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Hyperpolarisation of rat mesenteric endothelial cells by ATP-sensitive K^+ channel openers

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Abstract

Membrane potential responses of rat mesenteric endothelial cells were investigated in intact arteries using sharp electrodes. Levcromakalim, an activator of ATP-sensitive K^+ channels (K_{ATP}) induced concentration-dependent hyperpolarisation of the endothelial cells, which was reversible by glibenclamide and ciclazindol, inhibitors of K_{ATP}. Another K_{ATP} activator, diazoxide, also hyperpolarised the endothelial cells. Carbachol induced endothelial hyperpolarisation that was inhibited by combinations of apamin and charybdotoxin, but not Ba^{2+} and ouabain. Prior stimulation with levcromakalim inhibited carbachol-induced responses, and this inhibitory effect was also sensitive to glibenclamide. 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one (NS 1619), an activator of large conductance, Ca^{2+}-activated K^+ channels (BK_{Ca}), induced only small hyperpolarisations of the endothelial cells. Preincubation of tissues with 18α- or 18β-glycyrrhetinic acid, inhibitors of gap junction communication, increased the input resistance and depolarised the endothelial cells, and inhibited the hyperpolarising effect of levcromakalim. It is concluded that activation of K_{ATP} causes hyperpolarisation of rat mesenteric endothelial cells, probably through gap junctional transfer of smooth muscle hyperpolarisation, and that this may represent an important modulator of endothelial function.

Keywords: Mesenteric artery, rat; Endothelium; Levcromakalim; Hyperpolarisation; 18α-Glycyrrhetinic acid; Carbachol

1. Introduction

ATP-sensitive K^+ channels (K_{ATP}) have been found on vascular endothelial cells (Janigro et al., 1993; Katnik and Adams, 1997) and activation of these channels may hyperpolarize the endothelium. This would increase the gradient for Ca^{2+} influx (Lückhoff and Busse, 1990; Langheinrich et al., 1998) and may therefore modulate the release of endothelium-derived factors. Although it has been known for some time that direct activation of smooth muscle K_{ATP} causes vasorelaxation, recent studies have suggested that the vascular effects of K_{ATP} activation might also show endothelium dependence. Indeed, activation of endothelial K_{ATP} may contribute to shear stress-induced release of endothelium-derived nitric oxide in the rabbit aorta (Hutcheson and Griffith, 1994), and may also mediate the vasodilation induced by hyperosmolarity in coronary microvessels (Ishizaka and Kuo, 1997). Furthermore, adenosine dilates porcine coronary arteries by activating endothelial K_{ATP} and hence stimulating release of nitric oxide by the endothelium (Kuo and Chancellor, 1995). The vasorelaxation elicited in some tissues by K^+ channel activating agents (such as levcromakalim and diazoxide) may also be dependent on the release of endothelium-derived factors; for example in the dog epicardial coronary artery (Drieu La Rochelle et al., 1992), the rat isolated mesenteric artery (White and Hiley, 1997b) and the rat perfused mesenteric bed (Feleder and Adler-Graschinsky, 1997).

In rat mesenteric arteries, the vasorelaxant effect of levcromakalim is modulated by endothelium-derived nitric oxide (White and Hiley, 1998a) and possibly also endothelium-derived hyperpolarising factor (EDHF; White and Hiley, 1997b). However, it now also seems likely that K^+ channel activating agents may themselves modulate the release of endothelium-derived factors, and in particular EDHF (McCulloch and Randall, 1997; White and Hiley,

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1998b). Although the identity of EDHF remains to be established, and may well vary between vascular beds, very recent findings have suggested that endothelium-derived hyperpolarisation may result from conduction of the agonist-induced hyperpolarisation of the endothelium to the smooth muscle (Bény, 1997), either through gap junction communication (Chaytor et al., 1998; Dora et al., 1999, Yamamoto et al., 1999) or by the effect of K^+ ion efflux (Edwards et al., 1998), although subsequent findings render it unlikely that the latter mechanism is universal (Quignard et al., 1999). It is therefore possible that activation of endothelial K_{ATP} channels could induce smooth muscle hyperpolarisation and relaxation through analogous mechanisms, and indeed this has been hypothesised by other investigators (Ishizaka and Kuo, 1997).

The aim of the present study was to determine the effects of K^+ channel activating agents on the membrane potential of endothelial cells in an intact arterial preparation, and the interactions of these agents with the endothelium-dependent vasodilator, carbachol, at the level of the endothelium. This is of considerable importance, since previous electrophysiological studies into the actions of the K^+ channel activating agents on endothelial cells have been carried out in isolated cells, and therefore have been unable to take into account any interaction between the endothelium and the underlying smooth muscle cells.

2. Materials and methods

2.1. Myograph studies

Male Wistar rats (250–350 g; Tucks, Rayleigh, Essex, UK) were killed with an overdose of sodium pentobarbitone (120 mg kg^{-1}, i.p., Sagatal, Rhone Merieux, Harlow, Essex). The mesentery was removed and placed in ice-cold, gassed (95% O_2/5% CO_2) Krebs–Henseleit solution of the following composition (mM): NaCl, 118; KCl 4.7; $MgSO_4$, 1.2; KH_2PO_4 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; D-glucose, 10. Segments (2 mm in length) of third order branches of the superior mesenteric artery were removed and mounted in a Mulvany–Halpern myograph (Model 500A, Danish Myo-technology, Aarhus, Denmark) as described in White and Hiley (1997a). Vessels were maintained at 37°C in Krebs–Henseleit solution, containing indomethacin (10 μM) and bubbled with 95% O_2/5% CO_2. After equilibration, vessels were normalised to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mm Hg (Mulvany and Halpern, 1977). The mean vessel diameter under these conditions was 328 ± 12 μm ($n = 14$).

After normalisation, the endothelium was removed by rubbing the intimal layer of the vessels with a human hair. The successful removal of endothelium was then demonstrated by contracting vessels with methoxamine (10 μM) and adding carbachol (10 μM); a relaxation of $< 10\%$ was indicative of endothelial removal.

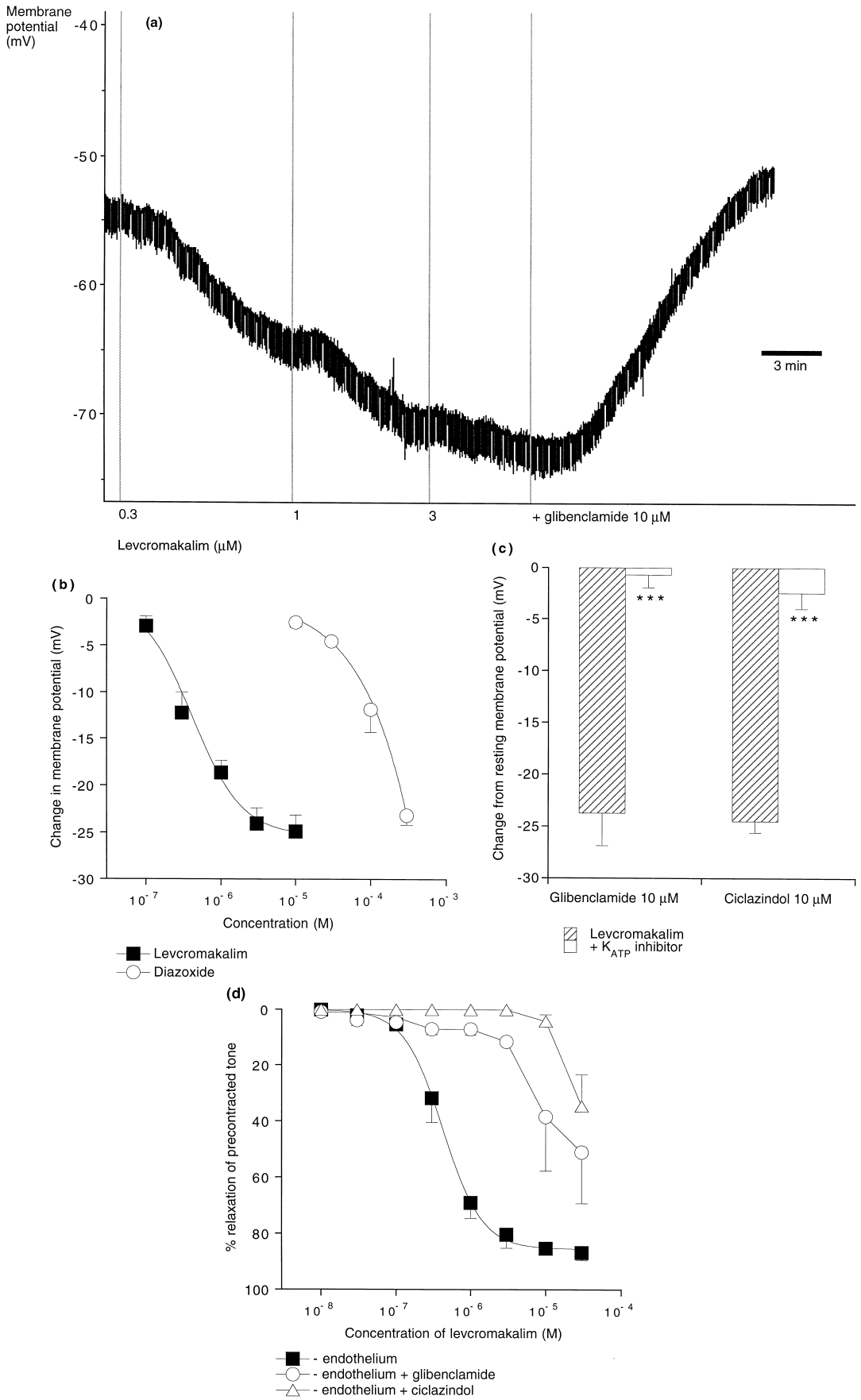
The vasorelaxant effect of levcromakalim was tested by precontracting vessels with methoxamine (10 μM) and then cumulatively adding levcromakalim. In some experiments, the K_{ATP} channel inhibitors, glibenclamide (10 μM) or ciclazindol (10 μM) were added for 30 min before precontraction of the vessels. The mean contraction induced by 10 μM methoxamine was 19.9 ± 2.2 mN in control vessels ($n = 10$), 14.9 ± 3.9 mN in vessels pre-incubated in the presence of glibenclamide ($n = 4$), and 17.2 ± 1.7 mN in the presence of ciclazindol ($n = 4$).

2.2. Electrophysiological studies

After the rat had been killed as described above, the main mesenteric artery was isolated and adherent fat and connective tissue removed. A 5-mm-long segment of the artery was cut and transferred to a 0.5-ml organ bath for electrophysiological experiments. The segment of artery was cut open longitudinally and pinned to the silicone rubber base of the organ bath with the intimal surface exposed.

Sharp electrodes were pulled from borosilicate glass capillaries (GC120F-10, Clark Electromedical Instruments, Reading, Berks.) using a Flaming–Brown type horizontal puller (P-80, Sutter Instruments, San Rafael, CA, USA) in order to produce electrodes with a long taper (tip resistance 60–100 MΩ when filled with 1 M KCl). The electrodes were connected to the headstage of an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA, USA) and electrical responses at the tip of the electrode were monitored by injecting current pulses (0.1 nA, duration 40 ms) and monitoring changes in measured potential on an oscilloscope (Nicolet 3091). After immersing the tip in the bath solution, the bridge balance and capacitance neutralisation controls were used to compensate for the

Fig. 1. (a) Original recording showing concentration-dependent hyperpolarisation of rat mesenteric endothelial cells by levcromakalim and reversal by glibenclamide. The vertical deflections on the recording are caused by current injections through the electrode (0.1 nA). Vertical lines denote addition of drugs at the concentrations indicated. (b) Concentration–response curves for the effect of levcromakalim and diazoxide on endothelial cell membrane potential. Values are shown as mean and vertical lines indicate S.E.M. The curves drawn are those obtained from the curve-fitting procedure. Levcromakalim, $n = 3$–18; diazoxide, $n = 3$. (c) Effect of glibenclamide (10 μM) and ciclazindol (10 μM) on the hyperpolarisation induced by levcromakalim (1–10 μM). Filled bars indicate control responses, and open bars denote responses in the presence of a K_{ATP} blocker. Vertical lines indicate S.E.M. $n = 4$ for all data. *** Indicates $P < 0.001$ compared to control in the absence of the K_{ATP} blocker. (d) Concentration–response curves for the relaxant effect of levcromakalim in myograph-mounted rat small mesenteric arteries in the absence of endothelium. The curves drawn are those obtained from the curve-fitting procedure. Control, $n = 10$; in the presence of glibenclamide or ciclazindol, $n = 4$.


(a)
Membrane potential (mV)
-40
-50
-60
-70
3 min
0.3
1
3
+ glibenclamide 10 μM
Levcromakalim (μM)
(b)
Change in membrane potential (mV)
0
-5
-10
-15
-20
-25
-30
10- 7
10- 6
10- 5
10- 4
10- 3
Concentration (M)
Levcromakalim
Diazoxide
(c)
Change from resting membrane potential (mV)
0
-5
-10
-15
-20
-25
-30
***
***
Glibenclamide 10 μM
Ciclazindol 10 μM
Levcromakalim
+ K ATP inhibitor
(d)
% relaxation of precontracted tone
0
20
40
60
80
100
10- 8
10- 7
10- 6
10- 5
10- 4
Concentration of levcromakalim (M)
- endothelium
- endothelium + glibenclamide
- endothelium + ciclazindol

electrode resistance and capacitance and the measured potential was set to zero. The electrode, viewed under a light microscope, was then advanced slowly towards the intimal surface of the arterial segment at an angle of approximately 40° using a Huxley type manipulator. Under these conditions, characteristic small deflections in voltage were seen as the electrode made contact with the surface of an endothelial cell, and when this was observed the 'buzz' button of the amplifier was used to attempt to penetrate the cell membrane. Successful impalements were seen as a sudden change in voltage to a stable, negative level, and cells were generally left for 5 min after impalement before addition of drugs. The input resistance of impaled cells was estimated through Ohm's law by observing the change in membrane potential induced by injecting current pulses (0.1 nA, duration 400 ms every 5 s). The duration of impalements was found to be very variable, ranging from less than 1 min to several hours.

If electrodes in contact with an endothelial cell were advanced further, a transient negative deflection was observed as the electrode moved through the cell, and the measured potential returned to zero. Due to the long fine taper of the electrode tip and the relatively shallow angle of approach, advancing the electrode still further never led to impalement of smooth muscle cells in this study. Instead, the elastic membrane caused the electrode to bend, which was visible under the light microscope, and also as very large negative deflections on the oscilloscope as the electrode resistance became very high. Advancing the electrode further beyond this point invariably caused it to break. Further evidence that the impaled cells were endothelial cells comes from the observation that voltage displacements and stable impalements characteristic of these cells were never observed if the intimal surface of the preparation was rubbed with a moistened cotton bud prior to experimentation. We were also able to functionally characterise the impaled cells as being endothelial cells, since it has recently been shown that carbachol-induced hyperpolarisation of smooth muscle cells is inhibited by a combination of barium chloride and ouabain, whereas the hyperpolarising effect of carbachol on the endothelium is resistant to this treatment (Edwards et al., 1998).

Upon removal of an electrode from an impaled cell, whether carried out manually or due to loss of the impalement, the membrane potential and electrode resistance were examined in order to ensure that there were no changes due to voltage offsets or blocking of the electrode tip during recording.

2.3. Drugs

Methoxamine, carbachol, L-N^G-nitroarginine methyl ester (L-NAME), barium chloride, apamin and charybdotoxin (all from Sigma) and ciclazindol (generously provided by Professor M.L.J. Ashford, Department of Biomedical Sciences, University of Aberdeen) were dissolved in distilled water. Indomethacin (Sigma) was dissolved in 5% (w/v) $NaHCO_3$ solution. Diazoxide (Sigma) was dissolved in methanol. Levcromakalim (a gift of SmithKline Beecham) was dissolved in ethanol. Glibenclamide, 18β-glycyrrhetinic acid (Aldrich) and 18α-glycyrrhetinic acid (Sigma) were dissolved in dimethyl sulphoxide. Ouabain (Sigma) was dissolved directly in the Krebs–Henseleit solution in order to reach the desired concentration.

2.4. Statistical analysis

Relaxation responses in myograph experiments are expressed as the percentage relaxation of the tone induced by methoxamine. Data are given as the mean ± S.E.M. EC_{50} values for cumulative responses were obtained from individual concentration–response curves by fitting the data to the logistic equation:

$$E = \frac{E_{max} \cdot A^{n_H}}{EC_{50}^{n_H} + A^{n_H}}$$

where E is the effect (reduction in tone or change in membrane potential), A is the concentration of the agonist, E_{max} is the maximum effect, n_H is the slope function and EC_{50} the concentration of relaxant giving half the maximal relaxation. The curve fitting was carried out using KaleidaGraph (Synergy Software, Reading, PA, USA) running on a Macintosh computer. Comparison of all data was by Student's unpaired *t*-test. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of K^+ channel activating agents on the membrane potential of rat mesenteric endothelial cells

In 60 endothelial cells, the mean resting membrane potential was -52.6 ± 0.7 mV, with a mean input resistance of 17.3 ± 1.2 MΩ. Levcromakalim caused concentration-dependent hyperpolarisation of rat mesenteric endothelial cells (Fig. 1a,b), and this was reversed by the addition of 10 μM glibenclamide (Fig. 1a,c) or 10 μM ciclazindol (Fig. 1c), two inhibitors of K_{ATP} channel opening. Hyperpolarisation of the endothelial cells was also observed in the presence of another K_{ATP} activating agent, diazoxide (Fig. 1b). The levcromakalim-induced hyperpolarisation exhibited a maximum response of 25.1 ± 1.5 mV and an EC_{50} of 0.36 ± 0.07 μM ($n = 3$–18; parameters derived from the curve-fitting procedure).

3.2. Relaxant effect of levcromakalim in rat mesenteric arteries denuded of endothelium

In myograph-mounted rat small mesenteric arteries denuded of endothelium, levcromakalim induced concentra-

tion-dependent relaxations of methoxamine-induced tone, with a maximum relaxation of 80.7 ± 2.4% and an EC_{50} of 0.36 ± 0.04 μM. The relaxation to levcromakalim was inhibited by the presence of either 10 μM glibenclamide or 10 μM ciclazindol (Fig. 1d).

3.3. Effect of K^+ channel blocking agents on carbachol-induced hyperpolarisation of rat mesenteric endothelial cells

In the presence of L-NAME, carbachol caused hyperpolarisation of rat mesenteric endothelial cells (Fig. 2a). Although a combination of 50 μM barium chloride and 1 mM ouabain caused depolarisation of the endothelial cells (by 5.7 ± 0.6 mV, $n = 7$), the response to carbachol was unaffected. In the presence of a combination of apamin (50 nM) with charybdotoxin (50 nM), the hyperpolarising response to carbachol was abolished, and small depolarisations to this agent were observed (Fig. 2a). These data are described quantitatively in Fig. 2b.

3.4. Effect of levcromakalim and glibenclamide on carbachol-induced hyperpolarisation of rat mesenteric endothelial cells

Fig. 3a shows that the endothelial cell hyperpolarisation induced by carbachol is inhibited by prior stimulation of the endothelium with levcromakalim (1–10 μM, causing hyperpolarisation from −49.3 ± 0.8 mV to −69.2 ± 2.1 mV; $n = 6$).

In rat mesenteric endothelial cells stimulated with levcromakalim (1–10 μM, causing hyperpolarisation from −51.2 ± 2.6 mV to −62.8 ± 5.6 mV; $n = 4$), addition of 10 μM glibenclamide reversed the hyperpolarising effect (to −50.2 ± 2.3 mV; $n = 4$) of the K^+ channel activator, and also prevented its inhibitory effect on a subsequent carbachol-induced response (Fig. 3b). Glibenclamide alone had no effect on the response to carbachol. These data are presented in quantitative form in Fig. 3c.

3.5. Effect of the BK_{ca} activator, NS 1619, on rat mesenteric endothelial cells

Addition of NS 1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-5-(trifluoromethyl)- 2 *H*-benzimidazol-2-one; 10 or 30 μM) to resting rat mesenteric endothelial cells (mean membrane potential −53.1 ± 1.7 mV; $n = 7$) evoked only small hyperpolarisations (10 μM, 2.5 ± 0.6 mV; 30 μM, 2.6 ± 1.2 mV, both $n = 5$).

3.6. Effect of 18α-glycyrrhetinic acid on the membrane potential and input resistance of resting and levcromakalim-stimulated rat mesenteric endothelial cells

Addition of 18α-glycyrrhetinic acid (50 μM) to resting endothelial cells caused a large depolarisation (onset typically after 5 min, from a resting membrane potential of −51.2 ± 2.0 mV to −27.9 ± 3.7 mV; $n = 8$). This was preceded by an earlier phase (3–5 min) of membrane hyperpolarisation (by 4.2 ± 1.0 mV; $n = 8$). The input

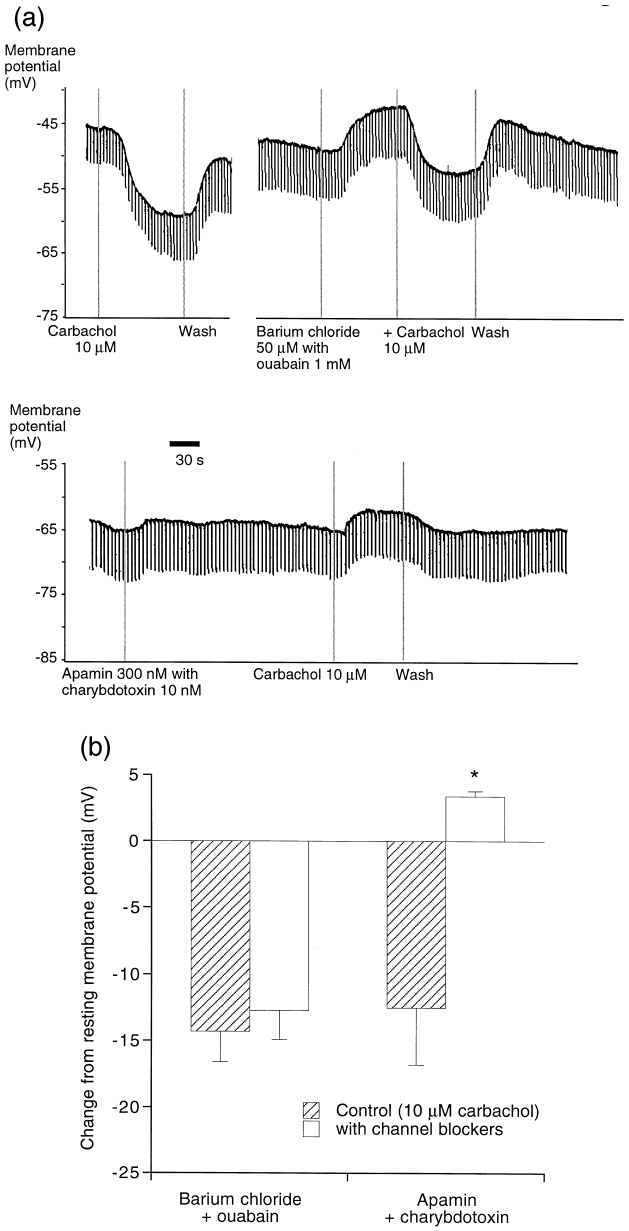
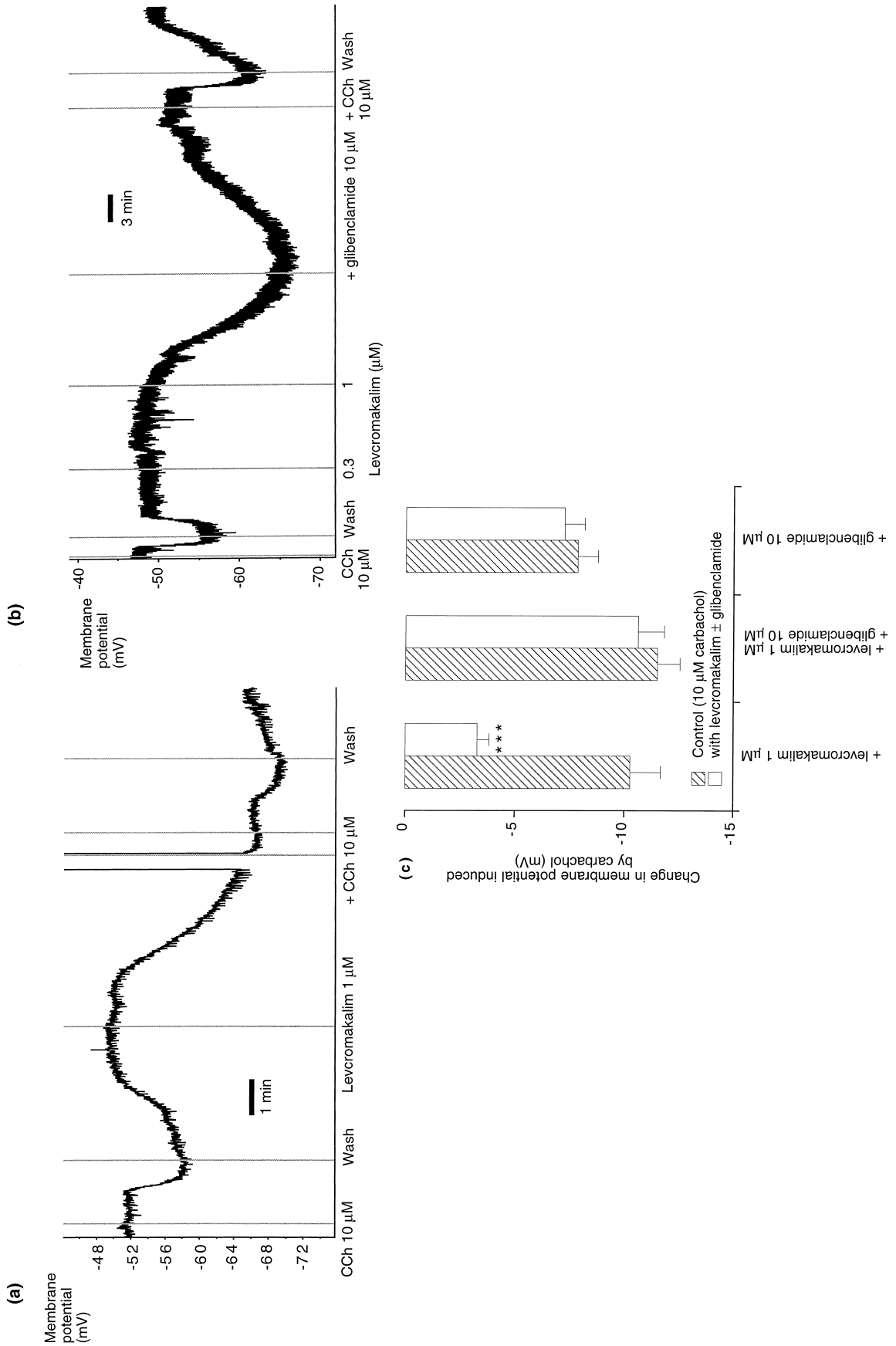


Fig. 2. (a) Original recording showing the hyperpolarising effect of carbachol on rat mesenteric endothelial cells, and the effect of preincubation with combinations of either $BaCl_2$ (50 μM) and ouabain (1 mM), or apamin and charybdotoxin (both 50 nM). The vertical deflections on the recording are caused by current injections through the electrode (0.1 nA). (b) Quantitative representation of the effects of barium chloride/ouabain or apamin/charybdotoxin on the hyperpolarisation of endothelial cells induced by carbachol (10 μM). Hatched bars indicate control responses, and open bars denote responses in the presence of inhibitors. Vertical lines indicate S.E.M. Control and test experiments for $BaCl_2$/ouabain, $n = 7$; control and test experiments for apamin/charybdotoxin, $n = 4$. * Indicates $P < 0.05$ compared to control in the absence of channel blockers.


(a)
Membrane potential (mV)
-48
-52
-56
-60
-64
-68
-72
1 min
CCh 10 µM
Wash
Levcromakalim 1 µM
+ CCh 10 µM
Wash
(b)
Membrane potential (mV)
-40
-50
-60
-70
3 min
CCh 10 µM
Wash
0.3
1
Levcromakalim (µM)
+ glibenclamide 10 µM
+ CCh 10 µM
Wash
(c)
Change in membrane potential induced by carbachol (mV)
0
-5
-10
-15
***
Control (10 µM carbachol) with levcromakalim ± glibenclamide
+ levcromakalim 1 µM
+ levcromakalim 1 µM + glibenclamide 10 µM
+ glibenclamide 10 µM

resistance of the cells also increased with a similar time course (from a resting resistance of 17.5 ± 1.8 MΩ to 228 ± 44 MΩ; $n = 8$). A typical trace is shown in Fig. 4a. The membrane potential of cells exposed to 18α-glycyrrhetinic acid was very unstable (mean lifespan of impalement after addition of 18α-glycyrrhetinic acid was 4.8 ± 1.2 min, $n = 8$), and it was generally necessary to re-establish impalements in order to evaluate the effects of 18α-glycyrrhetinic acid.

The membrane potential of endothelial cells exposed to levcromakalim (1–10 μM) was -69.8 ± 2.0 mV (from a resting membrane potential of -53.7 ± 2.0 mV; $n = 4$). Subsequent addition of 50 μM 18α-glycyrrhetinic acid depolarised the cells to a mean membrane potential of -48.4 ± 3.1 mV ($n = 4$), with little initial hyperpolarisation (1.5 ± 1.1 mV; $n = 4$). The depolarisation was accompanied by an increase in input resistance from 21.5 ± 3.6 MΩ to 258 ± 80 MΩ ($n = 4$; Fig. 4b). It should be noted that the membrane potential of levcromakalim-stimulated mesenteric endothelial cells was significantly ($P < 0.01$) more negative than that of unstimulated cells in both the absence and presence of 18α-glycyrrhetinic acid. The effects of 18α-glycyrrhetinic acid on endothelial cell input resistance and membrane potential are shown in Fig. 4c,d, respectively.

3.7. Effect of preincubation of vessels with 100 μM 18α-glycyrrhetinic acid or 30 μM 18β-glycyrrhetinic acid on levcromakalim-induced hyperpolarisation

In additional experiments, impalements of 15 rat mesenteric endothelial cells gave a resting membrane potential of -52.8 ± 1.1 mV and input resistance of 17.8 ± 1.7 MΩ. Fig. 5a shows that these cells were significantly hyperpolarised by 10 μM levcromakalim (from -52.0 ± 1.0 mV to -81.5 ± 2.3 mV; $n = 5$).

Following preincubation for 30–60 min with 100 μM 18α-glycyrrhetinic acid, subsequent impalements showed that endothelial cells had depolarised to -37.0 ± 4.5 mV, with an approximately 20-fold increase in input resistance ($n = 8$; Fig. 5b). In three cells which were impaled for sufficiently long times that responses to levcromakalim (10 μM) could be established, the effects of the K_{ATP} activator were essentially abolished (membrane potential -26.0 ± 4.6 mV, in the presence of 10 μM levcromakalim -25.5 ± 5.1 mV; $n = 3$; see Fig. 5c).

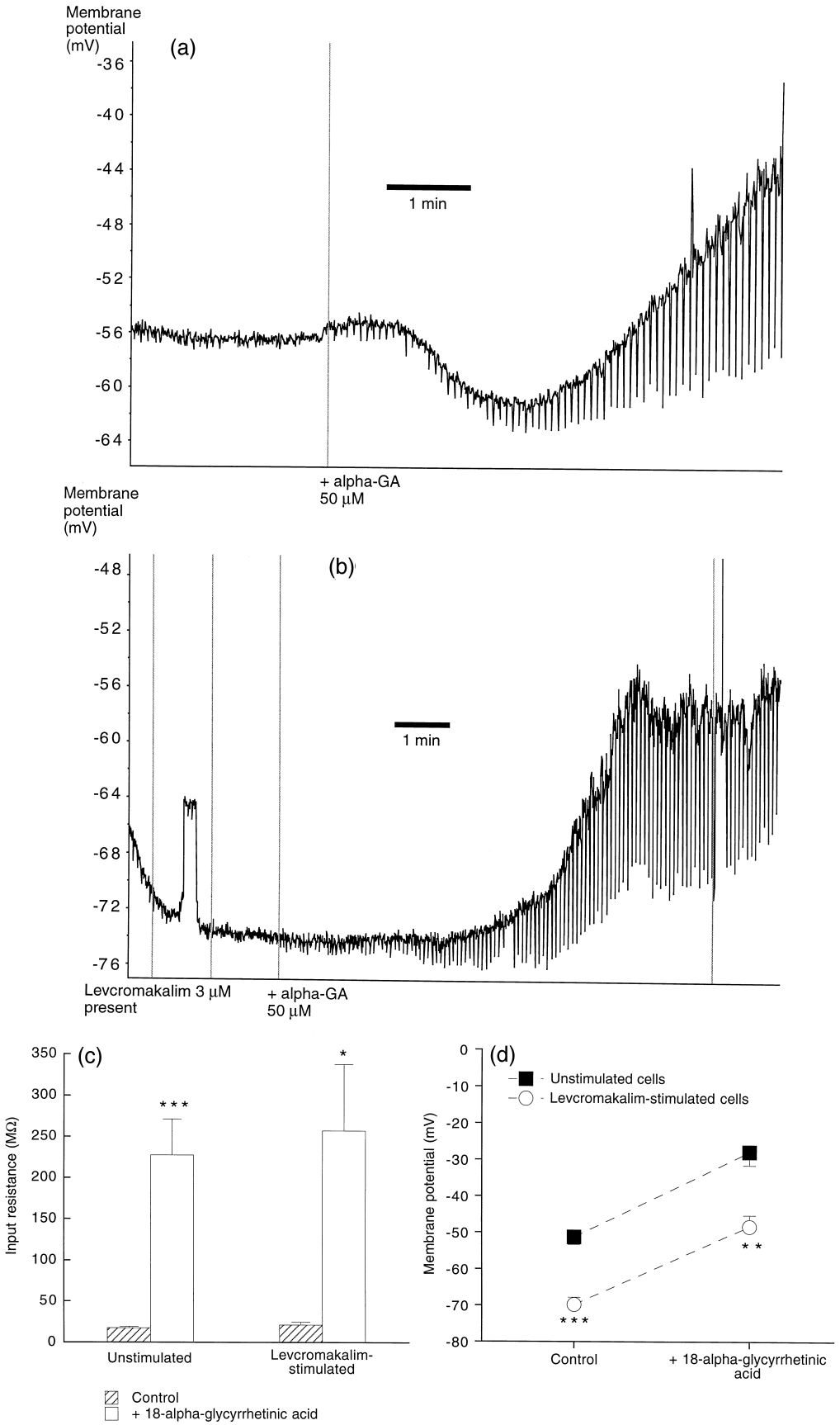
18β-glycyrrhetinic acid (30 μM), another inhibitor of gap junctions, also depolarised rat mesenteric endothelial cells (from a resting potential of -50.3 ± 2.6 mV to -31.7 ± 5.7 mV, $n = 5$) and caused approximately a 17-fold increase in input resistance (from 12.2 ± 2.6 MΩ to 211 ± 33 MΩ; $n = 5$; Fig. 5b). In three cells in which responses to levcromakalim could be evaluated, it caused only modest hyperpolarisation (from a membrane potential of -26.9 ± 3.7 mV in the presence of 18β-glycyrrhetinic acid to -31.1 ± 5.3 mV in the additional presence of 10 μM levcromakalim; $n = 3$; Fig. 5c).

4. Discussion

This study shows that activation of K_{ATP} causes hyperpolarisation of endothelial cells in an intact arterial preparation, and that this may occur through gap junctional transfer of smooth muscle hyperpolarisation to the endothelium.

Cholinergic agonists have been known for some time to cause hyperpolarisation of endothelial cells, and this occurs through inositol trisphosphate-dependent release of intracellular Ca^{2+} stores and subsequent Ca^{2+} influx (Chen and Cheung, 1992; Marchenko and Sage, 1993). The elevated intracellular Ca^{2+} levels then cause activation of Ca^{2+}-activated K^+ channels (Marchenko and Sage, 1996). Endothelium-dependent vasodilators may also cause hyperpolarisation of vascular smooth muscle through release of endothelium-derived hyperpolarising factor (EDHF; see Cohen and Vanhoutte, 1995, for review). In small arteries such as the rat mesenteric artery and rat hepatic artery, EDHF-mediated responses are abolished by a combination of apamin and charybdotoxin, whereas each toxin alone is ineffective (Waldron and Garland, 1994; Zygmunt and Hogestatt, 1996; White and Hiley, 1997a). It was widely assumed that K^+ channel blocking agents inhibited EDHF through an action at the level of the smooth muscle, however we previously provided evidence that these agents might also inhibit EDHF by acting on the endothelium (White and Hiley, 1997a). The present study shows that apamin and charybdotoxin do inhibit carbachol-induced

Fig. 3. (a) Original recording showing the inhibitory effect of levcromakalim prestimulation of rat mesenteric endothelial cells on subsequent responses to carbachol. The vertical deflections on the recording are caused by current injections through the electrode (0.1 nA). Vertical lines denote addition of drugs at the concentrations indicated. The impalement was lost transiently prior to the second carbachol response. (b) Original trace showing the reversal by glibenclamide (10 μM) of the inhibitory effect of pre-exposure to levcromakalim on subsequent responses to carbachol. The vertical deflections on the recording are caused by current injections through the electrode (0.1 nA). Vertical lines denote addition of drugs at the concentrations indicated. (c) Quantitative summary of the effects of levcromakalim and/or glibenclamide on the hyperpolarisation induced by carbachol (10 μM). Hatched bars indicate control responses, and open bars denote responses in the presence of the K_{ATP} modulators. Vertical lines indicate S.E.M. Control and test experiments for levcromakalim alone, $n = 6$; control and test experiments for levcromakalim and glibenclamide, $n = 5$; control and test experiments for glibenclamide only, $n = 4$. *** Indicates $P < 0.001$ compared to control in the absence of K_{ATP} modulators.


Membrane potential (mV)
(a)
-36
-40
-44
-48
-52
-56
-60
-64
1 min
+ alpha-GA 50 μM
Membrane potential (mV)
(b)
-48
-52
-56
-60
-64
-68
-72
-76
1 min
Levcromakalim 3 μM present
+ alpha-GA 50 μM
(c)
Input resistance (MΩ)
350
300
250
200
150
100
50
0
***
*
Unstimulated
Levcromakalim-stimulated
Control
+ 18-alpha-glycyrrhetinic acid
(d)
Membrane potential (mV)
0
-10
-20
-30
-40
-50
-60
-70
-80
Unstimulated cells
Levcromakalim-stimulated cells
***
**
Control
+ 18-alpha-glycyrrhetinic acid

hyperpolarisation by acting on the endothelium, and thus confirm more recent findings by Doughty et al. (1999) in the rat mesenteric artery and Edwards et al. (1998) in the rat hepatic artery. Carbachol-induced hyperpolarisation of mesenteric endothelial cells was not affected by a combination of barium ions and ouabain found by Edwards et al. (1998) to inhibit EDHF-mediated hyperpolarisation of rat mesenteric smooth muscle cells, thus providing functional evidence that the cells investigated in the present study were indeed vascular endothelial cells.

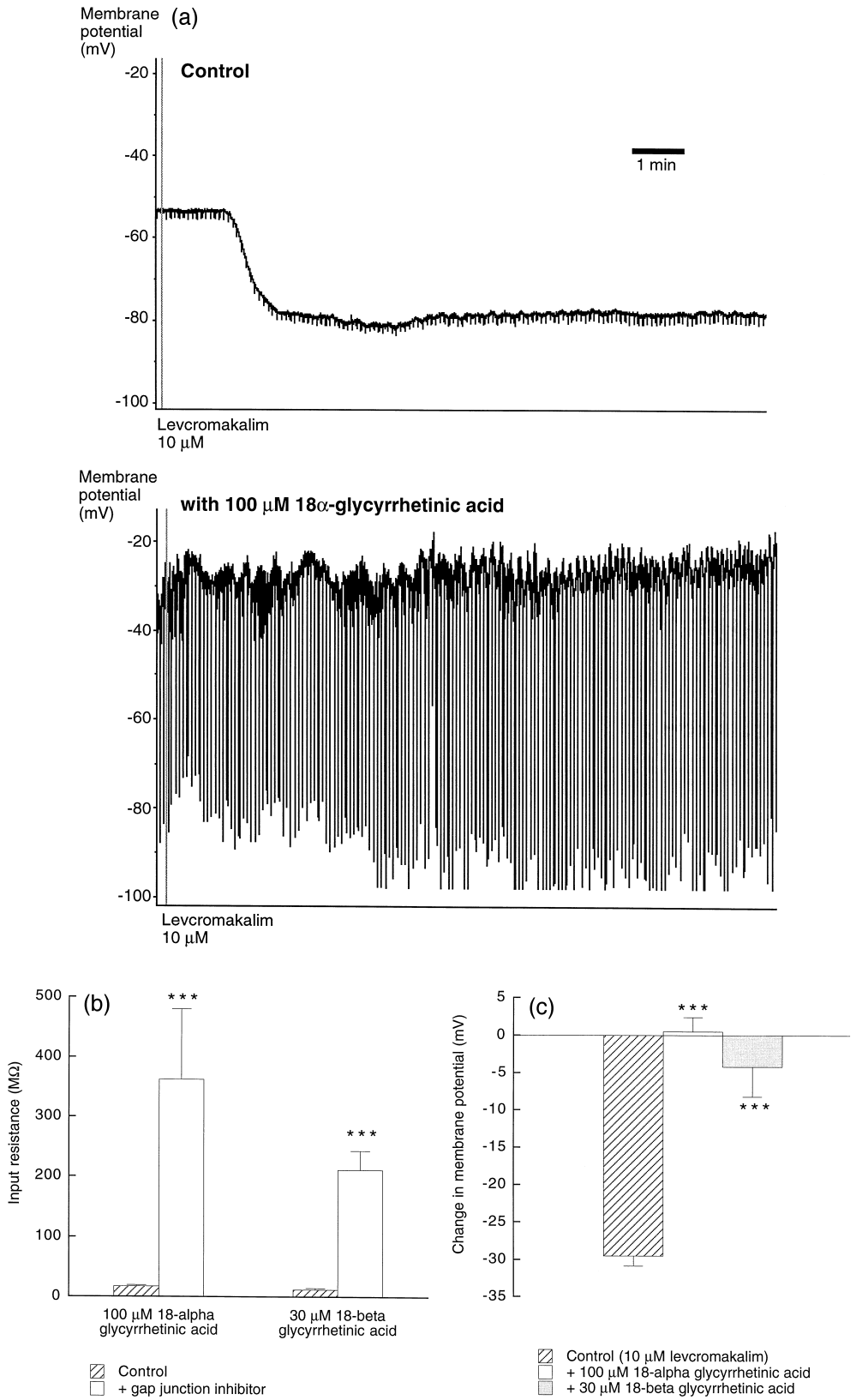
The K^+ channel activators, levcromakalim and diazoxide, both caused concentration-dependent hyperpolarisation of rat mesenteric endothelial cells, and the levcromakalim-induced hyperpolarisation was inhibited by the K_{ATP} channel blocking agents glibenclamide and ciclazindol. It is of note that the potency of levcromakalim in causing hyperpolarisation of the endothelial cells was very similar to its potency in inducing relaxation of rat small mesenteric arteries, which was also glibenclamide- and ciclazindol-sensitive. This suggests that hyperpolarisation of the endothelium may occur simultaneously with smooth muscle relaxation, therefore raising the possibility that endothelial hyperpolarisation may contribute to the relaxant effects of the K^+ channel activating agents. Recent findings have provided evidence that the relaxant effects of the K^+ channel openers may show endothelium-dependence (Feleder and Adler-Graschinsky, 1997; White and Hiley, 1997b; Tanaka et al., 1999). However, it is not clear whether this involves the release of endothelium-derived factors (Kuo and Chancellor, 1995; Feleder and Adler-Graschinsky, 1997; Hassessian et al., 1993), or direct coupling of endothelial hyperpolarisation to the underlying smooth muscle (Bény, 1997; Ishizaka and Kuo, 1997). It is also possible that the influence of the endothelium on the effects of K^+ channel openers reflects indirect interactions between the vasodilators (McCulloch and Randall, 1997; White and Hiley, 1998a) rather than direct actions of K^+ channel openers on the endothelial cells.

The present study shows that the hyperpolarising effect of carbachol is diminished in endothelial cells that have been pre-exposed to levcromakalim. The hyperpolarisation and inhibition of the carbachol response caused by levcromakalim are K_{ATP}-dependent, since both are blocked by glibenclamide, which alone had no effect on the response to carbachol. These observations confirm our previous findings in myograph-mounted rat small mesenteric artery (White and Hiley, 1998b) which led us to propose that the inhibitory effect of K_{ATP} channel openers on EDHF-mediated relaxation to carbachol was due to an action on the endothelium rather than the smooth muscle. Whether EDHF activity is due to electrical coupling through gap junctions of endothelial to smooth muscle cells (Bény and Pacicca, 1994; Bény, 1997; Chaytor et al., 1998; Taylor et al., 1998; Dora et al., 1999) or efflux of K^+ ions through endothelial channels (Edwards et al., 1998), it is clear that the inhibition by levcromakalim of carbachol-induced hyperpolarisation of the endothelial cell must lead to inhibition of subsequent, EDHF-mediated responses, such as vasorelaxation. It seems reasonable to speculate that it is the hyperpolarising effect of levcromakalim which inhibits subsequent responses to carbachol, although whether this is due to a change in driving force for K^+ entry through the channels activated by carbachol, or a change in opening probability of these channels, or even hyperpolarisation-induced inhibition of inositol trisphosphate-dependent mechanisms (Ito et al., 1991) is at present unclear.

It is now evident vascular endothelial cells may be functionally connected to the underlying smooth muscle cells through myoendothelial gap junctions. Indeed, it has recently been shown that the endothelium-dependent hyperpolarisation of vascular smooth muscle induced by cholinergic agonists may result from their hyperpolarising effects on the endothelium being conducted to the smooth muscle in a gap junction-dependent fashion (Chaytor et al., 1998; Taylor et al., 1998; Dora et al., 1999; Yamamoto et al., 1999). It is therefore equally possible that electrical responses in the smooth muscle may be conducted to the endothelial cells (Bény and Pacicca, 1994; Yamamoto et al., 1998), although electrical communication between the endothelium and smooth muscle may be unidirectional (Welsh and Segal, 1998). The present study used two means to test whether the hyperpolarisation induced by levcromakalim is due to a direct effect on the endothelium or an indirect effect (presumably gap junction communication) via the smooth muscle. Firstly, NS 1619, used at concentrations which have previously been shown to acti-

Fig. 4. (a) Original recording showing the effect of 50 μM 18α-glycyrrhetinic acid on rat mesenteric endothelial cells. The vertical deflections on the recording are caused by current injections through the electrode (0.1 nA). Vertical lines denote addition of drugs at the concentrations indicated. (b) Recording of the effect of 18α-glycyrrhetinic acid on rat mesenteric endothelial cells pre-exposed to levcromakalim (1–10 μM). The vertical deflections on the recording are caused by current injections through the electrode (0.1 nA). Vertical lines denote addition of drugs at the concentrations indicated. (c) Quantitative summary of the effects of 18α-glycyrrhetinic acid on the apparent input resistance of rat mesenteric endothelial cells. Hatched bars indicate control responses, and open bars denote responses in the presence of 18α-glycyrrhetinic acid. Vertical lines indicate S.E.M. Control and test experiments for unstimulated cells, $n = 8$; control and test experiments for levcromakalim-stimulated cells, $n = 4$. * Indicates $P < 0.05$ and $^{***}P < 0.001$ compared with control cells not exposed to 18-α-glycyrrhetinic acid. (d) Effect of 18α-glycyrrhetinic acid on the membrane potential of unstimulated cells (filled symbols) or levcromakalim-stimulated cells (open symbols). Values are shown as mean, and vertical lines indicate S.E.M. Control and test experiments for unstimulated cells, $n = 8$; control and test experiments for levcromakalim-stimulated cells, $n = 4$. ** Indicates $P < 0.01$ and $^{***}P < 0.001$ compared with control cells not exposed to levcromakalim.

vate BK_{Ca} channels in vascular smooth muscle (Holland et al., 1996) and cause iberiotoxin-sensitive relaxation of rat small mesenteric arteries (White and Hiley, 1998c), caused only small hyperpolarisations in mesenteric endothelial cells. If the response to levcromakalim were mediated through electrical transfer from the smooth muscle, then



NS 1619 would equally be expected to be a potent hyperpolarising agent on the endothelium. It is notable, however, that responses to NS 1619 showed considerable variation between vessels; in five cells exposed to 30 μM NS 1619, for example, two cells responded with hyperpolarisations of around 5 mV, whilst the other three hyperpolarised by less than 2 mV. The reason for this variation is unclear but may, for example, indicate some inhomogeneity of myoendothelial gap junction communication in this tissue.

In the second set of experiments, both 18α- and 18β-glycyrrhetinic acid, inhibitors of gap junctional communication (Davidson and Baumgarten, 1988), were found to cause an increase in input resistance and depolarisation of the endothelial cells, characteristic of an inhibition of electrical communication (Yamamoto et al., 1998). When 50 μM 18α-glycyrrhetinic acid was added after cells had been exposed to levcromakalim, however, the mean membrane potential of cells in the presence of levcromakalim was still significantly more negative than control endothelial cells, suggesting that inhibition of gap junction communication did not reverse the effect of levcromakalim. Nevertheless, preincubation of vessels with either 30 μM 18β-glycyrrhetinic acid or 100 μM 18α-glycyrrhetinic acid, which again caused significant depolarisation of endothelial cells as well as increasing the apparent input resistance of the cells, almost abolished the hyperpolarising effect of a subsequent addition of levcromakalim. It therefore seems likely that a relatively small proportion of the gap junction population needs to remain functional for significant transfer of hyperpolarisation from smooth muscle to endothelium.

The present study therefore provides evidence that K_{ATP} activating agents cause hyperpolarisation of endothelial cells in an intact vascular preparation, which may occur through gap junction-mediated transfer of smooth muscle hyperpolarisation to the endothelium. This may interfere with the effect of other endothelium-dependent vasoactive factors and may therefore represent an important modulator of endothelial cell function.

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Fig. 5. (a) Original recording showing the effect of 10 μM levcromakalim on the membrane potential in control endothelial cells (upper panel) and cells incubated for 30–60 min with 100 μM 18α-glycyrrhetinic acid (lower panel). The vertical deflections on the recording are caused by current injections through the electrode (0.1 nA). Vertical lines denote addition of drugs at the concentrations indicated. (b) Quantitative representation of the effects of preincubation for 30–60 min with 18α- or 18β-glycyrrhetinic acid on the apparent input resistance of rat mesenteric endothelial cells. Hatched bars indicate control responses, and open bars denote responses in the presence of gap junction inhibitor. Vertical lines indicate S.E.M. Control, $n = 15$ (18α-glycyrrhetinic acid) and $n = 5$ (18β-glycyrrhetinic acid). Test experiments, $n = 8$ (18α-glycyrrhetinic acid) and $n = 5$ (18β-glycyrrhetinic acid). *** Indicates $P < 0.001$ compared with control cells. (c) Effect of gap junction inhibitors on hyperpolarisation of rat mesenteric endothelial cells induced by 10 μM levcromakalim. Bars indicate control responses to 10 μM levcromakalim (hatched bars), and responses in tissues preincubated with either 100 μM 18α-glycyrrhetinic acid (open bars) or 30 μM 18β-glycyrrhetinic acid (stippled bars). Vertical lines indicate S.E.M. Control, $n = 5$; with 100 μM 18α- or 30 μM 18β-glycyrrhetinic acid, $n = 3$. *** Indicates $P < 0.001$ compared with control responses to levcromakalim.

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