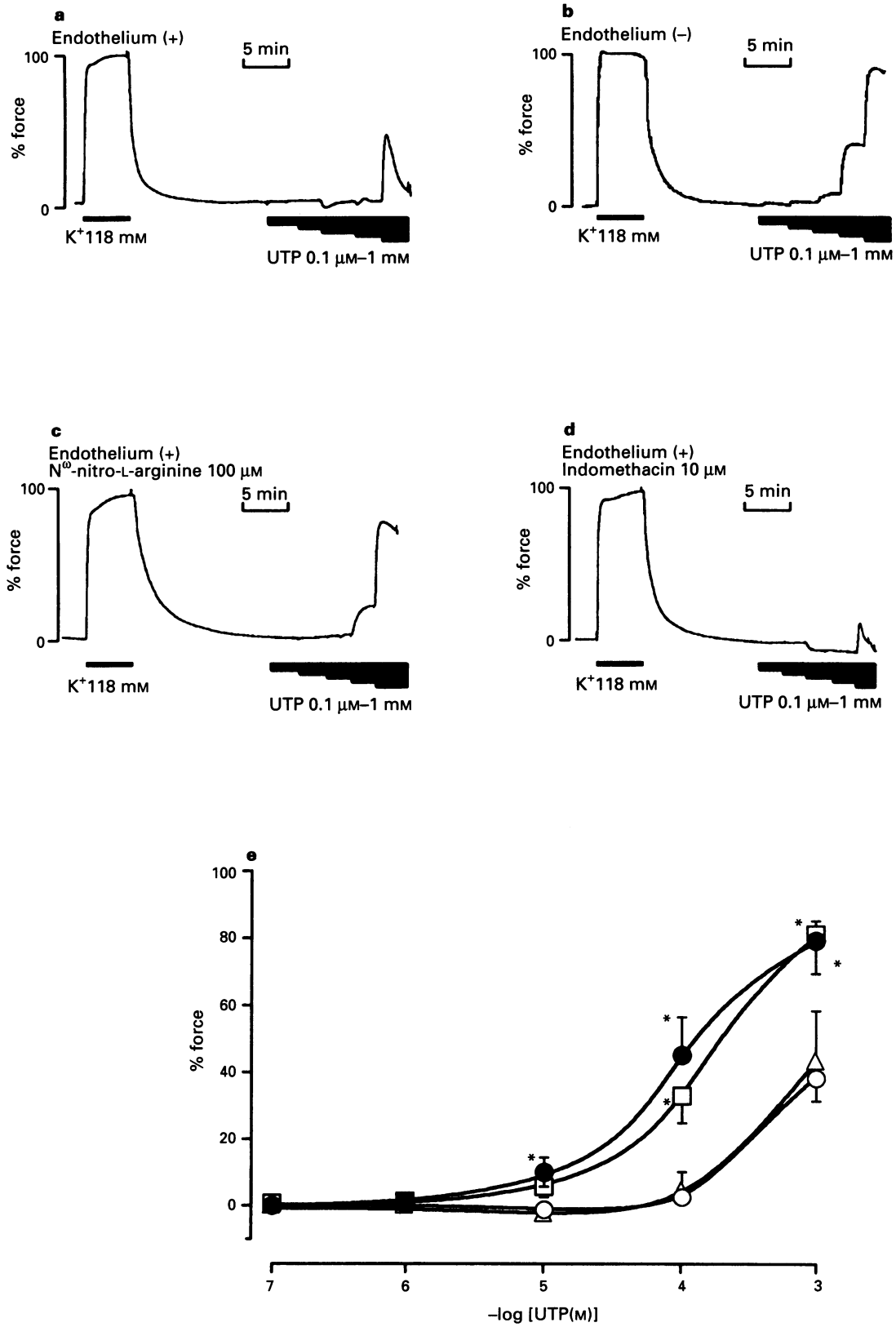

Figure 1 Representative recordings of the effect of cumulative applications of UTP (0.1, 1, 10, 100 μM, and 1 mM) on force cerebral arterial strips. (a) Endothelium-intact strips (control), (b) endothelium-denuded strips, (c,d) endothelium-intact strips pretreated with either 100 μM N^{ω}-nitro-L-arginine (L-NOARG) (c) or 10 μM indomethacin (Ind) (d). The pretreatments of arterial strips with L-NOARG or Ind were carried out 40 min before starting experiments. At the beginning of each measurement, the responses to 118 mM K^+-PSS were recorded and registered as 100%. (e) Summary of changes in force induced by cumulative applications of UTP. All data were obtained from 5 different experiments. The responses observed after pretreatment with L-NOARG or Ind and endothelium-denudation were compared statistically with those of the control; strips with intact endothelium (○); (●) endothelium-denuded; (□) pretreated with 100 μM; L -NOARG; (△) pretreated with 10 μM Ind. *$P<0.05$.

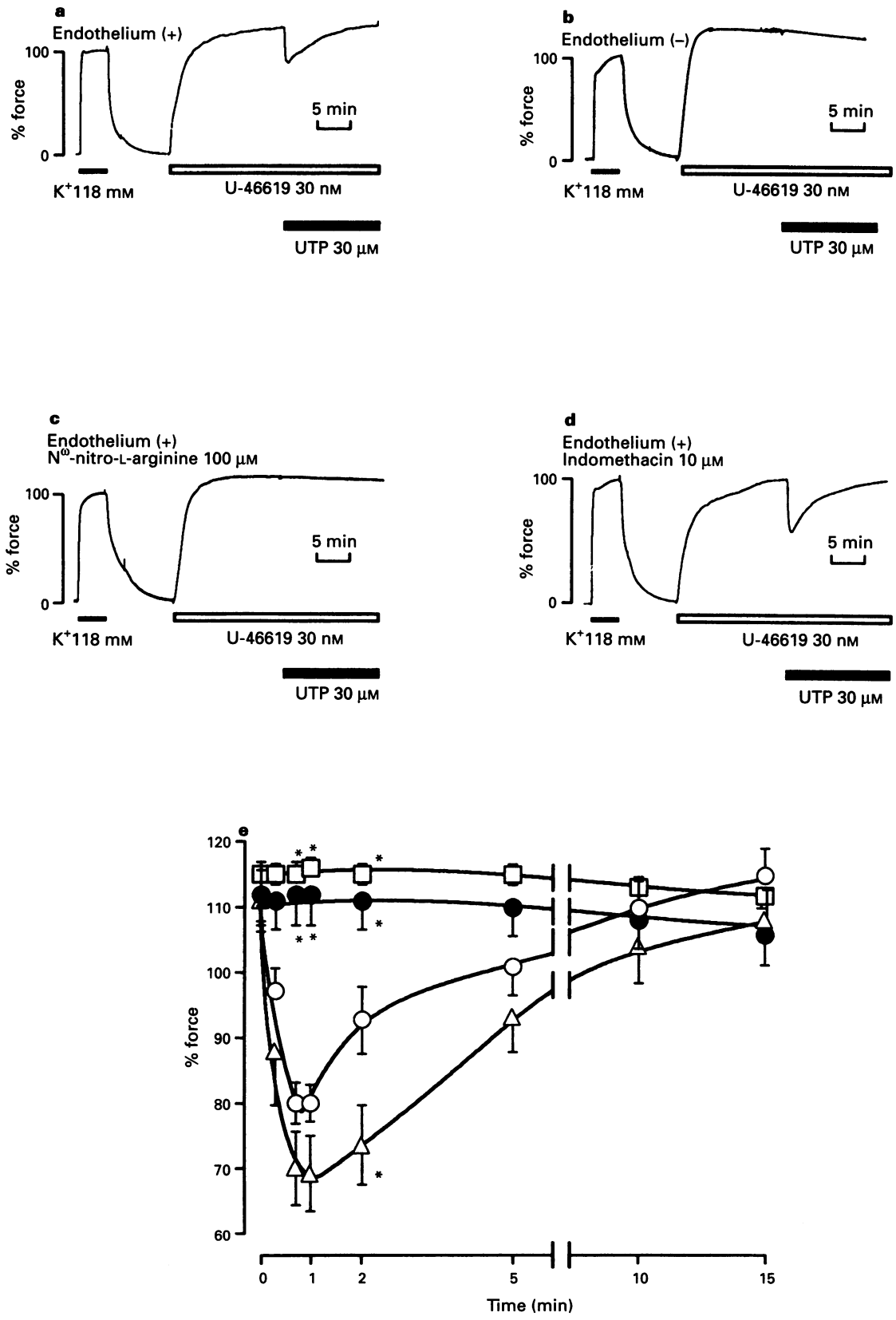

Figure 2 Representative recordings showing UTP (30 μM)-induced changes in force increased by pretreatment with U-46619 (30 nM). (a) Endothelium-intact strips (control), (b) endothelium-denuded strips, (c.d) endothelium-intact strips pretreated with either 100 μM N^{ω}-nitro-L-arginine (L-NOARG) (c) or 10 μM indomethacin (Ind) (d). The pretreatments of arterial strips with L-NOARG or Ind were carried out 40 min before starting experiments. At the beginning of each measurement, the responses to 118 mM K^+-PSS were recorded and registered as 100%. (e) Summary of the changes in force induced by 30 μM UTP of vascular strips precontracted with 30 nM U-46619. All data were obtained from 5 different experiments. The responses observed after pretreatments with L-NOARG or Ind and endothelium-denudation were compared statistically with those of the control strips with intact endothelium (○); (●) endothelium-denuded; (□) pretreated with 100 μM L-NOARG; (△) pretreated with 10 μM Ind. *$P<0.05$.

α,β-methylene-ATP (100 μM) abolished the second contractile response to further cumulative application of α,β-methylene-ATP (finally 200 μM). However, the subsequent application of either 1 mM UTP (Figure 3a) or 1 mM ATP (Figure 3b) induced significant contractions of the P_{2X}-desensitized strips. Subsequently, the second cumulative application of UTP (finally 2 mM) or ATP (finally 2 mM) on P_{2X}-desensitized strips did not elicit further contraction (Figure 3a,b), which indicated that both P_{2X} and UTP or other possible ATP receptors were desensitized. Under these conditions, the application of ATP or UTP did not elicit a further contraction of UTP- or ATP-desensitized strips, respectively (Figure 3a,b). Another potent agonist of the P_{2U} receptor, ATPγS (100 μM), also induced a potent contraction similar to UTP (Figure 3c). The second

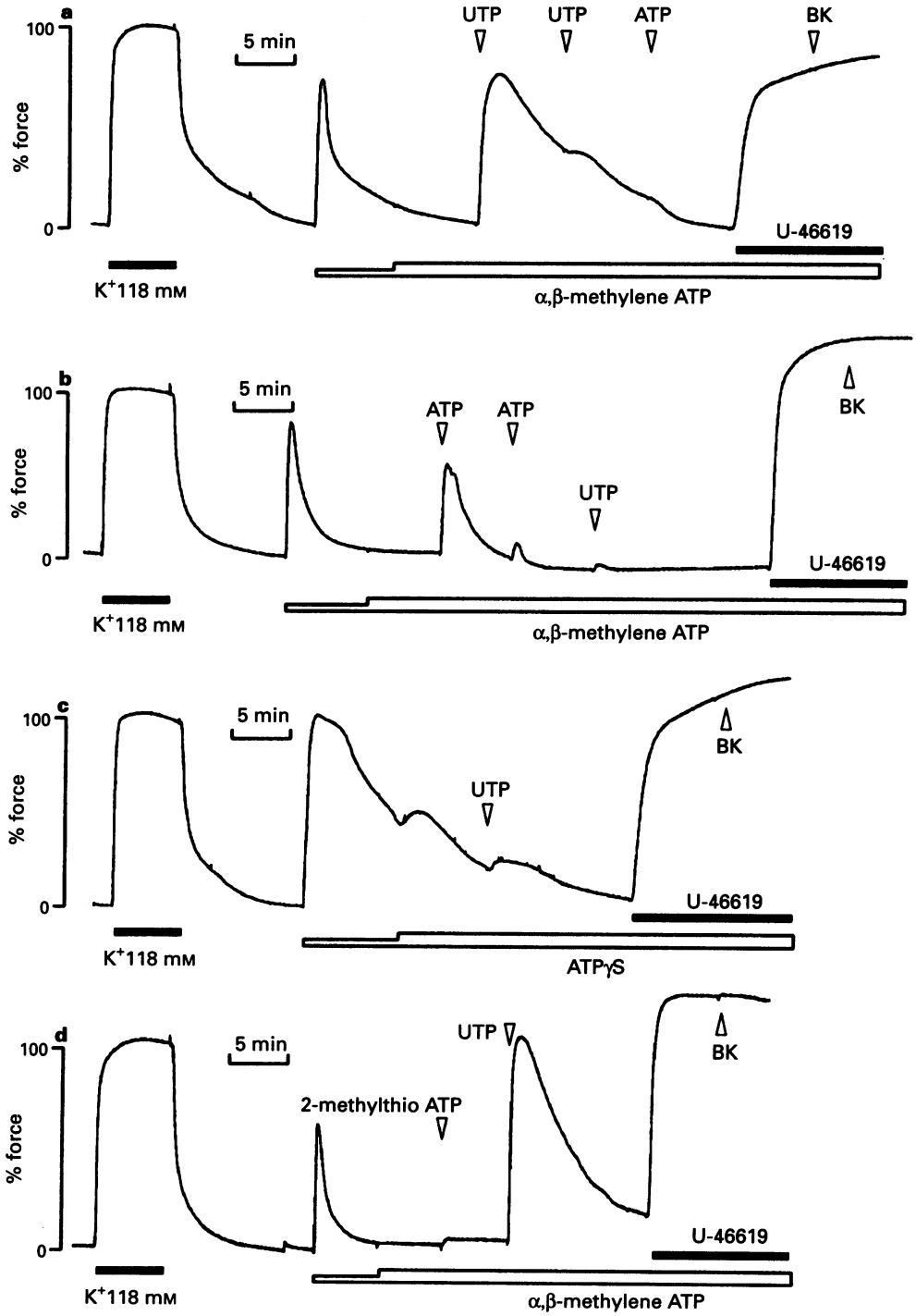

Figure 3 Cross-desensitization between ATP and UTP on the force development of endothelium-denuded cerebral arterial strips. After recording the control response to 118 mM K^+-PSS, nucleotides at the minimal concentrations required to induce maximal responses (1 mM ATP, 1 mM UTP, 100 μM α,β-methylene-ATP, and 100 μM 2-methylthio-ATP) were applied additively without any intervening washes. The nucleotides given at first were applied twice to confirm the complete desensitization to homologous nucleotides. The orders of the applications were: (a) two of α,β-methylene-ATP; two of UTP, and ATP; (b) two of α,β-methylene-ATP, two of ATP, and UTP; (c) two of ATPγS and UTP; and (d) two applications of α,β-methylene-ATP, 2-methylthio-ATP, and UTP. U-46619 (1 μM) and bradykinin (1 μM) were added at the end of each measurement to confirm the contractility and the success in denudation of endothelium of nucleotides-desensitized strips, respectively.

cumulative application of ATPγS (finally 200 μM) did not elicit further contraction, and in this condition, the subsequent appalication of UTP (1 mM) also did not induce further contractions of the ATPγS-desensitized strips. 2-Methylthio-ATP (100 μM), a potent agonist at the P_{2Y} receptor, induced a small and transient contraction of arterial strips (data not shown); however, it did not elicit further contraction of P_{2X}-desensitized strips, which suggests that 2-methylthio-ATP binds to the P_{2X} receptor in bovine cerebral arterial smooth muscle. Although 2-methylthio-ATP did not elicit any contraction in P_{2X}-desensitized strips, UTP elicited a further contraction (Figure 3d). At the end of each experiment, U-46619 (1 μM) and bradykinin (1 μM) were added to confirm that there was no nonspecific toxic condition in this protocol to affect the contractility of smooth muscle and that endothelial cells were successfully removed, respectively.

Mechanism underlying UTP-induced contraction of cerebral arterial smooth muscle

To investigate the direct effect of UTP on cerebral arterial smooth muscle, the changes in $[Ca^{2+}]_i$ and force of endothelium-denuded strips were simultaneously monitored. As shown in Figure 4, in the presence of extracellular Ca^{2+}, UTP induced an initial transient and subsequent sustained lower increase in $[Ca^{2+}]_i$ and force of endothelium-denuded arterial strips in a concentration-dependent manner. The peak levels of transient $[Ca^{2+}]_i$ elevation induced by 1 mM UTP reached 111±16.4% of the response to 118 mM K^+-PSS (=100%), and then, $[Ca^{2+}]_i$ gradually declined to lower sustained level (23.8±4.3%, at 10 min). The time courses of changes of UTP-induced force were similar to those of $[Ca^{2+}]_i$ (Figure 4).

In the absence of extracellular Ca^{2+}, basal levels of $[Ca^{2+}]_i$ and force decreased gradually below the resting level (−60±6.8% and −5.9±1.0%, respectively, Figure 4). In Ca^{2+}-free PSS, UTP induced a small rapid transient increases in $[Ca^{2+}]_i$ and force which were finally restored to the level before the application of UTP. The peaks of initial transient increases in $[Ca^{2+}]_i$ and force induced by UTP are significantly smaller than those in normal PSS (containing 1.25 mM extracellular Ca^{2+}). The sustained increases in $[Ca^{2+}]_i$ and force, which were observed in the presence of extracellular Ca^{2+}, were completely abolished by the removal of extracellular Ca^{2+}.

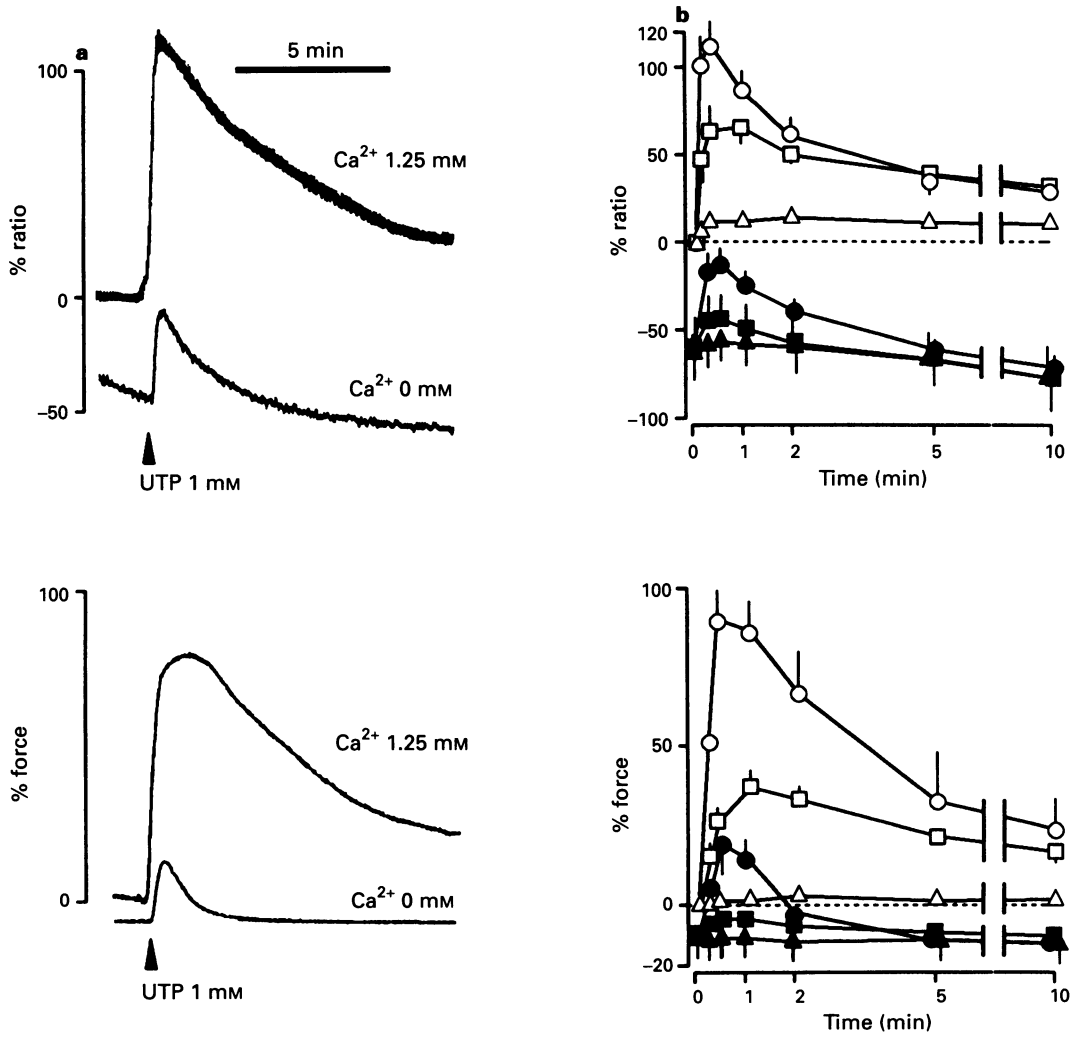

Figure 4 Effects of UTP on $[Ca^{2+}]_i$ and force of cerebral arterial strips without endothelium in the presence and absence of extracellular Ca^{2+}. At the beginning of each measurement, the responses to 118 mM K^+-PSS were recorded and registered as 100%. (a) Representative recordings of increases in $[Ca^{2+}]_i$ (upper panel) and in force (lower panel) induced by 1 mM UTP in the endothelium-denuded arterial strips in normal PSS (1.25 mM Ca^{2+}) and in Ca^{2+}-free PSS (containing 2 mM EGTA). (b) Summary of increases in $[Ca^{2+}]_i$ (upper panel) and in force (lower panel) induced by various concentrations (△,▲: 10 μM, □,■: 100 μM, ○,●: 1 mM) of UTP in the presence (open symbols) and the absence (closed symbols) of extracellular Ca^{2+}. Values are mean with s.e.mean (n=6). Dashed lines indicate the resting level (=0%) of $[Ca^{2+}]_i$ and force.

Effect of UTP on $[Ca^{2+}]_i$-force relationship of cerebral arterial smooth muscle

Many vasoconstrictor agonists (i.e. endothelin-1, histamine, U-46619, etc.) are known to induce smooth muscle contraction by three $[Ca^{2+}]_i$-related mechanisms, namely the influx of extracellular Ca^{2+}, the release of intracellular Ca^{2+}, and the sensitization to Ca^{2+} of contractile apparatus of smooth muscle (Somlyo & Somlyo 1994). We investigated the involvement of third mechanism, the $[Ca^{2+}]_i$-sensitization of contractile apparatus of smooth muscle in UTP-induced contraction. First, the effects of 1 mM UTP on the increases in $[Ca^{2+}]_i$ and force induced by cumulative applications of extracellular Ca^{2+} during membrane depolarization with 118 mM K^+-PSS were investigated. After the removal of extracellular Ca^{2+} with a series of incubations in Ca^{2+}-free PSS containing 2 mM EGTA for 10 min, in Ca^{2+}-free PSS (5.9 mM K^+) without EGTA for 5 min, and finally in Ca^{2+}-free 118 mM K^+-PSS for 5 min, extracellular Ca^{2+} was added cumulatively during depolarization with 118 mM K^+-PSS. The cumulative application of extracellular Ca^{2+} (10 μM–5 mM) induced stepwise increases in $[Ca^{2+}]_i$ and force due to membrane depolarization. When the effect of UTP and U-46619 were examined, UTP (1 mM) and U-46619 (1 μM) were added at 5 min after exchanging normal PSS for Ca^{2+}-free PSS (with 2 mM EGTA) (Figure 5a) and the same procedures were car-

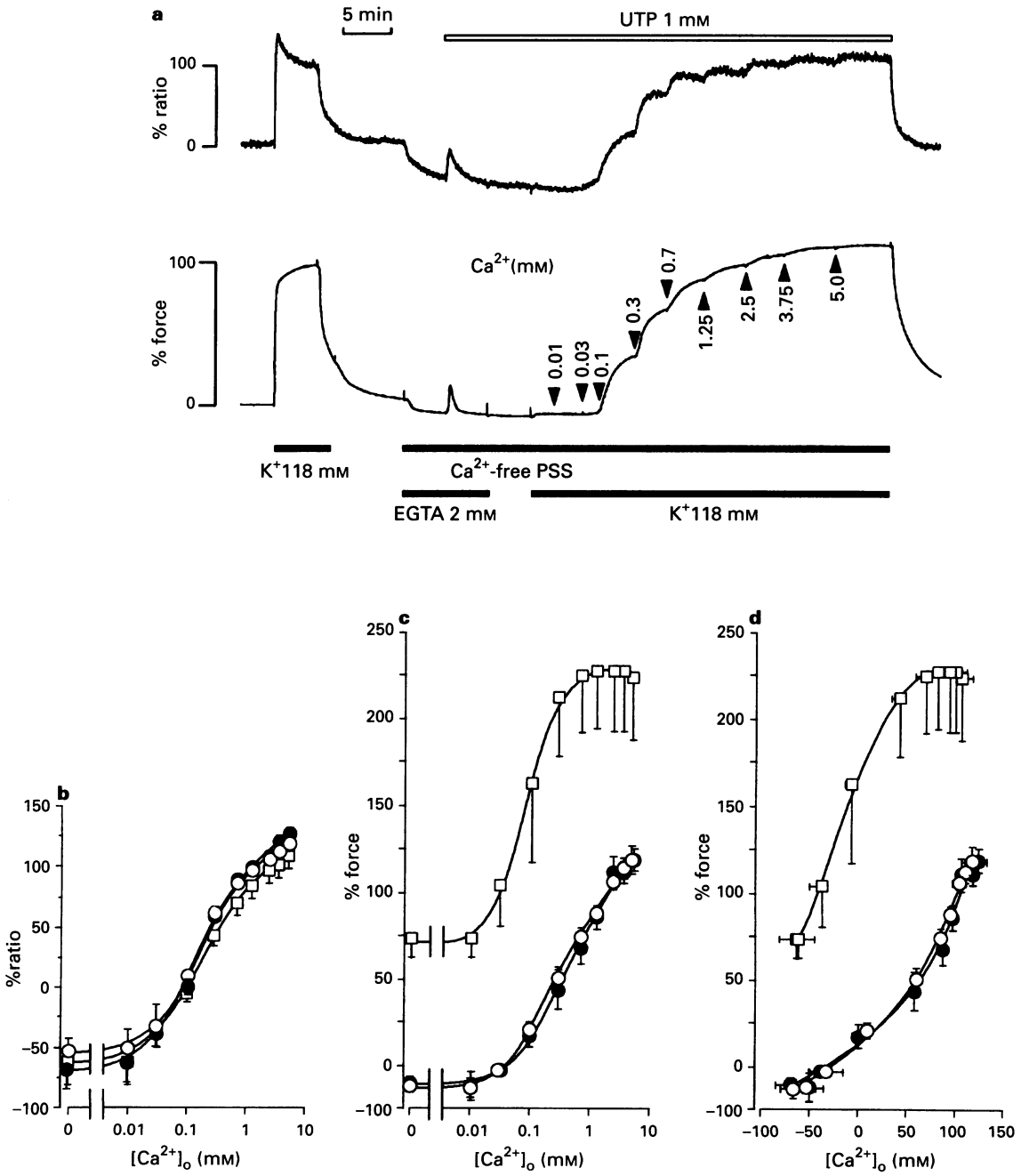

Figure 5 Effect of UTP and U-46619 on the $[Ca^{2+}]_i$-force relationship induced by cumulative applications of extracellular Ca^{2+} (0.01–5 mM) during 118 mM K^+-depolarization. (a) A representative recording of an increase in $[Ca^{2+}]_i$ (upper panel) and force (lower panel) induced by cumulative applications of extracellular Ca^{2+} (0.01–5 mM) during 118 mM K^+-depolarization in the presence of 1 mM UTP in the strips without endothelium. (b and c) Summaries of the changes in $[Ca^{2+}]_i$ (b) and force (c) in the absence (●) and the presence of either of 1 mM UTP (○) or 1 μM U-46619 (□). All values were the mean ± s.e.mean ($n=5$). (d) Effect of UTP and U-46619 on $[Ca^{2+}]_i$-force relationship of steady-state of contractions induced by increasing extracellular Ca^{2+} during 118 mM K^+-depolarization in the absence (●) and the presence of either 1 mM UTP (○) or 1 μM U-46619 (□). All values are the mean ± s.e.mean ($n=5$) and were reconstructed from the data shown in (b) and (c).

ried out in the presence of 1 mM UTP. The addition of UTP did not affect either stepwise increases in $[Ca^{2+}]_i$ or in force induced by cumulative applications of extracellular Ca^{2+} during 118 mM K^+-depolarization (Figure 5b and c, $P>0.05$). To analyze in detail the $[Ca^{2+}]_i$-force relationship, $[Ca^{2+}]_i$-force curves were reconstructed from results of simultaneously obtained changes in $[Ca^{2+}]_i$ and force. Figure 5d shows effects of 1 mM UTP on the $[Ca^{2+}]_i$-force relationship of steady-state contractions during depolarization with 118 mM K^+-PSS, the results of which were reconstructed from Figure 5b and c. The $[Ca^{2+}]_i$-force curve for steady-state high K^+-depolarization-induced contractions (basic $[Ca^{2+}]_i$-force curve) did not shift in the presence of 1 mM UTP ($P>0.05$, by analysis of covariance). In order to rule out the possible involvement of the hydrolysis of UTP by ectonucleotidase during prolonged exposure in this protocol (Figure 5a), α,β-methylene-ADP (100 μM), an inhibitor of ectonucleotidase, was added 3 min before the application of UTP. However, even in the presence of α,β-methylene-ADP, the $[Ca^{2+}]_i$-force relationship was not shifted by UTP (data not shown). In contrast to UTP, U-46619 (1 μM) shifted the $[Ca^{2+}]_i$-force relationship to the left (Figure 5d).

Discussion

In this study, we clarified for the first time that: (1) UTP induces a relaxation of cerebral artery mainly through endothelium-derived relaxing factor (EDRF); (2) UTP directly stimulates the P_{2U} receptor of arterial smooth muscle, thereby inducing $[Ca^{2+}]_i$ elevation (the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+}) and contraction, without affecting the Ca^{2+}-sensitivity of the contractile apparatus of smooth muscle. Although bovine cerebral artery has a vasoconstricting P_{2U} receptor, it seems not important in the regulation of vascular tone where the vasoconstriction is attenuated by EDRF simultaneously released from adjacent endothelial cells.

It was reported that UTP also induces PGI_2 release from smooth muscle cells (Demolle *et al.*, 1988) and endothelial cells (Needham *et al.*, 1987) and haemoglobin-insensitive endothelium-dependent relaxation of pial artery, which is compatible with the involvement of PGI_2 (Hardebo *et al.*, 1987). However, in our preparation, the endothelium-denuded strips failed to relax in response to UTP (Figure 2b), and the inhibition of PGI_2 formation with indomethacin in endothelial cells on arterial strips did not attenuate the endothelium-dependent relaxation induced by UTP (Figure 2d). These findings suggest that possible production of PGI_2 in smooth muscle or endothelium, if any, may not have a major role in the regulation of cerebrovascular tone by UTP. In perfused rat mesenteric arterial bed, N^{ω}-nitro-L-arginine methyl ester partially inhibited the UTP-induced endothelium-dependent decrease of perfusion pressure (Ralevic & Burnstock, 1991), suggesting the possible involvement of other mediators (PGI_2 or endothelium-derived hyperpolarizing factor) in the UTP-induced vasorelaxation. In our study, L-NOARG, an inhibitor of conversion pathway of L-arginine to nitric oxide, completely abolished the vasorelaxant effect of UTP both on resting tone (Figure 1) and on U-46619-raised tone (Figure 2) of vascular strips with intact endothelium. Therefore, it is concluded that EDRF is a major mediator in UTP-induced endothelium-dependent relaxation of bovine cerebral artery.

Another nucleotide, ATP also induces phasic contraction of vascular smooth muscle. Given the absence of selective antagonists, the subclassification of the ATP receptors (P_2 purinoceptors) was based on the rank order of agonist potency for structural analogues of ATP (Burnstock & Kennedy, 1985; O'Connor & Leff, 1990; O'Connor *et al.*, 1991). According to this classification, the purinoceptors regulating vascular tone are P_{2X} (α,β-methylene ATP > ATP > 2-methylthio-ATP) in smooth muscle and P_{2Y} (2-methylthio-ATP > ATP > α,β-methylene ATP) in endothelial cells. The activation of the P_{2X} purinoceptor induces Ca^{2+} influx through the ligand-gated Ca^{2+} channel (Benham, 1989), without the release of intracellular Ca^{2+}. In our study, UTP a pyrimidine nucleotide, induced a transient increase in $[Ca^{2+}]_i$ and force in the Ca^{2+}-free condition also; therefore it is unlikely that UTP-induced $[Ca^{2+}]_i$ mobilization may be mediated by P_{2X} purinoceptor, as being compatible with the desensitization study by von Kügelgen *et al.* (1990). Furthermore, UTP and ATP induced additive contractions in P_{2X}-desensitized strips (Figure 3a,b and d). In addition, in the P_{2X}-desensitized state, ATP and UTP showed cross-desensitization each other (Figure 3a and b), indicating the presence of the common receptor for ATP and UTP. Since it is clear that UTP activates one of the P_2 purinoceptors (UTP = ATP > ATPγS > ADP > 2MeSATP) associated with various biological responses (O'Connor *et al.*, 1991), this subtype has been named 'P_{2U}' or 'nucleotide receptor'. The desensitization to ATPγS, another agonist of the P_{2U} receptor, also markedly inhibited UTP-induced contraction (Figure 3c), supporting the involvement of the P_{2U} receptor in UTP-induced contraction. Although ATP induced an additive contraction in P_{2X}-desensitized strips, the additive contraction was smaller than that induced by UTP (Figure 3b). Because ATP is a nonselective agonist of P_2 receptors and is a precursor of P_1 receptor agonists, it is likely that ATP may stimulate the other subtypes, distinct from P_{2X} and P_{2U} receptors, or may change to AMP or adenosine that activates P_1 receptors, respectively. Therefore, the possible involvement of P_{2Y} or other P_2 receptors and P_1 receptors activation cannot be excluded in the attenuation of ATP-stimulated P_{2U}-contraction observed in this study (Figure 3b). Vascular endothelial cells have P_{2U} purinoceptors, which mediate both ATP- and UTP-induced phosphatidylinositol hydrolysis and release of intracellular Ca^{2+} in endothelial cells (Motte *et al.*, 1993). Although the existence of the P_{2U} purinoceptor has been suggested to induce $[Ca^{2+}]_i$ elevation and proliferation in smooth muscle cells (Tawada *et al.*, 1987; Erlinge *et al.*, 1993), the vasoconstriction mediated by the P_{2U} receptor has never been reported. Our findings that ATP and UTP induced cross desensitization to each other, indicates the first evidence of a vasoconstriction-mediating P_{2U} receptor.

In bovine middle cerebral arterial strips, the time-course of UTP-induced contraction included phasic and sustained components, which was very similar to the time course of human basilar artery (White & Robertson, 1987). Juul *et al.* (1992) observed a large sustained contraction induced by UTP in rat mesenteric artery and there was no cross-desensitization between UTP and ATP. In the present study, since the observed UTP-induced contractions are quite different in potency and time-course from those of Juul *et al.* (1992), there may also be subtypes of pyrimidine receptors, which activate the distinct signal transduction pathways leading to different types of contraction.

Some vasoconstrictor agonists are known to induce increases in $[Ca^{2+}]_i$ and force of smooth muscle through receptor/G-protein coupling mechanisms, phospholipase C activation and phosphoinositides break down. The increase in $[Ca^{2+}]_i$ is driven by two sources, namely the IP_3-induced release of intracellular Ca^{2+} (from intracellular storage site), and the influx of extracellular Ca^{2+}, and they initiate the force development of smooth muscle. In our study, UTP also induced increases in $[Ca^{2+}]_i$ from two sources (Ca^{2+} influx and Ca^{2+} release) similar to histamine (Abe *et al.*, 1990) and U-46619 (Miyagi *et al.*, 1995a). In addition to increase in $[Ca^{2+}]_i$, it is well established that the force of smooth muscle is also regulated by the Ca^{2+}-sensitivity of the contractile apparatus (Somlyo & Somlyo, 1994), the mechanism of which may involve some regulators: activation of protein kinase C (Nishimura *et al.*, 1988), and inhibition of myosin light chain phosphatase (Kitazawa & Somlyo, 1991) have been proposed. The agonists that release intracellular Ca^{2+} can also activate the phosphatidylinositol cascade to increase both IP_3 and diacylglycerol. Interestingly, the Ca^{2+}-sensitizing action (shift of Ca^{2+}-force curve during steady-state of contraction) was

not observed in the case of the UTP-induced contraction (Figure 5d). It is suggested that distinct G-proteins may be involved in Ca^{2+} release and Ca^{2+} sensitization (Kobayashi *et al.*, 1991) and the efficacy of a given agonist can differ greatly in activating IP_3 formation and diacylglycerol formation, which activates Ca^{2+} release and Ca^{2+} sensitization, respectively (Somlyo & Somlyo, 1994). Therefore, the absence of Ca^{2+}-sensitizing effect in P_{2U} purinoceptor activation, as observed in this study, can be explained by the selective involvement of G-protein(s) leading to Ca^{2+}-mobilizing signal transduction pathway, but not of the G-protein(s) regulating the Ca^{2+}-sensitization. U-46619 induces potent contraction even at $[Ca^{2+}]_i$ much lower than basal level, indicating the potent Ca^{2+}-sensitization of contractile apparatus of smooth muscle (Figure 5d). In contrast to U-46619, UTP-induced contraction corresponds well to the change in $[Ca^{2+}]_i$ (Figure 5d); thus the increases in both $[Ca^{2+}]_i$ and force induced by UTP showed a gradual decline finally reaching the much lower sustained level (Figure 4), where no modulation of $[Ca^{2+}]_i$-force relationship was observed. These findings suggest two possibilities; one is rapid desensitization of the UTP-mediating receptor and/or subsequent signalling pathway including Ca^{2+} influx, and the other is the rapid hydrolysis of extracellular UTP. However, the latter (rapid hydrolysis of UTP in 10 min) is unlikely because the UTP concentration used was sufficiently high (1 mM) in a 6 ml organ bath. In addition, pretreatment with α,β-methylene-ADP, which was reported to inhibit ecto-nucleotidase (Williams & Braunwalder, 1986) and hydrolysis for UTP to UDP (Richards *et al.*, 1993), did not affect the action of UTP on the $[Ca^{2+}]_i$-force relationship. These findings suggest that the hydrolysis of extracellular UTP, if it occurs, does not have a major role in the relatively phasic contraction by UTP (Figure 4).

Recently, evidence has been increasing of the physiological importance of nitric oxide (EDRF or neurotransmitter) in the regulation of regional cerebral blood flow (Kovách *et al.*, 1992). Nitric oxide diffuses into smooth muscle cells and stimulates the accumulation of cyclic GMP (Rapoport & Murad, 1983) and the activation of cyclic GMP-dependent protein kinase (Ignarro & Kadowitz, 1987). Nitric oxide-induced activation of cyclic GMP-dependent protein kinase decreases $[Ca^{2+}]_i$ and Ca^{2+}-sensitivity of the contractile apparatus of vascular smooth muscle, leading to vasorelaxation (Abe *et al.*, 1990). Haemoglobin, an inhibitor of nitric oxide, inhibits endothelium-dependent relaxation of vascular smooth muscle (Martin *et al.*, 1985). In the pathological condition such as subarachnoid haemorrhage, where cerebral arteries are exposed to many direct or indirect vasoactive substances including haemoglobin, the dysfunction of endothelium-dependent vasodilatation can be the significant cause of cerebral vasospasm (Fujiwara *et al.*, 1986). UTP, as observed in the present study, is one of the vasoactive substances that have dual effects on vascular tone, although UTP generates contraction only at high concentrations in the strips with intact endothelium. Therefore, a physiological role of extracellular UTP may be to act as a vasodilator in the regulation of local vascular tone, mainly by an endothelium-dependent mechanism rather than by a direct constrictor mechanism in smooth muscle. However, once the endothelial function is degenerated or is damaged (e.g. arteriosclerosis, subarachnoid haemorrhage), the vasoconstrictor action of UTP may appear, causing cerebral vasospasm. Hardebo *et al.* (1987) also suggested that the site of UTP release *in situ* (luminal or abluminal side of vascular wall) may decide the direction of vasomotor action of UTP.

The present observations clarified the underlying mechanisms of vascular tone regulation by UTP in bovine middle cerebral artery. Namely, UTP induces endothelium-dependent relaxation mediated exclusively by EDRF. At high concentrations, UTP induces contraction of cerebral arterial smooth muscle through the P_{2U} receptor, the activation of which leads to the modest release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+}, without affecting the Ca^{2+}-sensitivity of the contractile apparatus in smooth muscle. However, UTP elicits a relaxant action rather than a contractile action under physiological (endothelium-intact) conditions in the cerebral artery.

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