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Oestradiol-induced relaxation of rabbit basilar artery by inhibition of voltage-dependent Ca channels through GTP-binding protein

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1 Effects of oestradiol on the electrical and mechanical properties of the rabbit basilar artery were investigated by use of microelectrode, patch-clamp and isometric tension recording methods.

2 Oestradiol (10 nM – 100 μM) relaxed arterial tissue pre-contracted by excess $[K]_o$ solution (30 mM) in a concentration-dependent manner. In Ca-free solution, histamine (10 μM) and caffeine (20 mM) each produced a phasic contraction, but oestradiol (10 μM) did not significantly affect their amplitude.

3 Oestradiol ($\leqslant$100 μM) did not change the resting membrane potential of the artery whether in the presence or absence of TEA (10 mM). Action potentials observed in the presence of 10 mM TEA were abolished by oestradiol (100 μM).

4 Oestradiol (1 μM – 100 μM) inhibited the voltage-dependent Ba current in a concentration-dependent manner. Oestradiol (100 μM) inhibited the Ba current observed in the presence of nicardipine (1 μM) more than that in the absence of nicardipine (to 31.0% vs 62.0% of control).

5 GTPγS (30 μM) in the pipette enhanced the inhibitory actions of oestradiol on the Ba current. On the other hand, with GDPβS (1 mM) in the pipette, oestradiol failed to inhibit the Ba current. Pertussis toxin (PTX 3 μg ml^{-1}) in the pipette totally prevented the inhibitory action of oestradiol on the Ba current.

6 Oestradiol ($\leqslant$ 100 μM) had no significant effect on the outward K currents evoked by a membrane depolarization.

7 These results strongly suggest that oestradiol relaxes arterial tissue by inhibition of voltage-dependent Ca channels and that it inhibits both nicardipine-sensitive and -resistant Ca currents via a PTX-sensitive GTP-binding protein. The main target of oestradiol among the arterial Ca channels seems to be the nicardipine-resistant Ca channel, rather than the nicardipine-sensitive one.

Keywords: Oestradiol; basilar artery; Ca channel; Ca currents; sex hormone; Ca antagonist; oestrogen receptor; GTP-binding protein

Introduction

It is well known that treatment with oestrogen reduces the mortality due to cerebro- and cardiovascular diseases in post-menopausal women (Stampfer *et al.*, 1985; 1991; Bush *et al.*, 1987; Henderson *et al.*, 1988; Knopp, 1988; Paganini-Hill *et al.*, 1988). Modulation of metabolic processes, such as lipid metabolism, at the level of the gene might form part of these actions of oestrogen and thus oestrogen may reduce the risk of atherosclerosis in the coronary artery (Sarrel *et al.*, 1994).

In addition to its chronic actions, oestrogen has been reported to reduce vascular resistance and increase blood flow in various tissues within a few minutes of its administration (Magness & Rosenfeld, 1989; Williams *et al.*, 1990). These effects of oestrogen were thought not to be related to its genomic actions, because tamoxifen, an inhibitor of the genomic actions of oestrogen, had no antagonizing action on such acute responses (Han *et al.*, 1995). Shamma *et al.* (1992) reported that plasma oestrogen levels were directly correlated with cerebral blood flow and, according to Pines *et al.* (1991), oestrogen increases coronary blood flow through cardiac inotropic and vasodilator actions. With regard to the mechanisms involved in vasodilatation, it is known that the endothelium contributes partly to the vascular relaxation induced by oestradiol, though the main part of this effect is not dependent on the endothelium (Miller *et al.*, 1988; Gisclard *et al.*, 1988).

Oestradiol has been reported to inhibit the voltage-dependent Ca channel in cardiac and A7r5 cells (Jiang *et al.*, 1992; Zhang *et al.*, 1994) and to reduce the cytosolic Ca concentration and the contraction induced by various stimulants (Han *et al.*, 1995). These results directly support the idea that oestradiol acts as a Ca channel blocker (Collins *et al.*, 1993). However, a detailed analysis of the actions of oestradiol on membrane currents has not yet been reported. In the present experiments, we focused on the mechanisms involved in the vasorelaxant actions of oestradiol on freshly dispersed cells from the rabbit basilar artery using tension recording, membrane potential recording and voltage-clamp methods.

Methods

Female albino rabbits (Nippon White; 1.7–2.0 kg) were anaesthetized with sodium pentobarbitone by intravenous injection (40 mg kg^{-1}; Tokyo Kasei, Tokyo) and exsanguinated. The basilar artery was dissected and isolated by removal of the surrounding connective tissue.

Preparation for mechanical recording

A ring preparation of the basilar artery (1 mm in length) was

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held horizontally by a pair of thin wires (100 μm in diameter) in a small organ bath. One of the wires was fixed to the wall of the organ bath and the other to the force displacement transducer (TB-612T; Nihon Koden, Tokyo). Each preparation was equilibrated in warmed Krebs solution for 30 min. In some experiments, endothelium-denuded tissue was prepared by scrubbing the inner vessel wall with fine threads.

Preparation for microelectrode experiments

The basilar artery (10 mm in length) was fixed onto a rubber plate with small pins. Fine glass tubes (100–200 μM in diameter) were inserted into the lumen of the artery to prevent contraction. The membrane potential was measured following impalement with a glass-microelectrode (50–80 MΩ; filled with 3 M KCl) from the adventitial side using a micromanipulator (MP-1, Narishige Sci. Lab., Tokyo).

Preparation for patch-clamp experiments

The procedure for cell dispersion was similar to that described by Oike *et al.* (1990) except for differences in the enzymes used. In the present experiments, the basilar artery (5 mm in length) was incubated for 30 min at 35°C in warmed Ca-free solution containing 0.2% collagenase (Wako Pure Chem., Osaka, Japan), 0.05% papain (Sigma Chem., St. Louis, USA), 0.2% trypsin inhibitor (type IIS, Sigma Chem.), 0.2% bovine serum albumin (Fraction V, essentially fatty acid free; Sigma Chem.) and 0.2% dithiothreitol (DTT; Sigma Chem.). After enzyme treatment, the tissue was transferred to fresh Ca-free solution (35°C) and single cells were dispersed by gentle agitation with blunt-tipped pipettes. Dispersed cells were stored at 10°C in a fresh solution containing 0.5 mM Ca and 0.5 mM Mg. Experiments were performed at room temperature (25–28°C).

Recording of signals

The volume of the organ bath was 0.5 ml. For tension and membrane potential recording, Krebs solution pre-warmed to 32°C was superfused at a rate of 3 ml min^{-1}. The pH of the solution was maintained at 7.3–7.4 with 5% CO_2 and 95% O_2. The experimental procedure in Ca-free solution was as follows: after the preparation had been incubated in Ca-free solution containing 1 mM EGTA for 20 min, it was exposed to either 20 mM caffeine or 10 μM histamine for 3 min. This process was repeated several times to deplete calcium from the intracellular storage sites. Then (as indicated in Figure 2), the preparation was loaded with 2.5 mM Ca for 6 min or 20 min (for caffeine or histamine experiments, respectively) and rinsed with the Ca-free solution for 3 or 10 min before application of 20 mM caffeine or 10 μM histamine, respectively. After it had been confirmed that each drug produced a constant amplitude of phasic contraction, the effect of oestradiol was observed.

For the study of the effect of oestradiol on the action potential, the tissue was pretreated with 10 mM tetraethylammonium (TEA). For electrical stimulation, short electrical pulses (1–10 ms duration; 10–50 V intensity) were applied through Ag-AgCl wire electrodes (0.5 mm in diameter).

For the recording of K or Ca channel currents, a physiological salt solution (PSS) or 10 mM Ba solution, respectively, was superfused in the bath and the pipette was filled with a high-K solution or high-Cs solution, respectively. Membrane currents were recorded by a whole-cell voltage-clamp method (Hamill *et al.*, 1981). Patch electrodes (3–5 MΩ) were prepared with an electrode puller and heat polisher (PP-83 & MF-83, Narishige Sci. Inst. Lab., Tokyo, Japan) and manipulated with a three-dimensional water driven manipulator (MHW-3, Narishige Sci. Inst. Lab.). Data acquisition was carried out using an IBM-compatible PC (Deskpro 386/25m; Compaq, Houston, TX, U.S.A.) and the analysis was performed with pCLAMP software (Axon Inst., Burlingame, CA, U.S.A.). A hard copy was obtained with a laser printer (LP-880S; Kyocera, Kyoto, Japan).

Solutions

For tension and membrane potential recording, a modified Krebs solution was used with the following ionic composition (mM): NaCl 121.9, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 15.5, KH_2PO_4 1.2 and glucose 11.5. The pH of the solution was adjusted to 7.3–7.4 with 5% CO_2 : 95% O_2. For patch-clamp experiments, PSS of the following ionic composition was used (mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.3 and glucose 12. The nominally Ca-free solution used for cell dispersion was prepared by removal of 2.3 mM $CaCl_2$ from PSS. High K solution of the following ionic composition was in the pipette for recording outward currents (mM): KCl 120, pyruvate 20, glucose 20, $MgCl_2$ 5, adenosine 5′-triphosphate (ATP) 5 and ethyleneglycol-*bis*[β-aminoethylether]-*N,N,N′,N′*-tetraacetic acid (EGTA) 0.1 or 4. For recording the Ba current, 10 mM Ba solution and high-Cs solution (see below) were used in the bath and pipette, respectively. Ba solution (mM): $BaCl_2$ 10, NaCl 128, KCl 5.4 and glucose 11. High Cs solution (mM): CsCl 120, pyruvate 20, EGTA 4, $MgCl_2$ 5, ATP 5 and glucose 20. The pH of the solutions was adjusted to 7.37 ± 0.02 with 10 mM 4-[2-hydroxyethyl]-1-piperazine ethansulphonic acid (HEPES; Dojin Kagaku, Kumamoto, Japan) titrated with NaOH (PSS, Ba solution and Ca-free solution), KOH (high-K solution) or tris [hydroxymethyl] aminomethane (high-Cs solution).

Drugs

Drugs used in the present experiments were 17β-oestradiol, 17α-oestradiol (Sigma Chem., St. Louis, MO, U.S.A.), nicardipine (Yamanouchi Pharmac., Tokyo, Japan), 2-hydroxypropyl-β-cyclodextrin (Sigma Chem.), indomethacin (Sigma Chem.), methylene blue (Tokyo Kasei, Tokyo, Japan), GTPγS (Böehringer) and pertussis toxin (PTX; List Biological Labs, Campbell, GA, U.S.A.). In the present experiments, a water-soluble type of 17β-oestradiol which was encapsulated into 2-hydroxypropyl-β-cyclodextrin was mainly used. However, for comparisons between the actions of 17β-oestradiol and 17α-oestradiol, a water soluble type was used. This type of 17β-oestradiol, as well as the 17α-oestradiol, was dissolved in ethanol and diluted with Krebs solution. 2-Hydroxypropyl-β-cyclodextrin (0.65 mg ml^{-1}) or ethanol (0.01 or 0.1%) was applied as control of water-soluble (100 μM 17β-oestradiol contained 0.65 mg ml^{-1} 2-hydroxypropyl-β-cyclodextrin) or -insoluble oestradiol. To investigate the PTX sensitivity of the actions of oestradiol, a solution containing PTX was used in the pipette. PTX (100 μg ml^{-1}) was reconstituted in 10 mM phosphate buffer (pH 7) and stored at 4°C. PTX (6 μg ml^{-1}) was activated in Cs internal solution with 10 mM DTT for 20 min at 37°C. Then, PTX solution was added to an equal volume of Cs internal solution containing 10 mM ATP and 2 mM NAD^+ to yield PTX and ATP concentrations of 3 μg ml^{-1} and 5 mM respectively. For control experiments, we used a solution made by the same procedure, but without PTX as pipette solution.

Statistics

The results are expressed as mean values with standard deviation. Statistical significance was assessed with Student's *t* test and *P* values less than 0.05 were considered to be significant.

Results

Effects of oestradiol on the K-induced contraction

17β-Oestradiol (< 100 μM) had no effect on the resting tone of endothelium-intact or -denuded rabbit basilar artery. When 30

mM K solution was superfused, a sustained contraction was evoked following the transient contraction. Oestradiol (10 nM–100 μM) relaxed this sustained contraction in endothelium-intact basilar artery in a concentration-dependent manner (Figure 1). An evoked reduction in muscle tone occurred 1 min or less after the application of oestradiol and, after removal of oestradiol, the contraction was restored within 10 min (Figure 1a). Such an inhibitory action was not observed with 2-hydroxypropyl-β-cyclodextrin, used to increase the water solubility of 17β-oestradiol (data not shown).

When 17β-oestradiol was dissolved in ethanol, oestradiol produced a larger inhibitory effect on the 30 mM K-induced concentration (1 μM oestradiol in 0.01% ethanol, by 39.8 ± 10.1% of control, $n = 4$; in cyclodextrin, by 23.2 ± 10.7%, $n = 12$; $P < 0.05$). A higher concentration of ethanol (0.1%) itself produced muscle relaxation (by 36.2 ± 1.9% $n = 4$), and 17β-oestradiol in 0.1% ethanol (10 μM) completely inhibited the 30 mM K-induced contraction (by 97.5 ± 9.2% $n = 4$).

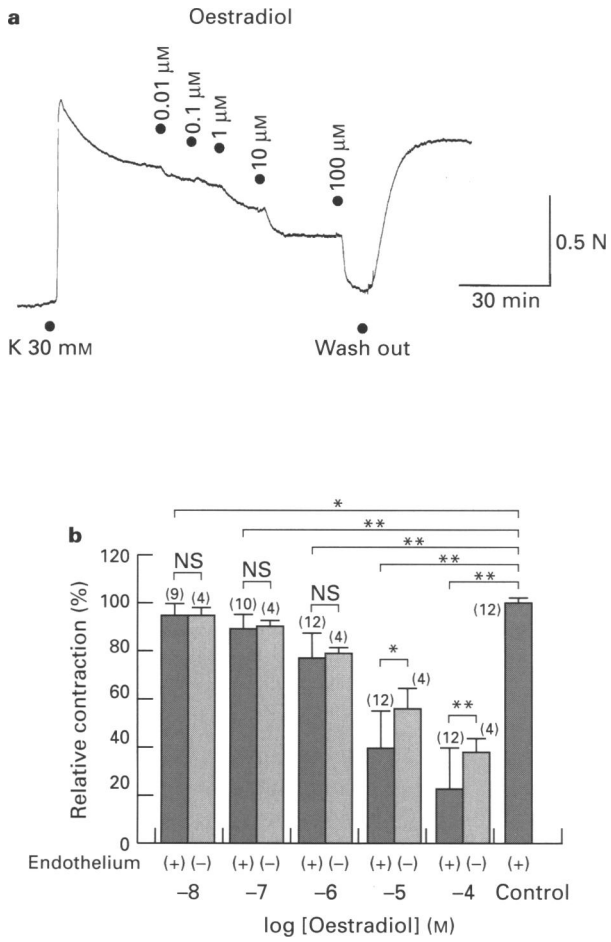

Figure 1 Effect of oestradiol on the amplitude of 30 mM K-induced contraction of the rabbit basilar artery. (a) An example of the inhibitory action of oestradiol on the tonic contraction induced by 30 mM K solution. Oestradiol (10^{-8}–10^{-4} M) was applied cumulatively as indicated by dots. Note that 30 mM K had produced a sustained contraction for 20–30 min. (b) Relationship between oestradiol concentration (10^{-8}–10^{-4} M) and relative amplitude of contraction induced by 30 mM K solution in the endothelium-intact and -denuded preparations. Right hand columns(−): endothelium-denuded preparation. Left hand columns(+): endothelium-intact preparation. Each column indicates mean value with s.d. The amplitude of the tonic component of the 30 mM K-induced contraction was normalized as 100% in each case. Control (extreme right column) indicates contraction observed in the presence of 2-hydroxypropyl-β-cyclodextrin (0.65 mg ml^{-1}) without oestradiol. * and ** indicate $P \leq 0.05$ and 0.01, respectively. NS: no statistical significance.

Compared to 17β-oestradiol, 17α-oestradiol inhibited the 30 mM K-induced contraction to a lesser extent (1 μM in 0.01% ethanol, by 17.9 ± 2.1% $n = 4$; 10 μM in 0.1% ethanol, by 56.3 ± 7.2% $n = 4$).

To investigate whether the inhibitory actions of oestradiol depended on the endothelium, similar experiments were performed on endothelium-intact and -denuded basilar arteries (Figure 1b). At higher concentrations (> 10μM), oestradiol produced relaxation to a greater extent in the endothelium-intact preparations. For example, in the endothelium-intact preparation, oestradiol (100 μM) relaxed the tissue to 21.5 ± 18.3% of control, while the same concentration of oestradiol relaxed endothelium-denuded tissue to only 37.6 ± 5.9% of control (in each case, the amplitude of the tonic contraction induced by 30 mM K in the absence of oestradiol was normalized as 100%). Similar experiments were performed on basilar arteries isolated from male, rather than female rabbits. Exactly the same relationship was obtained, thereby indicating that there was no sex-related difference in the relaxing actions of oestradiol.

Methylene blue (10 μM) and/or indomethacin (100 μM) did not affect the oestradiol (1–100 μM)-induced muscle relaxation (data not shown).

Effects of oestradiol on caffeine- and histamine-induced contractions

In Ca-free solution, caffeine (20 mM) and histamine (10 μM) each produced a phasic contraction of constant amplitude, when applied according to the protocol described in the Methods and shown in Figure 2. To investigate its effects on the intracellular Ca store, the effect of oestradiol on the caffeine- and histamine-induced contractions was observed in endothelium-denuded preparations in Ca-free solution (Figure 2a,b). Pretreatment of the tissue with oestradiol slightly suppressed the amplitude of both the caffeine- and histamine-induced contractions in some preparations; however, the effects were not statistically significant (caffeine $P > 0.05$, histamine $P > 0.05$; Figure 2c).

In Krebs solution (2.5 mM Ca), caffeine (20 mM) and histamine (10 μM) each produced phasic and tonic contractions. Oestradiol (10 μM) did not affect the amplitude of the phasic contraction, but suppressed the amplitude of the tonic contraction induced by either stimulant by about 30% (data not shown).

Effects of oestradiol on membrane and action potentials

The mean value for the resting membrane potential of the smooth muscle cells of the rabbit basilar artery was −48.6 ± 2.7 mV ($n = 13$) and spontaneous electrical membrane activity was not observed. Oestradiol (10 and 100 μM) had no effect on this resting potential (10 μM −47.2 ± 1.6 mV $n = 9$; 100 μM −46.8 ± 1.8 mV $n = 5$; $P > 0.05$). Furthermore, electrical field stimulation (EFS) with short pulses (1–10 ms pulse duration, 10–50 V intensity, bursts of 3 pulses at 20 Hz) did not evoke an action potential whether in the presence or absence of oestradiol (10 or 100 μM).

To evoke action potentials on which to observe the effects of oestradiol, the tissue was pretreated with 10 mM TEA. TEA (10 mM) depolarized the membrane to −41.0 ± 4.6 mV ($n = 17$) and generated spontaneous action potentials in some preparations (Figure 3a–c). Oestradiol did not affect the membrane depolarization induced by TEA (10 μM −40.6 ± 5.7 mV $n = 8$; 100 μM, −41.9 ± 4.0 mV $n = 12$; $P > 0.05$) but first prolonged the interval between spontaneous action potentials in the presence of TEA and then suppressed their generation (10 μM $n = 8$; 100 μM $n = 10$; Figure 3b,c). Removal of oestradiol (10–100 μM) restored the spontaneous generation of action potentials within a few minutes (Figure 3c). In some preparations, TEA (10 mM) failed to produce spontaneous action potentials, but EFS (1–10 ms duration; 10–50 V intensity) did evoke action potentials, the amplitude of which was then inhibited by oestradiol without changing the resting

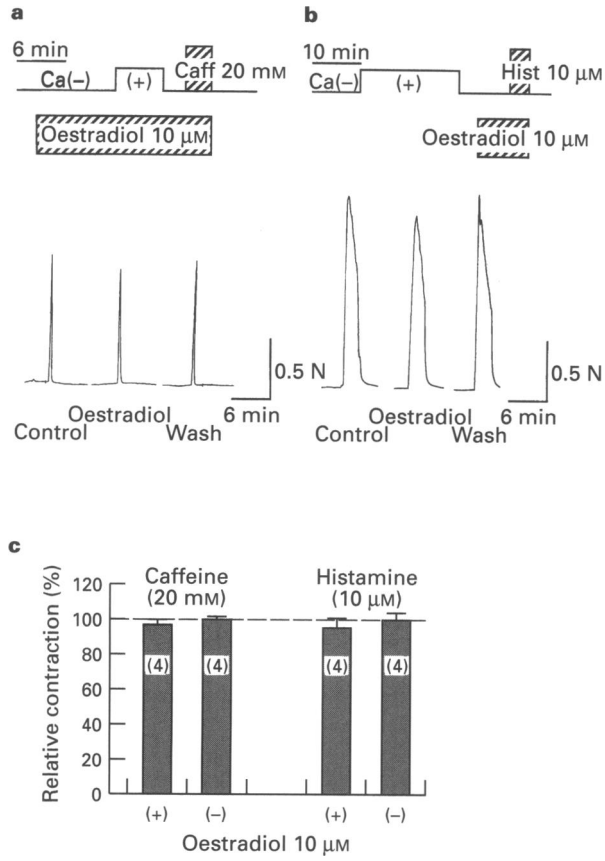

Figure 2 Effect of oestradiol (10 μM) on caffeine- and histamine-induced contractions in Ca-free solution. (a) and (b): Protocol and actual traces of caffeine (20 mM) or histamine (10 μM) -induced contractions before, during application of, and after removal of oestradiol. (c) Relative amplitude of contraction induced by 20 mM caffeine- (left-hand columns) or 10 μM histamine- (right-hand columns) during application of, and after removal of 10 μM oestradiol. The peak amplitude of contraction before application of oestradiol was normalized as 100% in each case.

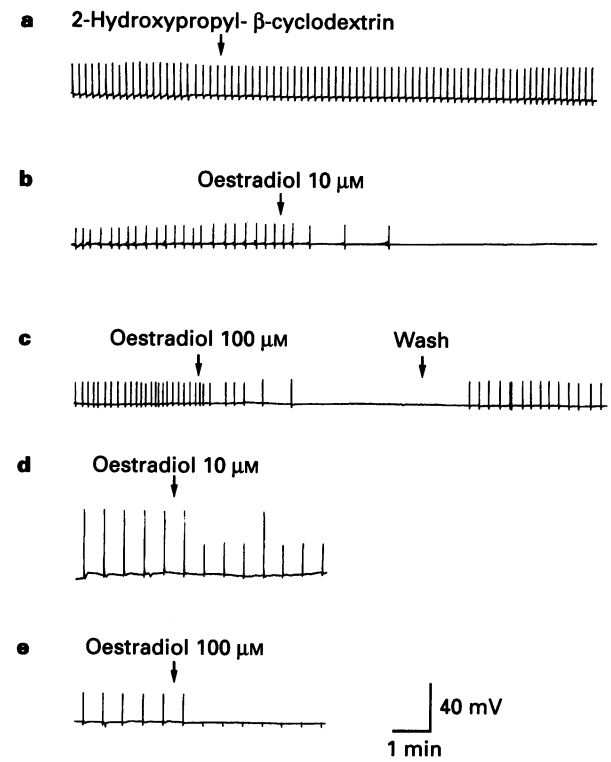

Figure 3 Effects of oestradiol and 2-hydroxypropyl-β-cyclodextrin on spontaneously generated (a–c) or EFS-evoked (d and e) action potentials in the presence of 10 mM TEA. Oestradiol and 2-hydroxypropyl-β-cyclodextrin were applied at the times indicated by arrows.

membrane potential (10 or 100 μM n = 4; Figure 3d,e). The vehicle, 2-hydroxypropyl-β-cyclodextrin, had no effect on the membrane potential or on action potential generation (n = 4; Figure 3a).

Effect of oestradiol on inward Ba currents

To help us study the mechanisms underlying the inhibitory action of oestradiol on the action potential, we recorded inward Ba currents from isolated and dispersed arterial cells and observed the effects on them of oestradiol. With the pipette filled with high-Cs solution, inward Ba currents were recorded in response to step or ramp voltage pulses in 10 mM Ba solution. For example, when the membrane was held at −80 mV and a ramp voltage pulse applied (from −110 to +50 mV; 300 ms duration), a V-shaped inward current with its peak at 20 mV was observed. Application of 30 μM oestradiol reduced the amplitude of this Ba current and, 10 min after removal of the drug, the current was almost restored to control amplitude (Figure 4a). No clear voltage-dependence was observed in the inhibitory action of oestradiol (1–100 μM) on the Ba current evoked by a ramp voltage pulse. However, oestradiol tended to reduce the amplitude of the Ba current measured at the end of the step pulse (100 ms) more than the peak amplitude (1 μM 88.5 ± 10.1% at peak, 78.4 ± 19.4% at the end; $P > 0.05$, 10 μM 77.4 ± 8.9% at peak, 68.3 ± 19.8% at the end; $P > 0.05$, 100 μM 62.0 ± 10.8% at peak, 42.3 ± 10.2% at the end; $P < 0.05$). Figure 4b shows the concentration-inhibition relationship for the effect of oestradiol on the peak Ba inward current evoked by a ramp voltage pulse, the maximum amplitude of the Ba current before application of oestradiol being normalized as 100% (control). This shows that oestradiol ($\geq$ 1 μM) inhibited the inward current in a concentration-dependent manner.

Application of 1 μM nicardipine reduced the amplitude of the Ba inward current evoked by a ramp voltage pulse from −130 to +50 mV; holding potential −80 mV) to 56% of control. Oestradiol (10 μM) further reduced the amplitude of the Ba current to 20% of control (Figure 5a). Nicardipine (1 μM) also reduced the amplitude of the inward current induced by a step voltage pulse (to 50% of control), though an increase in concentration (to 10 μM) did not further reduce the current amplitude (Figure 5b), indicating the presence of nicardipine-sensitive and -resistant components.

Figure 5c shows a typical effect of oestradiol (10 μM) on the nicardipine-resistant Ba current evoked by step depolarizing pulses (from the holding potential of −80 mV to −30, −10 and +10 mV). The peak amplitude of the Ba currents evoked by depolarizing pulses to −10 or +10 mV was inhibited by nicardipine (1 μM) to 51% or 40% of their respective controls in this particular cell. Additional application of 10 μM oestradiol further inhibited these Ba currents to 22% or 8% of their respective control values. When the amplitude of the Ba current in the presence of 1 μM nicardipine (centre panel, Figure 5c) was given the value 100%, oestradiol (10 μM) inhibited this nicardipine-resistant Ba current, evoked by step depolarizing pulses to −10 or +10 mV, to 40% or 20% of their respective control values. Figure 5d shows the relationship between the concentration of oestradiol and the relative amplitude of the current observed in the presence or absence of 1 μM nicardipine, where the amplitude of the Ba currents in the presence or absence of nicardipine was each normalized as 100%. To evaluate the actions of oestradiol, experiments were

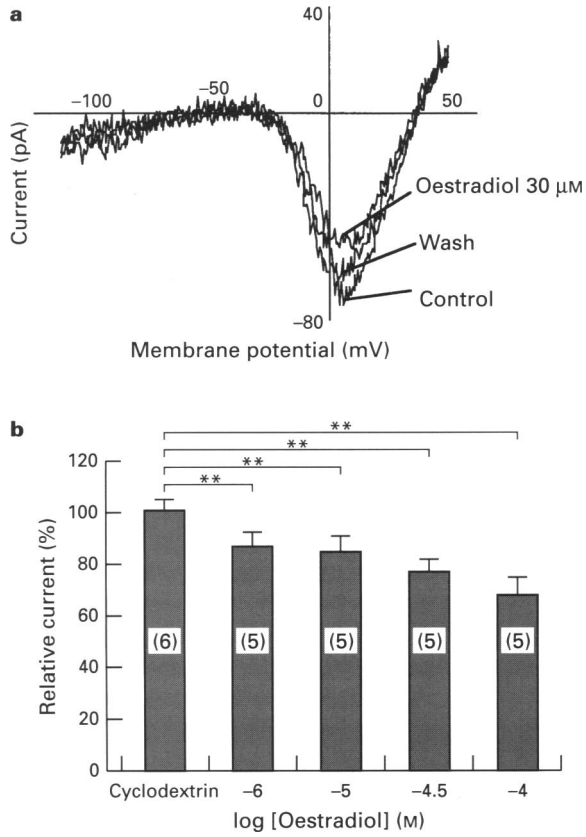

Figure 4 Effect of oestradiol on the Ba inward current evoked by a ramp voltage pulse. (a) Ba current evoked by a ramp pulse (from −110 to +50 mV; 300 ms duration). Ba currents recorded before, during application of, and after removal of oestradiol (30 μM) are superimposed. (b) Relationship between concentration of oestradiol and relative amplitude of inward Ba current. The maximum amplitude of the Ba current before oestradiol was normalized as 100%. Each column indicates the mean value of 5–6 observations with s.d. Leak current was subtracted by extrapolation of the linear component in each trace. ** $P \leqslant 0.01$.

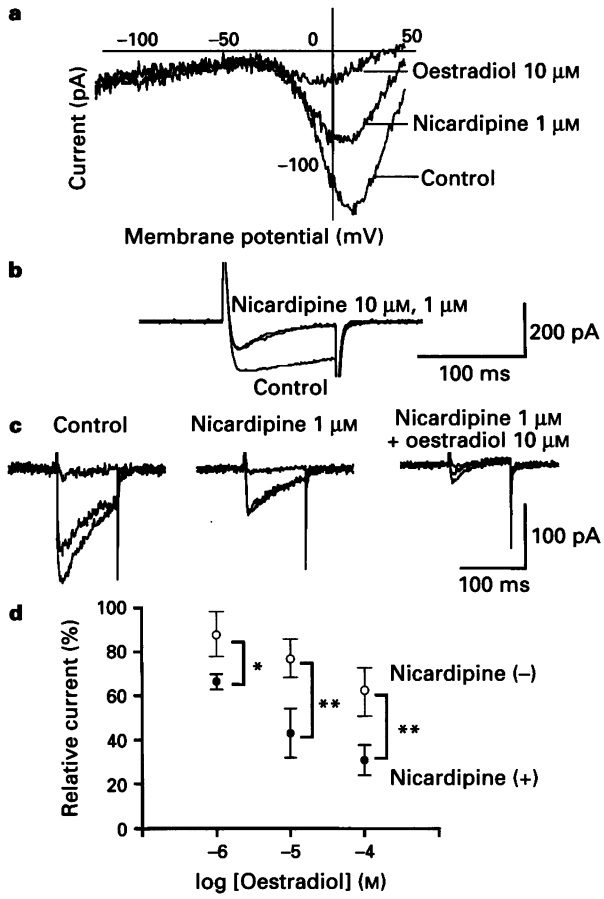

Figure 5 Effects of nicardipine and oestradiol on the Ba current. (a) Effect of oestradiol (10 μM) on the Ba currents evoked by a ramp depolarizing pulse (from −130 mV to +50 mV; holding potential −80 mV) in the presence of nicardipine (1 μM). Oestradiol was applied 10 min after application of nicardipine. The currents were recorded, respectively, before, 6 min after application of nicardipine and 6 min after application of oestradiol. (b) Effect of nicardipine (1 and 10 μM) on the Ba current evoked by a step voltage pulse to −10 mV (100 ms duration; from a holding potential of −80 mV). Currents in the presence of nicardipine were obtained 6 min after application of the drug. (c) Effect of oestradiol (10 μM) on the Ba current in the presence of 1 μM nicardipine. Depolarizing pulses were applied from the holding potential of −80 mV to −30, −10 and +10 mV; 100 ms duration. The currents were recorded before, 8 min after application of nicardipine and 7 min after application of oestradiol in the presence of nicardipine. (d) Relationship between the concentration of oestradiol and the relative amplitude of the Ba current evoked by a depolarizing pulse to −10 mV from a holding potential of −80 mV in the presence (●) and absence (○) of 1 μM nicardipine. The amplitude of the Ba current in the presence or absence of nicardipine were each normalized as 100%. Each symbol indicates the mean with s.d. of 4–6 observations. *$P \leqslant 0.05$; ** $P \leqslant 0.01$.

performed on cells in which the amplitude of the nicardipine-resistant current was more than one-third of control (mean value 62%). In nicardipine-treated cells, oestradiol (1–100 μM) suppressed the Ba current to a greater extent than in the nicardipine-untreated cells. Thus, as shown in Figure 5d, in treated cells it took only 1 μM oestradiol to suppress the Ba current to the extent achieved by 100 μM in untreated cells.

The magnitude of the inhibitory effect of nicardipine on the Ba current differed from one cell to another. For example, in the cell shown in Figure 6a, nicardipine (1 μM) almost completely suppressed the Ba current, while the same concentration of nicardipine only marginally suppressed the current in the cell shown in Figure 6b. Even though these cells represent extreme examples, we felt it was of interest to examine the actions of oestradiol on the nicardipine-sensitive and -resistant components of the Ba current in these cells. In the cell shown in Figure 6a oestradiol (100 μM) had little or no effect on the amplitude of the nicardipine-sensitive Ba current, while, in the cell shown in Figure 6b, it markedly reduced the amplitude of the nicardipine-resistant current.

Involvement of GTP-binding protein in the oestradiol-induced responses

When GTPγS (30 or 100 μM) was added to the Cs solution in the pipette, the inhibitory action of oestradiol was markedly enhanced. For example, 1 μM or 10 μM oestradiol inhibited the Ba current evoked by a ramp pulse (from −110 to 70 mV; holding potential −80 mV; 300 ms) to 70% or 43%, respectively, of control in the presence of 30 μM GTPγS (Figure 7a). As already shown (Figure 4b), in the absence of GTPγS, these concentrations of oestradiol suppressed the amplitude to only 86 or 84%, respectively. We confirmed the enhancing action of GTPγS on the inhibitory action of oestradiol using a short depolarizing pulse (50 ms). Figure 7b shows the effects of 1 and 10 μM oestradiol on the Ba current in the presence of 30 μM (filled circles) or 100 μM (open circles) GTPγS. When 100 μM GTPγS was in the pipette solution, the inhibitory action of oestradiol on the Ba current was greatly enhanced (Figure 7b). On the other hand, with 1 mM GDPβS in the pipette, oestradiol (100 μM) had little or no effect on the Ba current evoked by a ramp pulse (−110 mV to +50 mV; holding potential −80 mV; Figure 7c).

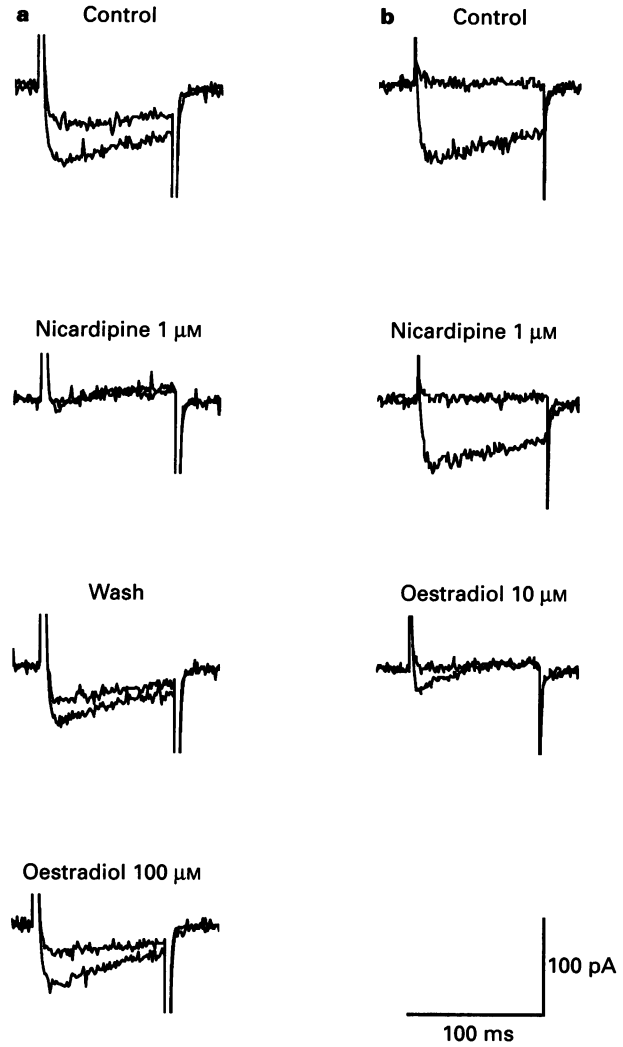

Figure 6 Effect of oestradiol on the Ba current in nicardipine-sensitive (a) and -resistant (b) cells. Depolarizing pulses to −10 and +10 mV (a) and to −30 and −10 mV (b) were applied from a holding potential of −80 mV. (a) Traces were recorded before, 5 min after application of nicardipine (1 μM), 10 min after removal of nicardipine and 7 min after application of oestradiol (100 μM). Oestradiol was applied 10 min after removal of nicardipine. (b) Before, 10 min after application of nicardipine (1 μM) and 9 min after application of oestradiol (10 μM). In (b), oestradiol was applied in the presence of nicardipine.

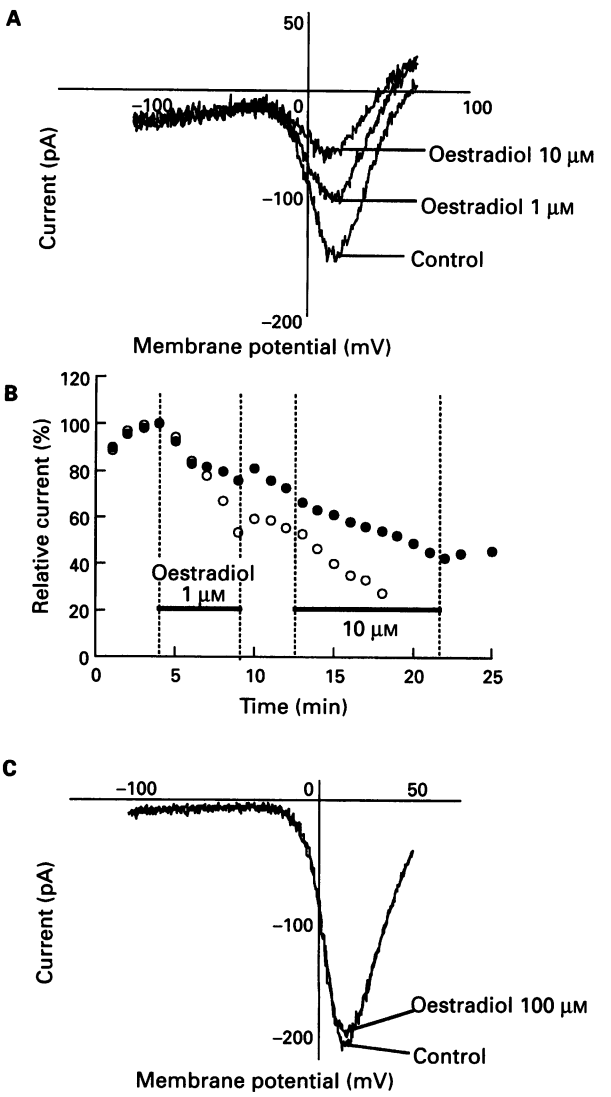

Figure 7 A typical example of the actions of oestradiol on the Ba current with GTPγS (30 μM and 100 μM) or GDPβS (1 mM) in the pipette. Oestradiol was applied cumulatively. (a) Effect of 30 μM GTPγS. A ramp voltage pulse (from −110 to +70 mV at a holding potential of −80 mV) was applied. The traces were obtained before, 7 min after application of 1 μM oestradiol and 10 min after application of 10 μM oestradiol. (b) The peak amplitude of the Ba current evoked by a step pulse (to −10 mV from the holding potential of −80 mV; 50 ms) was plotted. GTPγS at 30 (●) or 100 (○) μM was present in the pipette solution. The amplitude of the Ba current recorded before application of oestradiol was normalized as 100%. (c) Effect of GDPβS. A ramp voltage pulse (from −110 to +50 mV at the holding potential of −80 mV) was applied. Traces were obtained before and 7 min after application of oestradiol.

Figure 8a summarizes the effect of oestradiol on the amplitude of the Ba current in the presence and absence of 30 μM GTPγS and 1 mM GDPβS. Oestradiol (1 and 10 μM) inhibited the Ba current more strongly in the presence of GTPγS than in its absence. By contrast, no reduction in the amplitude of the Ba current was induced by 100 μM oestradiol when GDPβS (1 mM) was in the pipette.

Figure 8b shows the effect of PTX (3 μg ml^{-1}) on the inhibitory action of oestradiol (10 and 100 μM). As a control, the Ba current was measured with a pipette solution of exactly the same composition, except for the PTX. In this control situation, oestradiol inhibited the Ba current to much the same extent as it did with the ordinary pipette solution (see Figure 4). PTX prevented this action of oestradiol.

Effects of oestradiol on outward K currents

We also observed the effect of oestradiol on outward K currents. With the pipette filled with high K and 4 mM EGTA solution, an outward current was recorded in response to a ramp voltage pulse (from −110 to −70 mV; holding potential −70 mV) applied in nominally Ca-free solution. As shown in Figure 9a, the membrane currents evoked by a ramp pulse were the same before, during the application of, and after removal of oestradiol.

Large outward currents were evoked by step depolarizing pulses at various intensities in 2.3 mM Ca solution, with the pipette containing high K and 0.1 mM EGTA solution. Oestradiol did not affect these large outward currents (Figure 9b). Figure 9c shows the relationship between the amplitude of the membrane current and the intensity of the depolarizing pulse before, during the application of, and after removal of oestradiol. These results indicate that oestradiol has no effect on the Ca-dependent and -independent K outward currents in the rabbit basilar artery.

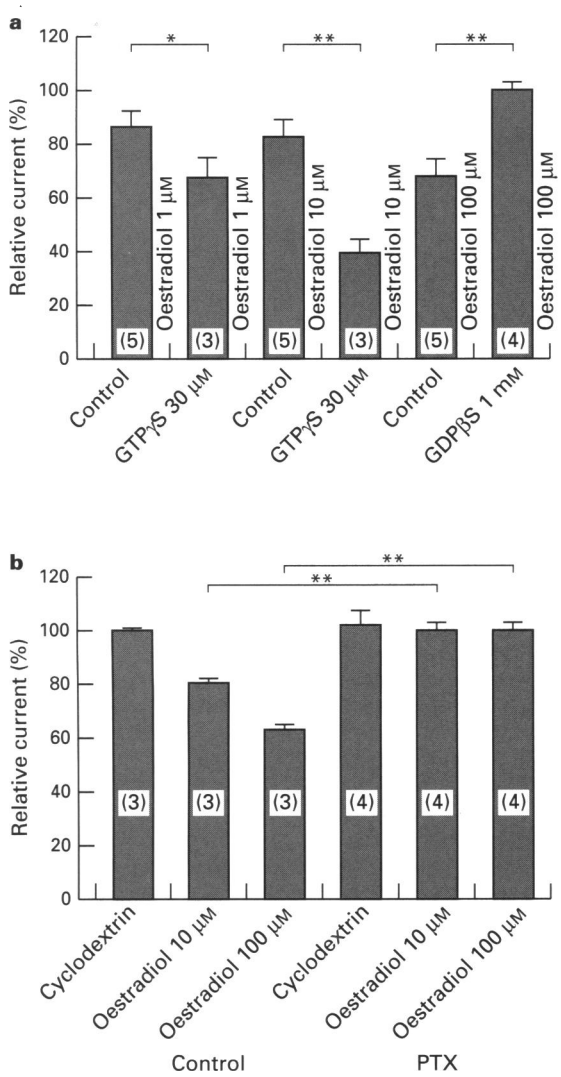

Figure 8 Effects of oestradiol on the Ba current with 30 μM GTPγS or 1 mM GDPβS (a) or with 3 μg ml^{-1} PTX (b) in the pipette. The peak amplitude of the Ba current evoked by a ramp pulse (from -110 to 50 mV; at the holding potential of -80 mV) was measured. The peak amplitude before application of oestradiol was normalized as 100% and those in the presence of oestradiol are expressed in a relative manner. In each cell, we applied only one concentration of oestradiol. The amplitude of the current was obtained at 7–10 min after application of oestradiol. Each column indicates mean value with s.d. Number of observations is noted in each column. *$P \leqslant 0.05$; **$P \leqslant 0.01$.

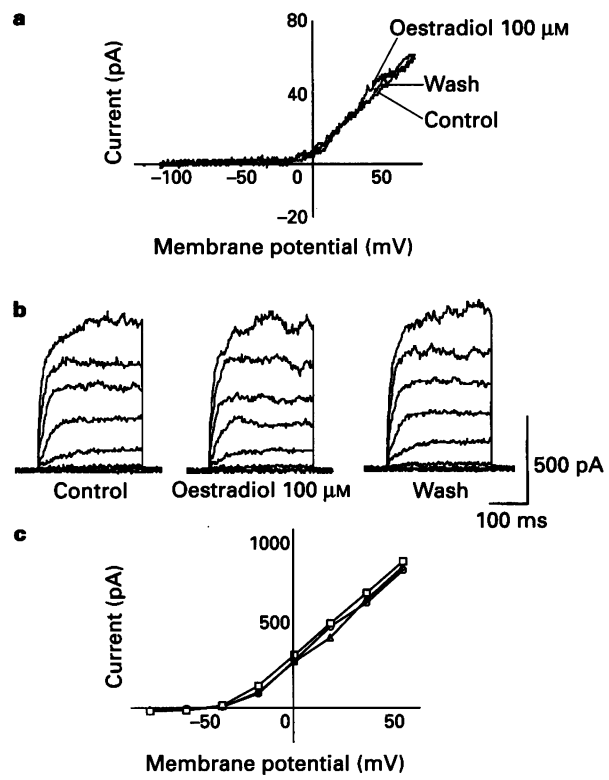

Figure 9 Effect of oestradiol on the outward K current evoked by ramp (a) and step (b) voltage pulses. (a) A ramp voltage pulse (from -110 to $+70$ mV at a holding potential of -70 mV; 300 ms duration) was applied before, 7 min after application of, and 10 min after removal of 100 μM oestradiol. EGTA (4 mM) was in the pipette solution. (b) Step depolarizing pulses (every 20 mV from -80 to $+60$ mV at a holding potential of -70 mV) were applied. The currents were recorded before, 10 min after application of, and 10 min after removal of 100 μM oestradiol. EGTA (0.1 mM) was in the pipette solution. (c) Relationship between membrane current and membrane potential during a depolarizing pulse in the presence or absence of oestradiol (100 μM): (○) control; (△) in the presence of oestradiol; (□) after removal of oestradiol.

Discussion

In the present experiments, we found that (1) oestradiol relaxes the rabbit basilar artery through endothelium-dependent and -independent pathways; (2) the direct inhibitory action of oestradiol may be related to an inhibition of the voltage-dependent Ca channel; (3) oestradiol inhibits the nicardipine-resistant component of the Ca current more strongly than the nicardipine-sensitive component and (4) these actions of oestradiol on the Ca channel involve PTX-sensitive GTP-binding protein.

Several lines of evidence have already suggested that the acute inhibitory actions of oestradiol in vascular tissues might be exerted via an inhibition of the voltage-dependent Ca channel (Stice *et al.*, 1987; Jiang *et al.*, 1991). In ventricular cells, Jiang *et al.* (1992) have found direct evidence of Ba current inhibition by oestradiol (10–30 μM). They speculated that the target of oestradiol in cardiac cells was the L-type Ca channel since oestradiol inhibited the Ba current evoked with the holding potential set at -40 mV. They also showed that the inhibitory effect of 10 μM oestradiol was holding-potential-dependent. For example, at holding potentials of -80 or -40 mV, 10 μM oestradiol suppressed the current amplitude to 83 or 61%, respectively, of the control value. In the rabbit basilar artery, we found that oestradiol reduced the amplitude of the Ba current and inhibited the nicardipine-resistant Ba current more strongly than it did the nicardipine-sensitive current. As oestradiol inhibits both T- and L-type Ca channels in A7r5 cells (Zhang *et al.*, 1994), inhibition of both dihydropyridine (DHP) -sensitive and -resistant Ca channels seems likely to be a common action of oestradiol in vascular smooth muscle cells. In the present experiments, we cannot exclude the possibility that nicardipine may potentiate the action of oestradiol.

Oike *et al.* (1990) reported that two types of Ba channel current, namely DHP-sensitive (23 pS) and -resistant (8 pS) channel currents, could be recorded in the rabbit basilar artery. However, these two components could not be separated clearly on the basis of their current-voltage relationships, because current inactivation of the DHP-resistant component was not fast (Oike *et al.*, 1990). In the present experiments, 1 μM and 10 μM nicardipine inhibited the Ba current to about the same extent, suggesting that 1 μM nicardipine induced maximum or near-maximum inhibition of the Ba current. These results indicated that, by use of 1 μM nicardipine, the Ba current could be classified into nicardipine-sensitive and -resistant components.

We found that, with GDPβS in the pipette, oestradiol did

not significantly inhibit the Ba current. On the other hand, in the presence of GTPγS (30 μM or 100 μM), oestradiol (1 μM) produced a much more effective inhibition of the Ba current than that observed in the absence of GTPγS. Furthermore, the inhibitory action of oestradiol was prevented by pretreatment with PTX. These results strongly indicate that oestradiol modulates the voltage-dependent Ca channel via a PTX-sensitive GTP-binding protein. There have been several reports concerning the modulation of DHP-sensitive Ca channels by various receptor agonists in vascular cells (noradrenaline, Droogmans *et al.*, 1987; Benham & Tsien, 1988; Loirand *et al.*, 1990; ATP, Xiong *et al.*, 1991; histamine and angiotensin II, Oike *et al.*, 1992). However, the DHP-resistant Ca current was reported not to be coupled with noradrenaline receptors in the rabbit ear artery (Benham & Tsien, 1988). On the other hand, Pacaud *et al.* (1987) reported that noradrenaline enhanced the amplitude of the fast Ba current observed in the presence of Ca antagonist in rat portal vein. GDPβS prevented the inhibitory action of oestradiol, suggesting that both nicardipine-sensitive and -resistant Ca channels might be associated with GTP-binding protein.

It is known that 17α-oestradiol, an inactive form of oestrogen, has no effect on the genomic oestrogen response (Girasole *et al.*, 1992). On the other hand, 17α-oestradiol, but not oestrone (our unpublished observation) had similar vasorelaxant actions to 17β-oestradiol, though to a lesser extent in the present experiments. Furthermore tamoxifen, an antagonist of the cytosolic oestrogen receptor, did not change the action of oestrogen in porcine coronary artery (Han *et al.*, 1995). These results suggest that the acute inhibitory actions of oestradiol differ from its chronic actions via genomic pathways. Recently, Matsuda *et al.* (1993) reported that 17β-oestradiol binds specifically to the 185 kDa transmembrane glycoprotein encoded by a protooncogene, c-erbB2, and increases tyrosine-kinase activity. It is interesting that 17α-oestradiol was able to bind to the 185 kDa protein with a lower affinity than 17β-oestradiol, while oestrone bound only weakly to this protein. They also reported that the binding affinities of various oestrogens correlated well with their autophosphorylation activity via a tyrosine-kinase action. We cannot be sure from the present experiments whether 17β-oestradiol inhibits the voltage-dependent Ca channels through GTP-binding protein, as other receptor agonists do, or through some metabolic pathways dependent on GTP-binding protein. However, it seems likely that 17β-oestradiol acted on an oestrogen binding site and modulated Ca channel activity through GTP-binding protein, rather than acting on the Ca channel itself, since GDPβS did not prevent the actions of Ca antagonists (our unpublished observation). Our results clearly show that oestradiol did not act as a Ca antagonist on voltage-dependent Ca channels.

Chronic treatment of arterial tissues with oestradiol enhances endothelium-dependent relaxation induced by acetylcholine and adenosine diphosphate (rabbit aorta and carotid artery; Miller *et al.*, 1988). However, the acute action of oestradiol is not dependent on the presence of endothelium (Jiang *et al.*, 1991). In our experiments on the rabbit basilar artery, acute administration of a high concentration of oestradiol (100 μM) relaxed the artery through endothelium-dependent and -independent mechanisms, although the relaxation induced by lower concentrations of oestradiol was the same whether endothelium was present or not. Furthermore, a high concentration of oestradiol did not significantly inhibit the caffeine- or histamine-induced contraction evoked in Ca-free solution, indicating that oestradiol acts on the contraction mediated by Ca influx through the membrane. Therefore, we strongly postulate that oestradiol, especially at low concentrations, interacts directly with a pathway related to Ca influx through the membrane. When the rabbit basilar artery was superfused with Krebs solution (2.5 mM Ca), caffeine (20 mM) and histamine (10 μM) produced both phasic and tonic contractions. As nicardipine abolished the tonic contractions induced by both caffeine and histamine, we concluded that the inhibitory effects of oestradiol on the tonic contractions induced by caffeine and histamine were not related to an inhibition of receptor-operated ion channels.

In the present experiments, relatively high concentrations of oestradiol were required to inhibit the 30 mM K-induced contraction in comparison to the effect observed by others in rabbit coronary artery (40% inhibition in coronary artery at 1 μM oestradiol vs 20% inhibition in basilar artery at 1 μM; Jiang *et al.*, 1991 and this study). In the mesenteric artery, the effect of oestradiol on the 30 mM K contraction was quantitatively the same as in the basilar artery (our unpublished observation). However, when we used 17β-oestradiol dissolved in ethanol, as Jiang *et al.* (1991) did, a much stronger inhibition was recorded than with the water soluble type of 17β-oestradiol encapsulated into 2-hydroxypropyl-β-cyclodextrin. Therefore, we conclude that oestradiol probably relaxes basilar and coronary arteries with similar potencies. It is not yet clear why 17β-oestradiol in ethanol produced a larger relaxation than it did when in cyclodextrin. As ethanol (0.05 or 0.1%), but not cyclodextrin (0.065%), produced muscle relaxation in the rabbit coronary artery (Jiang *et al.*, 1991) and basilar artery (present experiments), ethanol might facilitate the actions of oestradiol. It is also possible that the effective concentration (free form) of oestradiol may well have been lower when cyclodextrin-encapsulated oestradiol was used than when ethanol was the solvent.

In the present experiments, we observed significant inhibitions by oestradiol of the peak Ba current only at concentrations of more than 1 μM, a hundred times higher than those needed to inhibit the 30 mM K contraction. This may be due to different conditions for current and tension recordings, such as temperature (25 vs 32°C) and ionic composition of the solution (10 mM Ba vs 2.5 mM Ca). Furthermore, oestradiol more strongly inhibited the Ba current at the end of pulses, suggesting that Ca entry due to continuous depolarization, such as that achieved with high K, would be more sensitive to oestradiol than the effect of a short depolarization.

We also found that oestradiol ($\geqslant$ 100 μM) had no effect on either the Ca-dependent or -resistant components of the K current. The findings that oestradiol produced no depolarization of the membrane supports the above result. It is worth while noting the discrepancy between the actions of oestradiol on the Ba current and the Ca-dependent K current. As we used electrodes with relatively high resistance (3–5 MΩ), Ca-buffering was not sufficiently induced by 0.1 mM EGTA. Indeed, we could not record an inward current following any membrane depolarization. Therefore, we speculated that the large Ca-dependent K current, recorded in the present experiments, might be caused by a sustained elevation in the Ca concentration in the cell rather than by a transient elevation in concentration through Ca channel activation. A sustained increase in Ca concentration might be due to the poor buffering conditions for Ca referred to above.

It is well known that postmenopausal oestrogen therapy reduces the risk of cardiovascular disease (Stampfer *et al.*, 1985; 1991; Bush *et al.*, 1987; Henderson *et al.*, 1988; Knopp, 1988). On the other hand, there is dispute as to the possible beneficial effects of oestrogen therapy on cerebrovascular disease. Paganini-Hill *et al.* (1988) reported that oestrogen treatment reduced the risk of stroke, while Stampfer *et al.* (1991) reported no such effect. However, Stampfer *et al.* (1991) did show that oestrogen therapy tended to reduce the risk of subarachnoid haemorrhage (adjusted relative risk 0.53). In *in vivo* experiments, Bass *et al.* (1990) demonstrated that acute administration of oestradiol increased cerebral blood flow in women. Furthermore, it has also been reported that a rapid elevation to 10 nM or so was correlated with an increase in cerebral blood flow within a few days of serum oestradiol being raised by hMG stimulation in fertile women (Shamma *et al.*, 1992). In the light of all this evidence, muscle relaxation through Ca channel inhibition by oestradiol may well lead to an increase in cerebral blood flow and contribute to the prevention of cerebrovascular disease.

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