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ROLE OF GTP-PROTEIN AND ENDOTHELIUM IN CONTRACTION INDUCED BY ETHANOL IN PIG CORONARY ARTERY

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SUMMARY

1. We examined the effects of ethanol on the contractility of strips of porcine coronary artery, with and without endothelium, and following permeabilization with α-toxin, and of aortic valvular endothelial cells, *in situ*. Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of the coronary artery smooth muscle cells and of the valvular endothelial cells were monitored using front-surface fluorometry of the calcium indicator dye, fura-2. In permeabilized preparations, $[Ca^{2+}]_i$ was clamped using 10 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 10 μM A23187 (a calcium ionophore).

2. The strips without endothelium were placed in normal physiological salt solution (normal PSS) in the presence of ethanol (100–1000 mM). There were dose-dependent increases in $[Ca^{2+}]_i$ and a rapid sustained rise in tension. In Ca^{2+}-free PSS, ethanol increased $[Ca^{2+}]_i$ and tension, similar to, but much smaller than, findings with normal PSS.

3. For a given change in $[Ca^{2+}]_i$ induced by ethanol, the developed tension was greater than that observed during contractions induced by high $[K^+]_o$. Thus, the $[Ca^{2+}]$–tension curve for ethanol was shifted to the left of that for high $[K^+]_o$. The $[Ca^{2+}]$–tension curve for the contraction induced by ethanol in the absence of extracellular Ca^{2+} was shifted further to the left from that obtained in the presence of $[Ca^{2+}]_o$.

4. The mechanisms involved in this Ca^{2+}-sensitizing effect of ethanol were investigated using α-toxin-permeabilized coronary medial strips. Ethanol increased the tension development, in a concentration-dependent manner, at a fixed concentration of Ca^{2+} ($pCa = 6{\cdot}3$) in the presence of guanosine-5′-triphosphate (GTP), an effect antagonized by guanosine-5′-*O*-(β-thiodiphosphate) (GDPβS), a non-hydrolysable GDP analogue.

5. With intact endothelium, the ethanol-induced tension development was markedly reduced, although inhibition in the increase in $[Ca^{2+}]_i$ was slight. The $[Ca^{2+}]$–tension relationship of this contraction overlapped with that obtained with high $[K^+]_o$-induced contraction and was shifted to the right from that obtained in

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the absence of the endothelium. This endothelium-dependent reduction of $[Ca^{2+}]_i$ and tension induced by ethanol was inhibited when the strips were exposed to N^G-monomethyl-L-arginine (L-NMMA).

6. Ethanol induced a gradual and sustained increase in $[Ca^{2+}]_i$ in normal PSS, and a transient, concentration-dependent increase in $[Ca^{2+}]_i$ in Ca^{2+}-free PSS in porcine aortic valvular endothelial cells *in situ*.

7. These results suggest that ethanol contracts the coronary artery both by increasing $[Ca^{2+}]_i$ and by raising Ca^{2+} sensitivity of the contractile apparatus, as mediated by GTP-binding protein (G-protein). When the endothelium is exposed to ethanol, this contraction is prevented, presumably by releasing endothelium-derived relaxing factor (EDRF).

INTRODUCTION

Ethanol has various effects on human physiological systems. Since the main target organs are the liver and brain (Lieber, 1988; Deitrich, Dunwiddie, Harris & Erwin, 1989), the effects of ethanol on these organs have been given much attention, particularly with regard to toxicity. There are also reports that ethanol constricts the coronary artery (Altura, Altura & Carella, 1983; Hayes & Bove, 1988).

Ethanol is reported to increase cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, in hepatocytes, platelets, pancreatic acini, alveolar macrophages, and erythrocytes (Hoek, Thomas, Rubin & Rubin, 1987; Ponnappa, Hoek, Waring & Rubin, 1987; Dorio, Hoek, Rubin & Forman, 1988; Rubin & Hoek, 1988; Rooney, Hager, Rubin & Thomas, 1989; Reinlib, Akinshola, Potter & Mezey, 1990). In these cell types, it has been shown that ethanol stimulates inositol-1,4,5 trisphosphate (IP_3) formation by activating phospholipase C (PLC). More recently it has been shown in cell-free *in vitro* studies that the ethanol-induced activation of PLC depends on the presence of guanine nucleotides (Rubin & Hoek, 1988; Rooney *et al.* 1989). Thus, in non-muscle cells, ethanol may stimulate GTP-binding protein (G-protein) to induce activation of PLC, formation of IP_3, and an increase in $[Ca^{2+}]_i$. One study showed that ethanol decreased $[Ca^{2+}]_i$ in rat aortic smooth muscle cells in culture (Zhang, Cheng & Altura, 1992).

We now report the first evidence that ethanol increases $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of the contractile apparatus, and induces contraction of the coronary artery smooth muscle. This increase in Ca^{2+} sensitivity is apparently mediated by the activation of G-protein. This is also the first report that ethanol releases endothelium-derived relaxing factor (EDRF) from the endothelium and induces the Ca^{2+} transient in endothelial cells *in situ*.

METHODS

Tissue preparation

Fresh pig hearts were obtained from a local slaughterhouse immediately after the animals had been killed. The hearts were placed in ice-cold physiological salt solution (PSS) and brought to our laboratory. Left circumflex coronary arteries (2–3 cm from the origin) were isolated and after removing the adventitia, the segments were cut longitudinally with surgical scissors for the medial strips, and the inner surface was rubbed off with cotton swabs. Preparations with or without the endothelium were cut into circular strips (approximately 1 mm wide, 5 mm long,

and, 0·1 mm thick). To obtain valvular strips with an intact endothelium, the aortic valves were dissected in a manner so as not to touch their surface. The valve leaflets were cut into strips in an axial direction (approximately 2 mm wide, 5 mm long, and 0·18 mm thick). The centre of each leaflet, *corpus arantii*, was not used.

Fura-2 loading

Vascular strips with or without the endothelium were loaded with $[Ca^{2+}]_i$ indicator dye, fura-2, by incubating in oxygenated (a mixture of 95 % O_2 and 5 % CO_2) Dulbecco's modified Eagle's medium containing 25 μM fura-2 AM (an acetoxymethyl ester form of fura-2) and 5 % fetal bovine serum for 4 h at 37 °C. The valvular strips were incubated in oxygenated (95 % O_2 and 5 % CO_2) Dulbecco's modified Eagle's medium containing 50 μM fura-2 AM, 1 mM probenecid (Di Virgilio, Steinberg & Silverstein, 1989) and 5 % fetal bovine serum for 1·5 h at 37 °C. After loading with fura-2, both vascular and valvular strips were incubated in normal PSS for at least 1 h before starting the measurement, in order to remove dye in the extracellular space and for purposes of equilibration. Loading the vascular strips with fura-2, *per se*, did not affect the contractility, as described elsewhere (Abe, Kanaide & Nakamura, 1990; Hirano, Kanaide, Abe & Nakamura, 1990). At the beginning of the fura-2 equilibration period, the vascular and valvular strips were mounted vertically in separate quartz organ baths.

Measurement of tension development

Vascular strips mounted vertically in a quartz organ bath were connected to a force transducer (TB-612T, Nihon Koden, Japan). During the fura-2 equilibration period, the strips were stimulated with 118 mM K^+ PSS every 15 min and the resting tension was increased stepwise. After equilibration, the resting tension was adjusted to 250 mg. The responsiveness of each strip to 118 mM K^+ PSS was recorded before the start of the experimental protocol. The developed tension was expressed in per cent, assuming the values in normal PSS (5·9 mM K^+) to be 0 %, and in 118 mM K^+ PSS to be 100 %.

Front-surface fluorometry

Changes in fluorescence intensity of the fura-2–Ca^{2+} complex in vascular strips with and without endothelium were monitored with a front-surface fluorometer specifically designed for fura-2 fluorometry (CAM-OF1), in collaboration with Japan Spectroscopic Co. (Tokyo, Japan), and experiments were carried out at 37 °C, as described previously (Kodama, Kanaide, Abe, Hirano, Kai & Nakamura, 1989; Abe *et al.* 1990; Hirano *et al.* 1990). In brief, strips were illuminated by alternating (400 Hz) the excitation light (340 and 380 nm) through quartz optic fibres arranged in a concentric inner circle. Surface fluorescence of the strips was collected by glass optic fibres arranged in an outer circle and introduced through a 500 nm band-pass filter into a photomultiplier. Ratio of the fluorescence intensities at 340 and 380 nm excitation was monitored and expressed as a percentage, assuming the values in normal (5·9 mM K^+) and 118 mM K^+ PSS to be 0 and 100 %, respectively. The absolute value of $[Ca^{2+}]_i$ of vascular strips was calculated according to the method of Grynkiewicz, Poenie & Tsien (1985), with a K_d (apparent dissociation constant) of the fura-2–Ca^{2+} complex of 225 nM (at 37 °C).

In experiments using aortic valvular strips with an intact endothelium, Ca^{2+} measurements were carried out in a manner similar to that used for vascular strips. However, the temperature during measurements was 25 °C to prevent leakage of the fluorescence dye, as described elsewhere (Aoki, Kobayashi, Nishimura, Yamamoto & Kanaide, 1991). Accordingly, the absolute value of $[Ca^{2+}]_i$ was calculated using the method of Grynkiewicz *et al.* (1985) with a K_d value of 162 nM, as determined spectroscopically in 30 mM piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (Pipes)-buffered (pH 7·1 with KOH at 25 °C) Ca^{2+}-EGTA solution containing 10 mM EGTA (Aoki *et al.* 1991).

When measuring the fluorescence intensity of vascular strips with an intact endothelium, the fura-2 signal may be coming from either the smooth muscle or the endothelium, or both (Sato, Ozaki & Karaki, 1990). The following conditions used in the present study made feasible recording of fura-2 signals exclusively from smooth muscle cells in porcine coronary arterial strips with intact endothelial cells.

Temperature. Simultaneous determinations of $[Ca^{2+}]_i$ and tension of the vascular strips with intact endothelium were carried out at 37 °C. At this temperature, smooth muscle cells almost

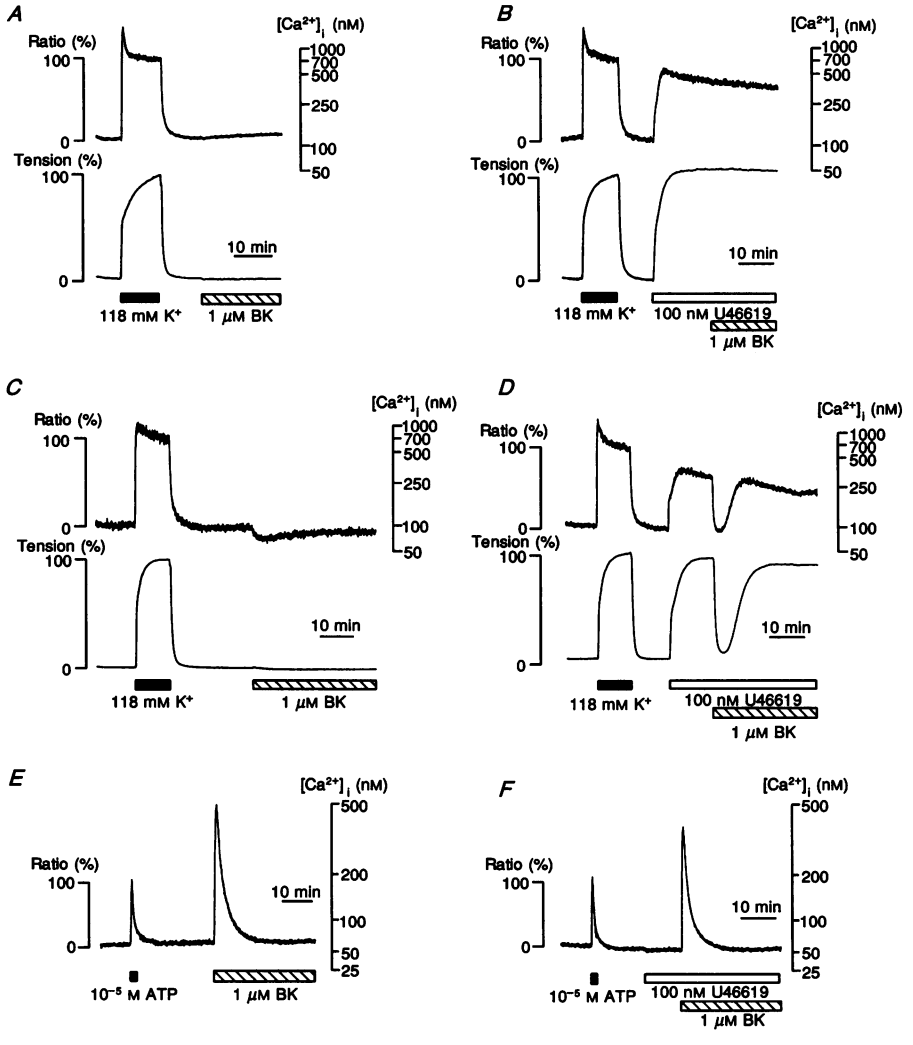

Fig. 1. Effect of bradykinin (BK) on $[Ca^{2+}]_i$ and tension in coronary arterial strips without (*A* and *B*) and with (*C* and *D*) endothelium, and on $[Ca^{2+}]_i$ in valvular endothelial cells (*E* and *F*). *A* and *B*, representative recordings of the effect of 118 mM K^+ and 1 μM BK on $[Ca^{2+}]_i$ (upper trace) and tension (lower trace) in coronary arterial strips without endothelium. BK was applied at rest in normal PSS (5·9 mM K^+) (*A*) and during the contraction induced by 100 nM U46619 (*B*). *C* and *D*, representative recordings of the effect of 118 mM K^+ and 1 μM BK on $[Ca^{2+}]_i$ and tension in coronary arterial strips with endothelium. BK was applied at rest in normal PSS (*C*) and during the contraction induced by 100 nM U46619 (*D*). *E* and *F*, representative recordings of changes in $[Ca^{2+}]_i$ induced by 10 μM ATP and by 1 μM BK in endothelial cells *in situ*. BK was applied in normal PSS (*E*) and during the treatment with 100 nM U46619 (*F*).

fully maintain, but endothelial cells rapidly and completely leak fura-2, as evidenced by fluorescence microscopic observations (data not shown). Thus, for example, bradykinin, which has no direct effect on $[Ca^{2+}]_i$ of vascular smooth muscle (Fig. 1*A* and *B*), raised the fura-2 fluorescence ratio in valvular endothelial cells markedly at 25 °C, both at rest (Fig. 1*E*) and

following pretreatment with U46619, an analogue of thromboxane A2 (Fig. 1*F*). These fura-2 signals from endothelial cells of aortic valves became weakened almost to extinction at 37 °C (Aoki *et al.* 1991). If the fluorescent signal from the endothelial cells had contributed to the observed fluorescent signal from coronary arterial strips with endothelium, a rise in the fluorescence ratio when bradykinin (BK) was applied would have resulted, in addition to the fluorescent signal from smooth muscle cells. This did not the occur. In porcine coronary arterial strips at 37 °C, with an intact endothelium, bradykinin induced only a slight decrease in $[Ca^{2+}]_i$ at rest (Fig. 1*C*) and a transient decrease in $[Ca^{2+}]_i$ and in force when applied to strips precontracted with U46619 (Fig. 1*D*). These changes in $[Ca^{2+}]_i$ and in force, induced by bradykinin, were abolished by removing the endothelial cells (data not shown). These observations suggest the presence of functional endothelial cells, which by releasing EDRF, decrease $[Ca^{2+}]_i$ and relax the adjacent smooth muscle. However, bradykinin-induced $[Ca^{2+}]_i$ elevation, as seen in endothelial cells (Fig. 1*E* and *F*), was never observed in porcine coronary arterial strips with an intact endothelium. Thus, at 37 °C, the fluorescent signal comes exclusively from the smooth muscle cells in porcine coronary arterial strips with intact endothelium under the present experimental conditions; the signal from the endothelium is negligible.

Probenecid. When fura-2-loaded vascular strips, with intact endothelium, were observed under a fluorescence microscope, the endothelial cells were stained with fura-2 only when probenecid, which prevents the leakage of fura-2 from endothelial cells (Aoki *et al.* 1991), was added during loading with fura-2 and the strips kept at 25 °C (data not shown). Thus, at 37 °C and without probenecid, fura-2 signals derived from the endothelial cells of the porcine coronary artery were negligible. Indeed, when the coronary strip with an intact endothelium was loaded with fura-2 in the presence of 1 mM probenecid, 10^{-6} M bradykinin caused a small, but significant elevation of fluorescence derived from the endothelium, at 25 °C (data not shown).

Membrane permeabilization of coronary arterial strips

Small strips (50–100 μm wide and 1–2 mm long) of the porcine coronary artery smooth muscle were dissected out, and isometric tension was measured with a force transducer (AE801; AME, Horten, Norway) in a well on a 'bubble' plate (Kobayashi, Kitazawa, Somlyo & Somlyo, 1989). The solution was changed by sliding the 'bubble' plate to an adjacent well. When steady responses to high K^+ were observed, the strips were incubated in normal relaxing solution (Ca^{2+}-free, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA)) for 5–10 min, and permeabilized by 60–75 min incubation at 22–25 °C in 5000–7500 units/ml (based on rabbit red blood cell haemolysis) of *Staphylococcus aureus* α-toxin in the relaxing solution (pCa 6·3, buffered with 10 mM EGTA). To deplete the sarcoplasmic reticulum of calcium, the permeabilized strips were treated with the calcium ionophore A23187 (10 μM) in relaxing solution for at least 10 min. This exposure to A23187 abolishes the intracellular Ca^{2+} release induced by caffeine, IP_3, or agonists, without affecting Ca^{2+} sensitivity of contractile apparatus in permeabilized strips and its modulation by agonists (Kobayashi, Gong, Somlyo & Somlyo, 1991).

Drugs and solutions

The composition of normal external PSS was as follows (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. High-K^+ PSS was prepared by replacing NaCl with equimolar KCl. The composition of Ca^{2+}-free PSS was the same as in normal PSS except that it contained 2 mM EGTA instead of 1·25 mM $CaCl_2$. PSS was bubbled with a mixture of 95 % O_2 and 5 % CO_2, the resulting pH being 7·4. The normal relaxing solution was (mM): potassium methanesulphonate, 74·1; magnesium methanesulphonate, 2; MgATP, 4·5; EGTA, 1; creatine phosphate, 10; Pipes, 30 (pH 7·1 with KOH at 20 °C). In activating solution (pCa 6·3), 10 mM EGTA was used, and a specified amount of calcium methanesulphonate was added to give a desired concentration of free Ca^{2+} ions. Ionic strength was kept constant at 0·2 M by adjusting the concentration of potassium methanesulphonate.

Fura-2 AM and EGTA were purchased from Dojindo Laboratories (Kumamoto, Japan). Ethanol, histamine dihydrochloride, methanesulphonic acid, creatine phosphate disodium and indomethacin were purchased from Wako (Osaka, Japan), and N^G-monomethyl-L-arginine (L-NMMA) was from Calbiochem (Frankfurt, Germany). Guanosine-5'-*O*-(β-thiodiphosphate) (GDPβS), guanosine-5'-triphosphate (GTP) and adenosine 5'-triphosphate (ATP) were purchased from Boehringer Mannheim (Germany), and *Staphylococcus aureus* α-toxin from Gibco BRL

(Gaithersburg, MO, USA). Bradykinin was obtained from Peptide Institute Co. Ltd (Osaka, Japan). Probenecid was purchased from Sigma (St Louis, MO, USA). KOH was obtained from Fluka (Buchs, Switzerland). All other chemicals were from Katayama Chemical (Osaka, Japan).

Data analysis

Values were expressed as means ± standard error of the mean (S.E.M.). Student's *t* test was used to determine statistical significance. *P* values less than 0·05 were considered to be significant. All data were collected using a computerized data acquisition system (MacLab, Analog Digital Instruments, Australia: Macintosh, Apple Computer, USA). Traces obtained in the measurement of $[Ca^{2+}]_i$ and tension were directly printed out from the computer to a laser printer (LaserWriter II NTX-J, Apple Computer, USA).

RESULTS

Effects of ethanol on $[Ca^{2+}]_i$ and tension of coronary arterial strips without endothelium

Figure 2*A* shows representative recordings of changes in $[Ca^{2+}]_i$ and tension induced by 118 mM K^+ depolarization, and then by 600 mM ethanol in normal PSS. When the strip was exposed to 118 mM K^+ PSS, $[Ca^{2+}]_i$ rapidly increased to a sharp peak, then decreased slightly to a plateau phase which was assumed to be a 100 % response. The tension also increased rapidly and reached the maximum plateau level within 10 min; this was also assumed to be a 100 % response. The values of $[Ca^{2+}]_i$ at rest (5·9 mM K^+) and the plateau phase of 118 mM K^+ depolarization were 108 ± 27 and 715 ± 103 nM, respectively (Hirano *et al.* 1990). After recovery to the resting level in normal PSS, 600 mM ethanol was applied. The $[Ca^{2+}]_i$ rose abruptly to reach a peak level after about 30 s (33·2 ± 4·7 %, $n = 4$), and then decreased to a steady level at about 15 min (20·2 ± 2·1 %, $n = 4$). This was sustained for as long as we observed. The tension also rapidly increased, reached the peak and was sustained at nearly the maximum plateau level (at about 15 min; 20·2 ± 1·9 %, $n = 4$). As shown in Fig. 2*B*, ethanol (100–1000 mM) induced, in a concentration-dependent manner, increases in $[Ca^{2+}]_i$ and tension of the coronary strips, without the endothelium. The higher the concentration of ethanol applied, the greater the peak levels and the extent of decline to reach sustained levels of $[Ca^{2+}]_i$. Figure 2*C* shows the relationship between $[Ca^{2+}]_i$ and tension during activation by ethanol and high-K^+ PSS. For a given change in $[Ca^{2+}]_i$, ethanol induced a greater tension development than did high-K^+ PSS. Thus, compared with the curve obtained by high-K^+ PSS, the curve during activation by ethanol shifted to the left. This would suggest that ethanol might increase Ca^{2+} sensitivity of the contractile apparatus.

Effects of ethanol on $[Ca^{2+}]_i$ and tension of vascular strips without endothelium in Ca^{2+}-free PSS

Figure 3*A* shows representative recordings of changes in $[Ca^{2+}]_i$ and tension induced by ethanol in Ca^{2+}-free PSS on coronary strips without endothelium. When the strips were exposed to Ca^{2+}-free PSS containing 2 mM EGTA, the $[Ca^{2+}]_i$ gradually decreased to reach a steady state (−26·5 ± 2·1 %, $n = 4$), yet there was no change in tension. Application of ethanol (600 mM) after 10 min incubation in Ca^{2+}-free PSS led to an abrupt elevation in $[Ca^{2+}]_i$, the peak being reached about 30 s after application (1·5 ± 3·6 %, $n = 4$); this was followed by a gradual decline to the

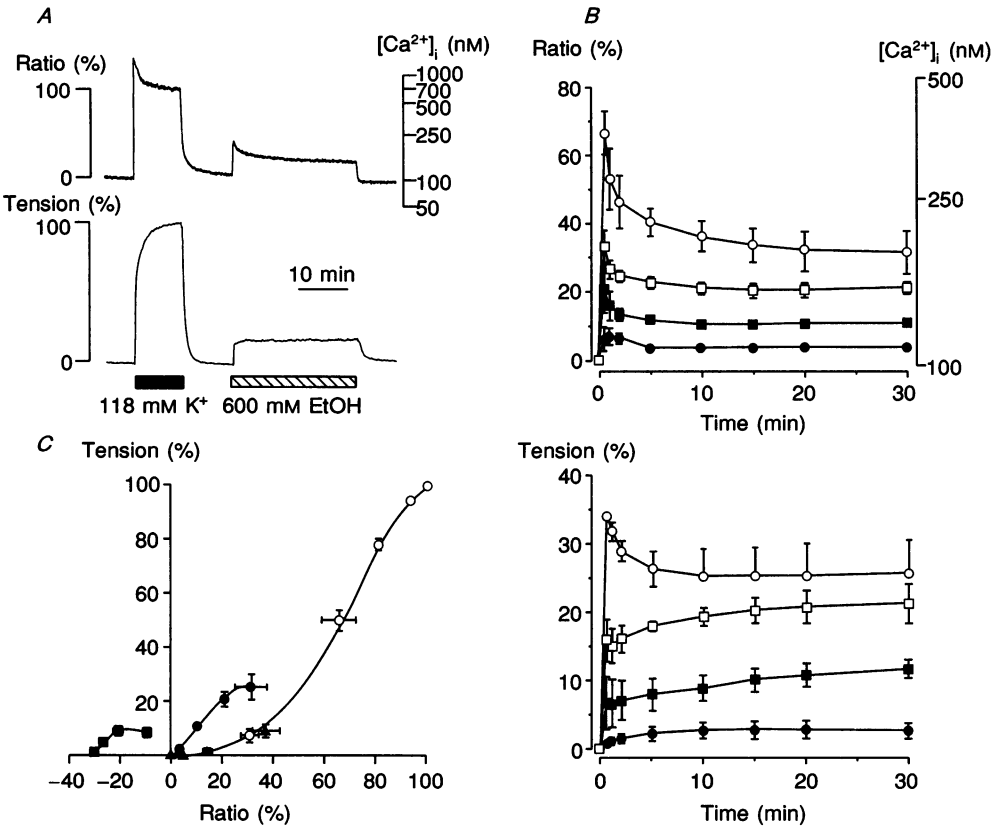

Fig. 2. Effect of ethanol on $[Ca^{2+}]_i$ and tension in coronary arterial strips without endothelium, in normal PSS (5·9 mM K^+). *A*, representative recording of the effect of 118 mM K^+ depolarization and 600 mM ethanol on $[Ca^{2+}]_i$ (upper trace) and tension (lower trace). *B*, effects of various concentrations of ethanol (●, 100 mM; ■, 300 mM; □, 600 mM; ○, 1000 mM) on $[Ca^{2+}]_i$ (upper panel) and tension (lower panel) ($n = 4$). $[Ca^{2+}]_i$ and tension were expressed as per cent, assuming values in normal PSS (5·9 mM K^+) to be 0 % and in 118 mM K^+ PSS to be 100 %, respectively. Values are expressed as means ± S.E.M. *C*, $[Ca^{2+}]_i$–tension relationships obtained at 15 min application (plateau level) of ethanol in the presence (●; from Fig. 2*B*) and the absence (■; from Fig. 3*B*) of extracellular Ca^{2+}. ▲, ethanol-induced $[Ca^{2+}]_i$–tension relationship of arterial strips with endothelium in the presence of extracellular Ca^{2+} (from Fig. 4). Control $[Ca^{2+}]_i$–tension relationship (○) was obtained at plateau levels of contractions induced by various concentrations of K^+ depolarization (5·9–118 mM K^+) in the presence of extracellular Ca^{2+}.

sustained level. The tension also rapidly increased to reach the peak level, and was sustained around this level (9·6 ± 1·8 %, 15 min, $n = 4$) for at least 30 min without decline. As shown in Fig. 3*B*, ethanol (100–1000 mM) increased both $[Ca^{2+}]_i$ and tension in Ca^{2+}-free PSS, in a concentration-dependent manner. At a given concentration of ethanol, the time courses of increases in both $[Ca^{2+}]_i$ and tension

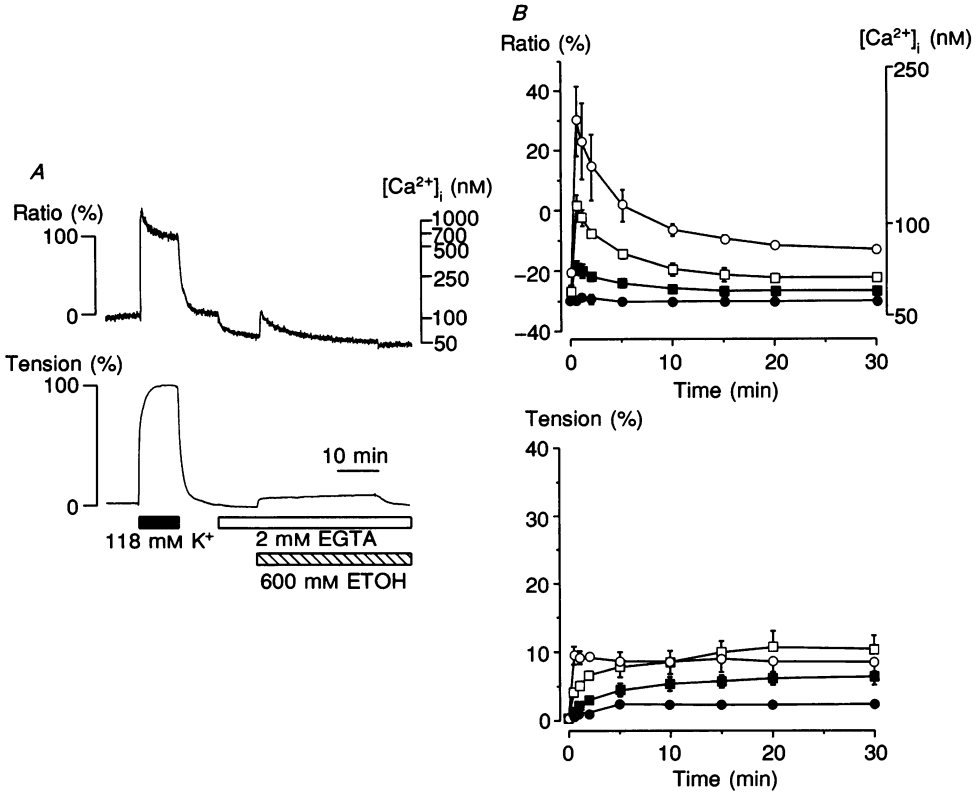

Fig. 3. Effects of ethanol on $[Ca^{2+}]_i$ and tension in coronary strips without endothelium in Ca^{2+}-free PSS containing 2 mM EGTA. *A*, representative recording of the effect of 600 mM ethanol on $[Ca^{2+}]_i$ and tension. Ethanol was applied 10 min after application of Ca^{2+}-free PSS containing 2 mM EGTA. *B*, effects of various concentrations of ethanol (●, 100 mM; ■, 300 mM; □, 600 mM; ○, 1000 mM) on $[Ca^{2+}]_i$ (upper panel) and tension (lower panel). Vertical bars show S.E.M. ($n = 4$).

observed in Ca^{2+}-free PSS (Fig. 3*B*) were similar to those seen in normal PSS (Fig. 2*B*), although the extent of the former were less than those of the latter. The curve for the relationship between $[Ca^{2+}]_i$ and tension during the contraction induced by ethanol in the absence of extracellular Ca^{2+} is shown in Fig. 2*C*. The curve shifted further to the left from that induced by ethanol in the presence of extracellular Ca^{2+}. It has to be noted that, in the absence of extracellular Ca^{2+}, ethanol developed tension (about 10 %), even though the peak of $[Ca^{2+}]_i$ increase was around the resting level (0 %).

Endothelium-related modification of ethanol effects on $[Ca^{2+}]_i$ and tension

In the following experiments, we examined the effects of ethanol on coronary strips with an intact endothelium, and compared the findings obtained with no endothelium. To confirm the preservation of functioning endothelial cells in the

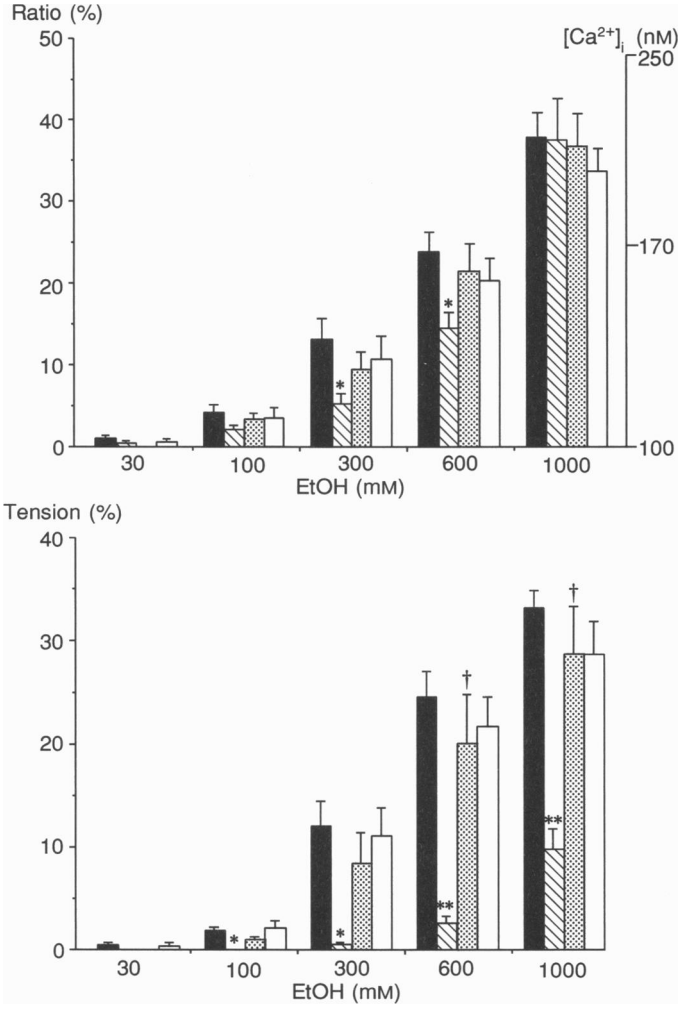

Fig. 4. Effects of ethanol on $[Ca^{2+}]_i$ and tension in coronary arterial strips with and without endothelium. Ordinate: $[Ca^{2+}]_i$ (upper panel) and tension (lower panel); abscissa: concentration of ethanol. ■, (− endothelium, − L-NMMA); ▨, (+ endothelium, − L-NMMA), ▩, (+ endothelium, + L-NMMA), □, (− endothelium, + L-NMMA). $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ (compared with value without endothelium and L-NMMA (■) at each concentration of ethanol, $n = 4$), $\dagger P < 0{\cdot}05$ (compared with value with endothelium but without L-NMMA (▨) at each concentration of ethanol, $n = 4$).

vascular strips, 10^{-6} M bradykinin was applied during activation by high-potassium PSS. Bradykinin induced no change in tension development of the strips without an endothelium; however, it did induce greater than 30% inhibition of tension development in strips with endothelium (data not shown). All the vascular strips with endothelium were pretreated for 80 min with 10^{-5} M

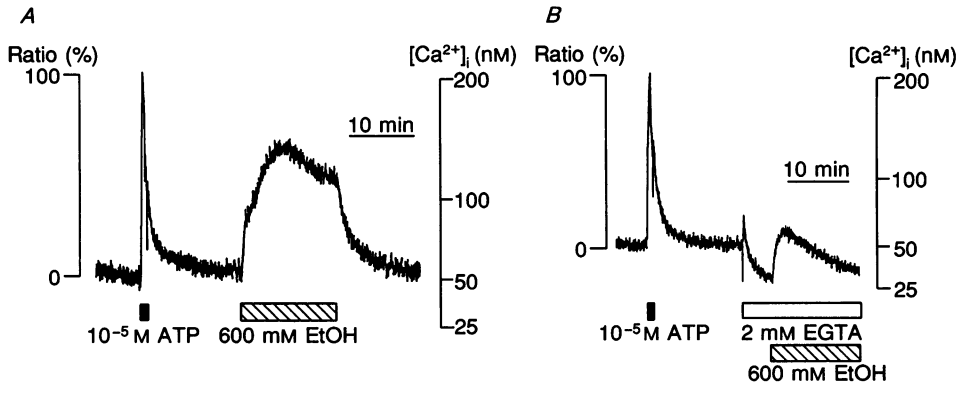

Fig. 5. Representative recording of changes in $[Ca^{2+}]_i$ induced by 10^{-5} M ATP and 600 mM ethanol in endothelial cells *in situ* in the presence (*A*) and the absence (*B*) of extracellular Ca^{2+}. The fluorescence ratio was expressed in per cent, assuming the resting level and the peak response induced by 10^{-5} M ATP to be 0 and 100 %, respectively.

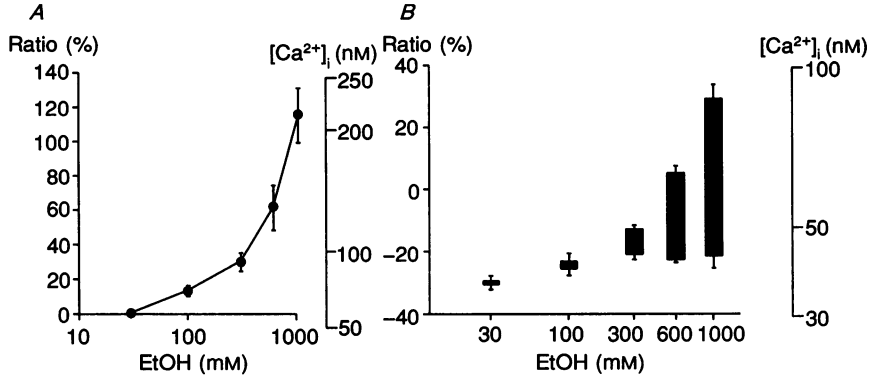

Fig. 6. Changes in $[Ca^{2+}]_i$ induced by various concentrations of ethanol in endothelial cells *in situ* in the presence (*A*) and the absence (*B*) of extracellular Ca^{2+}. *A*, the peak response of $[Ca^{2+}]_i$ induced by various concentrations of ethanol in normal PSS. *B*, the bottom and the top of each column show the concentrations of $[Ca^{2+}]_i$ after 5 min in Ca^{2+}-free PSS and just before stimulation, and the peak levels of $[Ca^{2+}]_i$ after stimulation, respectively. The fluorescence ratio of the resting level and the peak response induced by 10^{-5} M ATP were assumed to be 0 and 100 %, respectively. Vertical bars show S.E.M. of 3 measurements.

indomethacin to eliminate the possible effect of prostaglandin I_2. This pretreatment had no effects on $[Ca^{2+}]_i$ or tension development induced by ethanol (data not shown).

When the endothelial cells were intact, the contraction induced by ethanol was markedly reduced ($P < 0{\cdot}05$ in 100 and 300 mM ethanol, $P < 0{\cdot}01$ in 600 and 1000 mM ethanol), although the increase in $[Ca^{2+}]_i$ was slightly inhibited by the

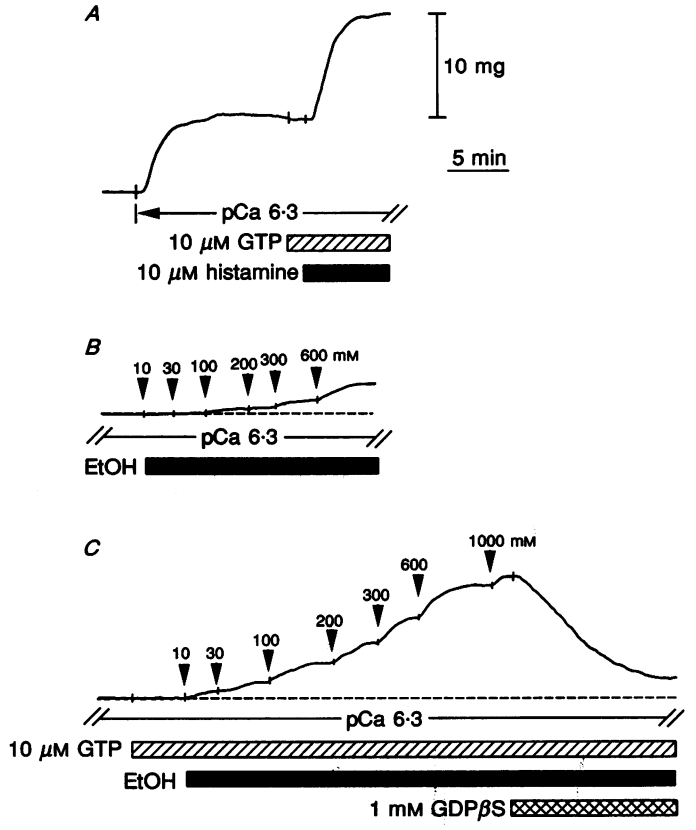

Fig. 7. Effect of guanine nucleotides on the contractile responses induced by ethanol in α-toxin-permeabilized coronary artery smooth muscle.The sarcoplasmic reticulum was depleted of calcium by treatment with 10 μM A23187. Traces in *A*, *B*, and *C* were obtained from the same strip, in this order. *A*, contractile response to 10 μM histamine and 10 μM GTP, superimposed on the contraction induced by highly buffered Ca^{2+} (pCa 6·3 with 10 mM EGTA). Note that 10 μM GTP, *per se*, had no contractile effect. The dashed lines in *B* and *C* indicate the level of steady contraction induced by constant Ca^{2+} (pCa 6·3). *B*, control responses to the cumulative application of various concentrations (10–600 mM) of ethanol in the absence of GTP, superimposed on pCa 6·3-induced contraction. *C*, responses to the cumulative application of various concentrations (10–1000 mM) of ethanol in the presence of 10 μM GTP, superimposed on pCa 6·3-induced contraction. The contraction induced by 1000 mM ethanol and 10 μM GTP was abolished by 1 mM GDPβS.

presence of endothelium ($P < 0·05$ in 300 and 600 mM ethanol) (Fig. 4). The $[Ca^{2+}]$–tension relationships of this contraction are shown in Fig. 2*C*; it shifted to the right from that observed in vascular strips without the endothelium, and almost overlapped that obtained in the case of a high-K^+-induced contraction. Thus, the increase in Ca^{2+} sensitivity of the contractile apparatus induced by ethanol was inhibited in vascular strips with the endothelium. This endothelium-

dependent reduction of the contraction was significantly eliminated by the addition of L-NMMA, an inhibitor of the production of endothelium-derived relaxing factor (EDRF) (Palmer, Ashton & Moncada, 1988) ($P < 0{\cdot}05$ in 600 and 1000 mM ethanol), as also shown in Fig. 4.

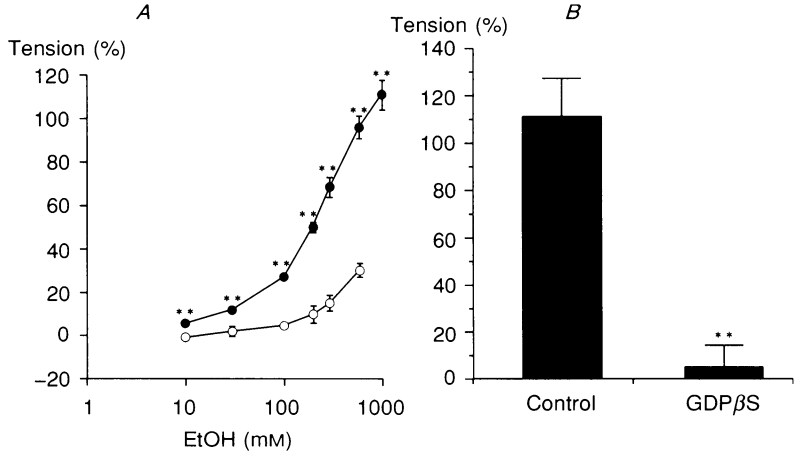

Fig. 8. Effects of GTP and GDPβS on contractions induced by ethanol at constant Ca^{2+} (pCa 6·3) in α-toxin-permeabilized coronary artery smooth muscle. The contractions were expressed in per cent, assuming that contractile responses to 10 μM histamine and 10 μM GTP at pCa 6·3 were 100 %. The data were obtained in experiments carried out using a protocol similar to that for Fig. 7. *A*, concentration–response curves for ethanol in the absence (○) and presence (●) of 10 μM GTP. Plots are mean of 6 experiments with S.E.M. shown by vertical bars. $^{**}P < 0{\cdot}01$ compared with ethanol-induced contraction in the absence of 10 μM GTP. *B*, contractions induced by 1000 mM ethanol and 10 μM GTP in the absence (control) and the presence of 1 mM GDPβS. Plots are mean of 6 experiments with S.E.M. shown by vertical bars. $^{**}P < 0{\cdot}01$ compared with control.

Effects of ethanol on $[Ca^{2+}]_i$ of aortic valvular endothelial cells in situ

Fluorescence signals disappeared when the surface of fura-2-loaded vascular strips were rubbed with a cotton swab; thus, the signals exclusively represented $[Ca^{2+}]_i$ changes in endothelial cells *in situ*. Figure 5*A* and *B* shows representative recordings of changes in $[Ca^{2+}]_i$ of aortic valvular endothelial cells *in situ* in the presence and the absence of extracellular Ca^{2+}. We used the responses to 10^{-5} M ATP as a control, because of reproducible, full responses to repetitive applications (Aoki *et al.* 1991). In each experiment, 10^{-5} M ATP was applied for 1 min followed by a 15 min recovery period. The fluorescence ratio of the resting level and the peak response to 10^{-5} M ATP were assumed to be 0 and 100 %, respectively. $[Ca^{2+}]_i$ levels at 0 and 100 % were 63·9 $\pm$ 7·4 and 176·7 $\pm$ 16·5 nM, respectively ($n = 5$). As shown in Fig. 5*A*, 600 mM ethanol induced a gradual and sustained increase in $[Ca^{2+}]_i$ in normal PSS. When the valvular strip was exposed to Ca^{2+}-free PSS containing

2 mM EGTA, $[Ca^{2+}]_i$ gradually decreased to reach a new steady state after 5 min. Subsequent application of 600 mM ethanol caused a transient elevation of $[Ca^{2+}]_i$, as shown in Fig. 5*B*. The peak responses induced by 600 mM ethanol in the presence and the absence of extracellular Ca^{2+} were $61{\cdot}0 \pm 13{\cdot}6$ and $4{\cdot}62 \pm 3{\cdot}0$ %, respectively.

Figure 6 summarizes the responses of $[Ca^{2+}]_i$ to various concentrations (30–1000 mM) of ethanol in endothelial cells *in situ* in the presence (Fig. 6*A*) and the absence (Fig. 6*B*) of extracellular Ca^{2+} ($n = 3$). Under both conditions, exposure to ethanol led to elevations of $[Ca^{2+}]_i$ in a concentration-dependent manner. The concentration of ethanol required to induce significant Ca^{2+} transients was similar in vascular and valvular strips, both in the presence and the absence of extracellular Ca^{2+}.

Contribution of G-protein to ethanol-induced Ca^{2+} sensitization of vascular smooth muscle

To determine whether or not the increase in Ca^{2+} sensitivity of the contractile apparatus induced by ethanol (Fig. 2C) is mediated through the activation of G-protein, we used receptor-coupled coronary arterial strips permeabilized with *Staphylococcus aureus* α-toxin. To determine the tension developed by ethanol and by histamine at constant $[Ca^{2+}]_i$, the sarcoplasmic reticulum was depleted of calcium by A23187, and $[Ca^{2+}]_i$ was buffered to maintain pCa 6·3 with 10 mM EGTA, as shown in Fig. 7. In the presence of 10 μM GTP, which itself had no effect on tension, 10 μM histamine enhanced the contractile response to constant cytosolic Ca^{2+} (pCa 6·3) (Fig. 7*A*). In the absence of GTP, ethanol *per se* had little effect on the force induced by constant Ca^{2+} (pCa 6·3), as shown in Fig. 7*B*. However, in the presence of 10 μM GTP, ethanol potentiated the contractile response to constant Ca^{2+} (pCa 6·3) in a concentration-dependent manner (Fig. 7*C*). The extent of an ethanol-induced increase in Ca^{2+} sensitization was comparable to that of the histamine-induced increase: at 1000 mM ethanol the increase was $110{\cdot}68 \pm 16{\cdot}59$ % ($n = 6$) of the 10 μM histamine-induced potentiation (with 10 μM GTP). GDPβS (1 mM) abolished ($P < 0{\cdot}01$) the enhanced contractile response to pCa 6·3 induced by 1000 mM ethanol and 10 μM GTP (Fig. 7*C*).

DISCUSSION

In the present study, we obtained the first evidence that ethanol induces sustained increases in $[Ca^{2+}]_i$ and tension both in the presence and the absence of extracellular Ca^{2+}, with a similar time course. Since ethanol induced an increase in $[Ca^{2+}]_i$ even in the absence of extracellular Ca^{2+}, not only Ca^{2+} influx but also Ca^{2+} release from intracellular store sites probably occurs. It has been reported that ethanol increases $[Ca^{2+}]_i$ in intact hepatocytes. Hoek *et al.* (1987) demonstrated the transient elevation of $[Ca^{2+}]_i$ induced by ethanol, and this elevation was prevented by depletion of Ca^{2+} pools. Reinlib *et al.* (1990) reported ethanol-induced increases in $[Ca^{2+}]_i$ and IP_3 in rat hepatocytes. To our knowledge, there has been only one such study using smooth muscle cells; Zhang *et al.* (1992) found that ethanol induced a decrease of $[Ca^{2+}]_i$ in rat aortic smooth muscle cells in culture.

It has been documented that ethanol constricts the coronary artery (Altura *et al.* 1983; Hayes & Bove, 1988); however, little is known of the underlying mechanisms. Detailed analysis of measurements of $[Ca^{2+}]_i$ and tension of the coronary arterial strips revealed that ethanol increases not only $[Ca^{2+}]_i$, but also Ca^{2+} sensitivity of the contractile apparatus, and that vasoconstriction occurs. The left-side shift of the $[Ca^{2+}]_i$ and tension relationship curve of ethanol from the one related to a high K^+-depolarization suggests that the ethanol-induced vasoconstriction may involve mechanisms which maintain high tension at low $[Ca^{2+}]_i$. Our results strongly suggest that ethanol increases Ca^{2+} sensitivity of the contractile apparatus.

Several agonists increase Ca^{2+} sensitivity of the contractile apparatus of vascular smooth muscles (Morgan & Morgan, 1984; Himpens & Somlyo, 1988; Rembold & Murphy, 1988; Sato, Ozaki & Karaki, 1988; Kodama *et al.* 1989) and this Ca^{2+} sensitization is mediated by the activation of G-protein, as demonstrated in vascular smooth muscle strips permeabilized with *Staphylococcus aureus* α-toxin (Nishimura, Kolber & van Breemen, 1988; Kitazawa, Kobayashi, Horiuti, Somlyo & Somlyo, 1989) and with β-escin (Kobayashi *et al.* 1989). Since the activation of G-protein is the only known mechanism which increases Ca^{2+} sensitivity of the vascular smooth muscle, we considered that the ethanol-induced vasoconstriction may involve the activation of G-protein. This hypothesis was supported by the evidence shown in Figs 7*C*, and 8*A* and *B*, in which we showed that ethanol requires the presence of cytosolic GTP to induce an increase in Ca^{2+} sensitivity of the contractile apparatus, and that a complete inhibition occurred with GDPβS. Consistent with this conclusion, it has been reported that in non-muscle cells, the activation of G-protein is involved in various effects of ethanol, including the activation of phospholipase C in turkey erythrocytes and human platelets (Rubin & Hoek, 1988; Rooney *et al.* 1989), and the activation of adenylate cyclase in lymphoma cells (Rabin & Molinoff, 1983). These biochemical studies were done *in vitro*. In the present study, we obtained evidence for the involvement of G-protein in ethanol-induced effects in *in situ* vascular smooth muscle cells.

In contrast to the ethanol-induced vasoconstriction, it was reported that ethanol induces increases in coronary blood flow and decreases in resistance, with no change in epicardial coronary dimensions in humans. They suggested that ethanol dilates intramyocardial resistance vessels (Cigarroa *et al.* 1990). This discrepancy can be explained by the physiological function of endothelial cells on smooth muscle cells. Knych, Guimaraes & Boivin (1984) reported that the mechanical removal of endothelial cells from rat aortic rings resulted in a significant shift of the ethanol dose–response curve to the right. In the present study, presence of the endothelial cells inhibited the ethanol-induced contraction of the coronary strips (Fig. 4), and the inhibitory effect of L-NMMA strongly supports the notion of involvement of EDRF in ethanol-induced endothelium-dependent inhibition of the ethanol-induced contraction of the coronary strips. Since the secretion of EDRF is mediated by increases in $[Ca^{2+}]_i$ in endothelial cells (Singer & Peach, 1982; Mayer, Schmidt, Humbert & Böhme, 1989), we considered that ethanol may increase $[Ca^{2+}]_i$ in endothelial cells. Indeed, such is the case, as shown in Figs 5 and 6, in which

ethanol induces the Ca^{2+} transient in the endothelial cells *in situ*. These results led to the conclusion that ethanol also has an effect on endothelial cells to modify the ethanol-induced contraction of the coronary artery, presumably through the action of EDRF.

We have data that show that the histamine-induced [Ca^{2+}]–tension curve shifted to the left from the one induced by K^+ depolarization, possibly mediated by G-proteins; however, nitroglycerin, an exogenous nitric oxide donor, made the former shift to the right and overlap with the latter (Abe *et al.* 1990) and this was also the case in the present study. The ethanol-induced [Ca^{2+}]–tension curve shifted to the left of that induced by K^+ depolarization, as mediated by G-proteins; however, in the presence of the endothelium, an endogenous nitric oxide donor, the former shifted to the right and there was an overlap with the latter in coronary arterial strips. Cyclic guanosine 5′-monophosphate, a product of the nitric oxide trans-signalling system, was found to induce Ca^{2+} desensitization of the contractile apparatus in vascular smooth muscle (Nishimura & van Breemen, 1989).

In conclusion, we suggest that ethanol contracts the coronary artery by a direct effect on vascular smooth muscle. Ethanol increases $[Ca^{2+}]_i$ and raises Ca^{2+} sensitivity of the contractile apparatus, as mediated by G-protein. Ethanol activates the endothelium to inhibit the ethanol-induced contraction of smooth muscle through the action of EDRF.

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REFERENCES

ABE, S., KANAIDE, H. & NAKAMURA, M. (1990). Front-surface fluorometry with fura-2 and effects of nitroglycerin on cytosolic calcium concentrations and on tension in the coronary artery of the pig. *British Journal of Pharmacology* **101**, 545–552.

ALTURA, B. M., ALTURA, B. T. & CARELLA, A. (1983). Ethanol produces coronary vasospasm: evidence for a direct action of ethanol on vascular muscle. *British Journal of Pharmacology* **78**, 260–262.

AOKI, H., KOBAYASHI, S., NISHIMURA, J., YAMAMOTO, H. & KANAIDE, H. (1991). Endothelin induces the Ca^{2+}-transient in endothelial cells *in situ*. *Biochemical and Biophysical Research Communications* **181**, 1352–1357.

CIGARROA, R. G., LANGE, R. A., POPMA, J. J., YUROW, G., SILLS, M. N., FIRTH, B. G. & HILLIS, L. D. (1990). Ethanol-induced coronary vasodilation in patients with and without coronary artery disease. *American Heart Journal* **119**, 254–259.

DEITRICH, R. A., DUNWIDDIE, T. V., HARRIS, R. A. & ERWIN, V. G. (1989). Mechanism of action of ethanol: initial central nervous system actions. *Pharmacological Reviews* **41**, 489–537.

DI VIRGILIO, F., STEINBERG, T. H. & SILVERSTEIN, S. C. (1989). Organic-anion transport inhibitors to facilitate measurement of cytosolic free Ca^{2+} with fura-2. In *Methods in Cell Biology*, ed. TARTAKOFF, A. M., pp. 453–462. Academic Press, San Diego.

DORIO, R. J., HOEK, J. B., RUBIN, E. & FORMAN, H. J. (1988). Ethanol modulation of rat alveolar macrophage superoxide production. *Biochemical Pharmacology* **37**, 3528–3531.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

HAYES, S. N. & BOVE, A. A. (1988). Ethanol causes epicardial coronary artery vasoconstriction in the intact dog. *Circulation* **78**, 165–170.

HIMPENS, B. & SOMLYO, A. P. (1988). Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *Journal of Physiology* **395**, 507–530.

HIRANO, K., KANAIDE, H., ABE, S. & NAKAMURA, M. (1990). Effects of diltiazem on calcium concentrations in the cytosol and on force of contractions in porcine coronary arterial strips. *British Journal of Pharmacology* **101**, 273–280.

HOEK, J. B., THOMAS, A. P., RUBIN, R. & RUBIN, E. (1987). Ethanol-induced mobilization of calcium by activation of phosphoinositide-specific phospholipase C in intact hepatocytes. *Journal of Biological Chemistry* **262**, 682–691.

KITAZAWA, T., KOBAYASHI, S., HORIUTI, K., SOMLYO, A. V. & SOMLYO, A. P. (1989). Receptor coupled, permeabilized smooth muscle: role of the phosphatidylinositol cascade, G-proteins and modulation of the contractile response to Ca^{2+}. *Journal of Biological Chemistry* **264**, 5339–5342.

KNYCH, E. T., GUIMARAES, C. M. S. & BOIVIN, S. (1984). Tolerance to ethanol-induced contractions of vascular smooth muscle: role of endothelium. *Life Sciences* **35**, 611–617.

KOBAYASHI, S., GONG, M. C., SOMLYO, A. V. & SOMLYO, A. P. (1991). Ca^{2+} channel blockers distinguish between G protein-coupled pharmacomechanical Ca^{2+} release and Ca^{2+} sensitization. *American Journal of Physiology* **260**, C364–370.

KOBAYASHI, S., KITAZAWA, T., SOMLYO, A. V. & SOMLYO, A. P. (1989). Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle: Physiological role of inositol 1,4,5-trisphosphate in pharmacomechanical coupling. *Journal of Biological Chemistry* **264**, 17997–18004.

KODAMA, M., KANAIDE, H., ABE, S., HIRANO, K., KAI, H. & NAKAMURA, M. (1989). Endothelin-induced Ca-independent contraction of the porcine coronary artery. *Biochemical and Biophysical Research Communications* **160**, 1302–1308.

LIEBER, C. S. (1988). Hepatic, metabolic and toxic effects of ethanol: a 1991 update. *Alcoholism: Clinical and Experimental Research* **15**, 573–592.

MAYER, B., SCHMIDT, K., HUMBERT, P. & BÖHME, E. (1989). Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. *Biochemical and Biophysical Research Communications* **164**, 678–685.

MORGAN, J. P. & MORGAN, K. G. (1984). Stimulus-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. *Journal of Physiology* **351**, 155–167.

NISHIMURA, J., KOLBER, M. & VAN BREEMEN, C. (1988). Norepinephrine and GTP-γ-S increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. *Biochemical and Biophysical Research Communications* **157**, 677–683.

NISHIMURA, J. & VAN BREEMEN, C. (1989). Direct regulation of smooth muscle contractile elements by second messengers. *Biochemical and Biophysical Research Communications* **163**, 929–935.

PALMER, R. M. J., ASHTON, D. S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**, 664–666.

PONNAPPA, B. C., HOEK, J. B., WARING, A. J. & RUBIN, E. (1987). Effect of ethanol on amylase secretion and cellular calcium homeostasis in pancreatic acini from normal and ethanol-fed rats. *Biochemical Pharmacology* **36**, 69–79.

RABIN, R. A. & MOLINOFF, P. B. (1983). Multiple sites of action of ethanol on adenylate cyclase. *Journal of Pharmacology and Experimental Therapeutics* **227**, 551–556.

REINLIB, L., AKINSHOLA, E., POTTER, J. J. & MEZEY, E. (1990). Ethanol-induced increases in $[Ca^{2+}]_i$ and inositol (1,4,5) triphosphate in rat hepatocytes. *Biochemical and Biophysical Research Communications* **173**, 774–780.

REMBOLD, C. M. & MURPHY, R. A. (1988). Myoplasmic $[Ca^{2+}]$ determines myosin phosphorylation in agonist-stimulated swine arterial smooth muscle. *Circulation Research* **63**, 593–603.

ROONEY, T. A., HAGER, R., RUBIN, E. & THOMAS, A. P. (1989). Short chain alcohols activate guanine nucleotide-dependent phosphoinositidase C in turkey erythrocyte membranes. *Journal of Biological Chemistry* **264**, 6817–6822.

RUBIN, R. & HOEK, J. B. (1988). Alcohol-induced stimulation of phospholipase C in human platelets requires G-protein activation. *Biochemical Journal* **254**, 147–153.

SATO, K., OZAKI, H. & KARAKI, H. (1988). Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura 2. *Journal of Pharmacology and Experimental Therapeutics* **246**, 294–300.

SATO, K., OZAKI, H. & KARAKI, H. (1990). Differential effects of carbachol on cytosolic calcium levels in vascular endothelium and smooth muscle. *Journal of Pharmacology and Experimental Therapeutics* **255**, 114–119.

SINGER, H. A. & PEACH, M. J. (1982). Calcium-and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension* **4**, suppl. II, 19–25.

ZHANG, A., CHENG, T. P. O. & ALTURA, B. M. (1992). Ethanol decreases cytosolic-free calcium ions in vascular smooth muscle cells as assessed by digital image analysis. *Alcoholism: Clinical and Experimental Research* **16**, 55–57.