Substance P and bradykinin activate different types of K_{Ca} currents to hyperpolarize cultured porcine coronary artery endothelial cells

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1. Substance P and bradykinin, endothelium-dependent vasodilators of pig coronary artery, trigger in endothelial cells a rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and membrane hyperpolarization. The aim of the present study was to determine the type of Ca^{2+}-dependent K^+ (K_{Ca}) currents underlying the endothelial cell hyperpolarization.

2. The substance P-induced increase in $[Ca^{2+}]_i$ was 30% smaller than that induced by bradykinin, although the two peptides triggered a membrane hyperpolarization of the same amplitude. The two agonists evoked a large outward K^+ current of the same conductance at maximal stimulation. Agonists applied together produced the same maximal current amplitude as either one applied alone.

3. Iberiotoxin (50 nM) reduced by about 40% the K^+ current activated by bradykinin without modifying the substance P response. Conversely, apamin (1 μM) inhibited the substance P-induced K^+ current by about 65%, without affecting the bradykinin response. Similar results were obtained on peptide-induced membrane hyperpolarization.

4. Bradykinin-induced, but not substance P-induced, endothelium-dependent relaxation resistant to N^G-nitro-L-arginine and indomethacin was partly inhibited by 3 μM 17-octadecynoic acid (17-ODYA), an inhibitor of cytochrome P450 epoxygenase. Similarly, the bradykinin-induced K^+ current was reduced by 17-ODYA.

5. Our results show that responses to substance P and bradykinin result in a hyperpolarization due to activation of different K_{Ca} currents. A current consistent with the activation of large conductance (BK_{Ca}) channels was activated only by bradykinin, whereas a current consistent with the activation of small conductance (SK_{Ca}) channels was stimulated only by substance P. The observation that a similar electrical response is produced by different pools of channels implies distinct intracellular pathways leading to K_{Ca} current activation.

Endothelial cells play a major role in the local control of the vascular tone by producing powerful vasoactive agents, like nitric oxide (NO), prostacyclin, and the still unidentified endothelium-derived hyperpolarizing factor (EDHF). The activation of endothelial cells by circulating mediators leads to an intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) rise, due to Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} influx from the extracellular space (Himmel *et al.* 1993). In many cases, this effect is associated with a membrane hyperpolarization (for review see Nilius *et al.* 1997), which plays an important role in the production of endothelial vasoactive substances by increasing the driving force for Ca^{2+} entry (Lückhoff & Busse, 1990).

The $[Ca^{2+}]_i$ rise seems to be directly responsible for the membrane hyperpolarization, as shown by the hyperpolarizing effect of Ca^{2+} ionophores (Colden-Stanfield *et al.* 1990; von der Weid & Bény, 1992), which strongly suggests the involvement of Ca^{2+}-dependent K^+ (K_{Ca}) channels. Furthermore, agonist-induced membrane hyperpolarization also results from the activation of K_{Ca} channels (for review see Nilius *et al.* 1997). Single channel analysis indicated that many types of K_{Ca} channels are stimulated by various agonists in endothelial cells (Nilius *et al.* 1997); however, the relative contribution of the different K^+ channels in endothelial cell hyperpolarization has been poorly studied.

The two peptides substance P and bradykinin are both endothelium-dependent vasodilators of the pig coronary artery, producing NO and EDHF as relaxing agents (Pacicca *et al.* 1992). In coronary artery endothelial cells, both peptides produced a transient hyperpolarization associated with an increase of $[Ca^{2+}]_i$ (Brunet & Bény, 1989; Sharma & Davis, 1994; Baron *et al.* 1996). The intracellular second

messenger cascade triggered by substance P- and bradykinin-receptor binding involves the activation of phospholipase C and the production of inositol 1,4,5-trisphosphate (IP_3), which in turn release Ca^{2+} from IP_3-sensitive Ca^{2+} stores (Farmer & Burch, 1992; Regoli *et al.* 1994). In this context, it is relevant to learn whether such a common pathway leads to the stimulation of different K_{Ca} channels. *d*-Tubocurarine, a non-specific inhibitor of K_{Ca} channels, differentially reduced the hyperpolarization provoked by substance P and bradykinin in pig coronary artery (von der Weid & Bény, 1992; Baron *et al.* 1996). This suggested that the hyperpolarization produced by both agonists possibly resulted from activation of different K_{Ca} channels. To test this hypothesis, we performed whole-cell patch clamp experiments, intracellular microelectrode membrane potential recordings, and $[Ca^{2+}]_i$ measurements in endothelial cells in primary culture. We took advantage of specific K_{Ca} channel inhibitors in order to discriminate between the different K^+ conductances stimulated by substance P and bradykinin.

METHODS

Endothelial cell primary culture

Left anterior descending branches of freshly killed domestic pig *Sus scrofa* coronary arteries were obtained at the slaughterhouse. The endothelial cells were collected by gentle rubbing of the internal face of the vessel with a scalpel, and centrifuged at 800 *g* for 8 min in culture medium consisting of: M199 medium (Gibco) supplemented with 20% fetal calf serum, 2 mM glutamine, non-essential amino acids (13 ml added to 1 l of M199; Gibco), MEM vitamin solution (13 ml added to 1 l of M199; Gibco) and gentamicin (50 mg l^{-1}). The cell pellet was resuspended in culture medium M199 and plated on collagen-coated culture Petri dishes or glass coverslips. Cells were cultured at 37 °C under 5% CO_2. Culture medium was changed 3 times a week. Cells were used after 2–5 days of primary culture. Endothelial cells were identified by their morphology, fusiform growing cells forming islets during 4 to 5 days, and a monolayer of polygonal cells (cobblestone-like) after 5–6 days of culture.

Whole-cell patch clamp recordings

We used the whole-cell configuration of the patch clamp technique (Hamill *et al.* 1981). Endothelial cells were observed with an inverted microscope (Nikon Diaphot 200, Tokyo, Japan). Borosilicate glass patch pipettes were pulled with a BB-CH-PC puller (Mecanex SA, Nyon, Switzerland) and had a resistance of 3–5 MΩ. Patch clamp recordings were made using a List EPC-7 amplifier (EPC7; List Medical, Darmstadt, Germany). Current was filtered with a low-pass filter at 1 kHz, digitized by an IT16 interface (Instrutech Corporation, Great Neck, NY, USA) and stored by a Macintosh II vx computer (Pulse; HEKA Electronik, Lambrecht, Germany). Recordings were performed on single cells, or small islets never exceeding four cells, to avoid space clamp problems.

To determine the current–potential relationship, repetitive 300ms voltage pulses were applied throughout the recording, usually reaching 30, 50 and 80 mV above the holding potential (varying between −60 and −20 mV). To normalize the results, we expressed the current conductance as density (pS pF^{-1}), and thus membrane capacitance was measured before each experiment by applying a 10 mV voltage step. The capacitive current transients were fitted with a single exponential (Pulsefit; HEKA Electronik), and the cell membrane capacitance (C_m) was calculated according to the following equations (de Roos *et al.* 1996):

$$R_a = V_p/I_o,$$
$$G_m = I_{ss}/(V_p - R_a I_{ss}),$$
$$C_m = \tau\,[(1/R_a) + G_m],$$

where R_a is the access resistance, V_p the applied voltage step (10 mV), I_o the peak current, I_{ss} the steady-state current, G_m the membrane conductance and τ the decay constant of the transient.

Experiments were performed at room temperature (20–22 °C). Cells were superfused with a solution containing (mM): 130 NaCl, 5·6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose, 8 Hepes (pH 7·5 with NaOH). In ionic selectivity experiments, the concentration of NaCl was changed to 95·6 mM and that of KCl was changed to 40 mM. In Ca^{2+}-free solution, $CaCl_2$ and $MgCl_2$ were omitted, and 2 mM EGTA added. The standard pipette solution contained (mM): 130 KCl, 1 $MgCl_2$, 5 MgATP, 10 Hepes (pH 7·3 with NaOH). $MgCl_2$ was omitted and 5 mM BAPTA added to assess the Ca^{2+} dependence of the activated currents.

Cytosolic Ca^{2+} measurements

Fura-2 loading. Cultured endothelial cells were loaded with fura-2 acetoxymethylester (fura-2 AM, 10 μM) for 1 h in a Hepes-buffered solution containing (mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 0·5 $MgSO_4$, 1 NaH_2PO_4, 20 Hepes and 10·1 glucose at pH 7·4 (37 °C), in the presence of pluronic F-127 (25%) and probenecid (1 mM) in order to improve fura-2 loading. Cells were then rinsed for half an hour with the same Krebs-Hepes solution used for experiments.

Fura-2 fluorescence measurements. Endothelial cells were observed on an inverted microscope (Diaphot TMD, Nikon). Cells were continuously perfused with an oxygenated Krebs-buffered solution containing (mM): 118·7 NaCl, 4·7 KCl, 2·5 $CaCl_2$, 1·2 $MgSO_4$, 1·2 KH_2PO_4, 24·8 $NaHCO_3$, 10·1 glucose, pH 7·4, gassed with a mixture of 75% N_2, 20% O_2 and 5% CO_2, since high O_2 concentrations greatly reduce the intensity of fura-2 fluorescence. Experiments were done at 32–33 °C. Images were recorded through an Extended Isis Intensified CCD camera (Photonic Science Ltd, East Sussex, UK) and interfaced with a Macintosh II fx computer. Fura-2 was excited with a high pressure mercury lamp at dual excitation wavelengths of 340 and 380 nm, via a rotating filter wheel. The emitted fluorescence was measured at 510 nm. 256-Greyscale images were stored, processed and pseudocoloured with the image analysis software IPLab Spectrum (Signal Analytics Corporation, Vienna, VA, USA). A pair of images was acquired every 3 s.

The process of ratioing was done on each pixel by dividing fluorescence obtained for 340 nm excitation by fluorescence obtained for 380 nm excitation.

Intracellular free calcium concentration was calculated from the fluorescence ratio according to the following equation (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}] = K_d[(R - R_{min})/(R_{max} - R)]\,(S_{f,2}/S_{b,2}),$$

where R_{min} and R_{max} are the minimal and maximal values of fluorescence ratio R when all the fura-2 is in the Ca^{2+}-free form or saturated with Ca^{2+}, respectively. The factor $S_{f,2}/S_{b,2}$ is the ratio of fluorescence intensity at 380 nm when all the fura-2 is in the Ca^{2+}-free form divided by the fluorescence intensity when the fura-2 is bound to Ca^{2+}. Like R_{min} and R_{max}, this factor was determined from calibration experiments. To perform a calibration, endothelial cells were exposed to 4-bromo-A23187 (1·7 μM), in the presence of

2·5 mM extracellular Ca^{2+} (R_{max}), and in a Krebs solution containing no Ca^{2+} and 2 mM EGTA (R_{min}). The K_d of fura-2 for Ca^{2+} was taken as 225 nM.

Membrane potential measurements by intracellular microelectrode

Endothelial cells were observed on an inverted microscope (Diaphot TMD, Nikon). Intracellular membrane potential measurements were made at room temperature in Krebs solution, pH 7·4, and gassed with a mixture of 95% O_2 and 5% CO_2. The cultured cell membrane potential was measured with a conventional glass microelectrode containing 3 M KCl (90–150 MΩ) and connected to an amplifier (WPI Intra707) with an Ag–AgCl half-cell. The membrane potential was monitored on a digital oscilloscope (Gould, type 1425) and recorded on a potentiometric recorder (W + W Electronics, USA 312). Criteria for accepting a record were a stable membrane potential below −20 mV, and a sharp penetration and withdrawal.

Tension measurements

The coronary artery lumina were rinsed by injection of cold, oxygenated (95% O_2–5% CO_2) Krebs solution. Segments of the coronary artery were cleaned of adherent tissue, and cut into rings of about 2 mm width. The rings were then cut to give strips of about 5 mm long. Ligatures were attached to both ends of the strips, which were mounted with a resting isometric tension of about 10 mN in an 85 μl tissue bath as previously described (Pacicca *et al.* 1992). Strips were precontracted with 10 μM prostaglandin F2α ($PGF_{2\alpha}$) in the presence of 1 μM N^G-nitro-L-arginine (L-NA) and 10 μM indomethacin in order to inhibit nitric oxide synthase and cyclo-oxygenase, respectively. Concentration–response curves were obtained by adding agonists in a non-cumulative manner. In order to block the cytochrome P450 epoxygenase activity, 17-octadecynoic acid (17-ODYA) was added for at least 25 min before the first application of substance P or bradykinin.

Chemicals and drugs

Bradykinin and substance P were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Pluronic F-127 was obtained from Calbiochem. Fura-2 AM, probenecid, $PGF_{2\alpha}$, indomethacin and 17-ODYA were purchased from Sigma. Apamin and iberiotoxin were purchased from Alomone Labs (Jerusalem, Israel).

Statistics

Experimental data were expressed as means ± 1 standard error of the mean (S.E.M.), n referring to the number of measurements. Student's t test was used to compare results, with $P < 0·05$ taken as the level of significance. The EC_{50} was calculated for each concentration–response curve by interpolating between two points on either side of the half-maximal response, and reading the corresponding concentration on the logarithmic scale.

RESULTS

Intracellular Ca^{2+} rise induced by substance P and bradykinin

Primary cultured endothelial cells showed a basal $[Ca^{2+}]_i$ level of 96·4 ± 8·3 nM ($n = 90$; 24 experiments). Substance P triggered an increase of $[Ca^{2+}]_i$ reaching a maximal amplitude of 702·3 ± 50·7 nM ($n = 19$; 5 experiments). Bradykinin-evoked increase of $[Ca^{2+}]_i$ reached a significantly higher peak value of 961·4 ± 32·0 nM ($n = 71$; 19 experiments; $P < 0·05$ *vs.* substance P). The response duration, estimated by the half-time of return to the basal Ca^{2+} level, was 211·1 ± 20·0 s ($n = 39$; 16 experiments) for bradykinin, and about 50% shorter, 112·7 ± 18·4 s ($n = 15$; 5 experiments; $P < 0·05$), for substance P. Figure 1*A* shows representative responses induced by substance P (100 nM, filled squares)

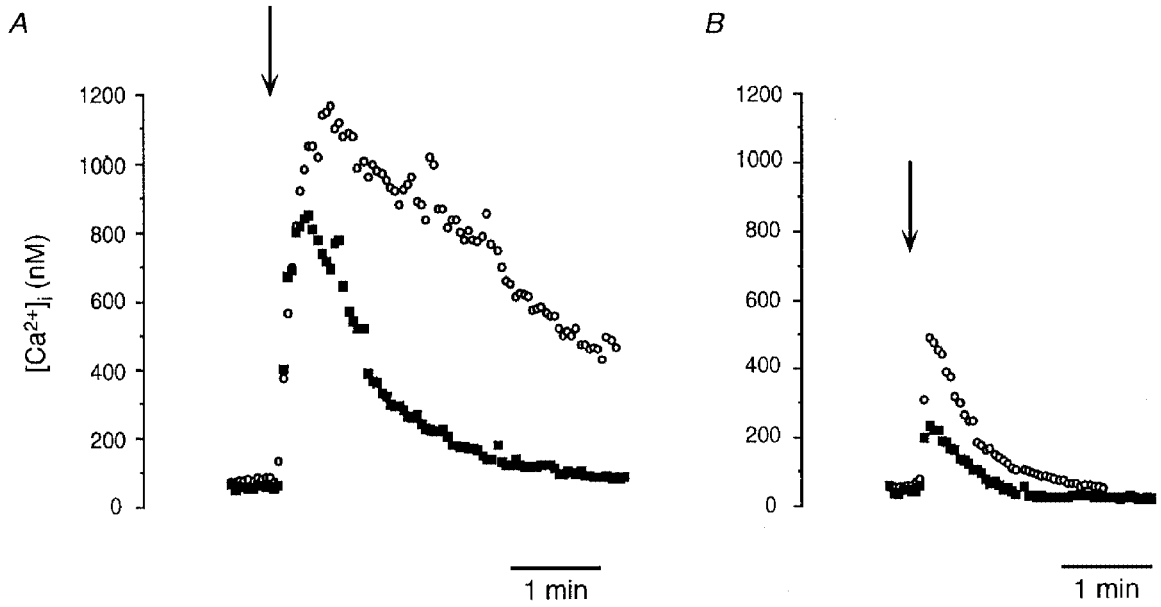

Figure 1. Substance P- and bradykinin-induced cytoplasmic Ca^{2+} rise in presence (*A*) and absence of extracellular Ca^{2+} (*B*)

Effect of substance P (100 nM, ■) and bradykinin (100 nM, ○) on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) measured with fura-2. Experiments were performed in the presence (*A*) and absence (*B*) of extracellular Ca^{2+}. Recordings were obtained from four different cells. Peptides were applied at the time indicated by the arrows and maintained until the end of the recordings.

and bradykinin (100 nM, open circles) obtained on two different cells. In the absence of extracellular Ca^{2+}, both responses became more transient with half-times of 35·7 ± 3·2 s and 60·0 ± 7·7 s, and reduced amplitudes of 314·4 ± 33·1 nM ($n = 16$; 4 experiments) and 474·4 ± 54·6 nM ($n = 18$; 7 experiments), for substance P and bradykinin, respectively (Fig. 1*B*), showing the importance of Ca^{2+} influx in the agonist-induced cellular Ca^{2+} rise.

It should be noted that the substance P-evoked cell stimulation declined with the age of the culture. The largest response to substance P was obtained after 2 days of culture with 85·6% of responding cells. The amplitude of the response decreased on day 3, and reached 267 ± 47 nM ($n = 4$) on day 4, with only 11% of responding cells. On the contrary, the response to bradykinin remained unchanged even after 4–5 days of culture (data not shown). Consequently, all subsequent experiments using substance P were performed on 2-day-old cultures.

K^+ current activated by substance P and bradykinin

Figure 2*A* and *B* shows typical currents induced by successive application of substance P (100 nM) and bradykinin (100 nM) on the same cell. Following the cell stimulation, an outward current developed rapidly and then gradually decreased with time. The maximal current evoked by the two peptides was of similar amplitude. Estimated by the half-time of return to the basal current level, the substance P response duration was 32·4 ± 3·9 s ($n = 28$) compared with 70·0 ± 10·7 s ($n = 32$) for bradykinin ($P < 0·05$), corresponding to a 54% reduction.

In order to compare the results obtained on different cells, the slope conductance of the activated currents was expressed as a function of the cell capacitance. The membrane capacitance was 25·8 ± 1·6 pF ($n = 30$) for a single cell, and 43·9 ± 2·3 pF ($n = 19$) and 84·0 ± 3·4 pF ($n = 22$) for two and three electrically coupled cells, respectively.

The current–potential curves of the maximal currents activated by substance P and bradykinin are represented in Fig. 2*C*. The slope conductances were 258·7 ± 17·7 pS pF^{-1} ($n = 40$) for substance P, and 268·5 ± 18·7 pS pF^{-1} ($n = 46$) for bradykinin, showing that the amplitude of the activated currents was not significantly different between responses ($P = 0·7$). With 5·6 mM extracellular K^+, the current reversal potentials were −72·9 ± 0·8 mV ($n = 40$) for substance P, and −73·7 ± 1·0 mV ($n = 46$) for bradykinin ($P = 0·4$), close to the K^+ equilibrium potential of −80 mV. Changing the ionic composition of the bathing solution confirmed that these currents were selective for K^+ ions. With 40 mM K^+ in the bath, the current reversal potentials were then −25·8 ± 0·7 mV ($n = 5$) for substance P, and −27·2 ± 1 mV ($n = 5$) for bradykinin ($P = 0·3$; bradykinin *vs.* substance P), for a calculated K^+ equilibrium potential of −30 mV.

In the absence of intra- and extracellular Ca^{2+} (2 mM EGTA in the bath and 5 mM BAPTA in the patch pipette), no currents were elicited by the two peptides (data not shown), thus indicating that K_{Ca} currents are implicated in endothelial cell stimulation.

Inhibitory effect of apamin and iberiotoxin on the K^+ current

In order to discriminate between the different K_{Ca} currents underlying the cellular activation, we tested different specific K_{Ca} channel inhibitors on the substance P and bradykinin responses. The application of apamin (1 μM), a well-known blocker of small conductance K_{Ca} (SK_{Ca}) channels (Latorre *et al.* 1989), strongly reduced the slope conductance of the substance P-evoked current to 92·8 ± 37·5 pS pF^{-1} ($n = 5$; $P < 0·05$ *vs.* control 258·7 ± 17·7 pS pF^{-1}) corresponding to an inhibition of about 65% (Fig. 3*A* and *B*). Iberiotoxin (50 nM), a selective blocker of large conductance K_{Ca} (BK_{Ca}) channels (Galvez *et al.* 1990), did not significantly modify the substance P response (249·4 ± 34·4 pS pF^{-1}; $n = 11$; $P = 0·8$ *vs.* control; Fig. 3*A* and *B*). The combination of apamin and iberiotoxin produced the same inhibition as apamin alone on the substance P-evoked current (100·1 ± 42·3 pS pF^{-1}; $n = 5$; $P = 0·9$ *vs.* apamin 92·8 ± 37·5 pS pF^{-1}; Fig. 3*B*).

Conversely, the bradykinin response was not significantly affected by apamin; the current slope conductance was then 280·0 ± 63·6 pS pF^{-1} ($n = 5$; $P = 0·8$ *vs.* control 268·5 ± 18·7 pS pF^{-1}; Fig. 3*C* and *D*), whereas iberiotoxin decreased the current conductance to 164·1 ± 24·2 pS pF^{-1} ($n = 6$; $P < 0·05$ *vs.* control; Fig. 3*C* and *D*), thus reducing by about 40% the K^+ current activated by bradykinin. Here again, apamin plus iberiotoxin did not produce a higher inhibition than iberiotoxin alone: 154·2 ± 35·7 pS pF^{-1} ($n = 7$; $P = 0·8$ *vs.* iberiotoxin 164·1 ± 24·2 pS pF^{-1}; Fig. 3*D*). Neither apamin nor iberiotoxin shifted the current reversal potential of the currents activated by substance P or bradykinin; when both inhibitors were applied, the current reversal potential of the substance P-induced outward current was slightly shifted from −73 mV to −66 ± 3·02 mV ($n = 5$; $P < 0·05$).

These results showed that substance P and bradykinin stimulated in a large proportion different types of K_{Ca} currents. However the stimulation of endothelial cells with a combination of substance P and bradykinin did not result in a larger outward current than either peptide alone, the slope conductance being 230·5 ± 38·2 pS pF^{-1} ($n = 20$; $P = 0·4$ *vs.* substance P 258·7 ± 17·7 pS pF^{-1} or bradykinin 268·5 ± 18·7 pS pF^{-1} alone). We thus speculate that one agonist produced an inhibition on the channels stimulated by the other. To test this hypothesis we applied simultaneously substance P and bradykinin in the presence of iberiotoxin, in order to block BK_{Ca} channels stimulated by bradykinin and putatively revealed an inhibition of the total current. In this condition the K^+ current was 144·3 ± 37·3 pS pF^{-1} ($n = 8$) corresponding to a reduction of about 40% compared with the K^+ current stimulated by substance P in the presence of iberiotoxin (249·4 ± 34·4 pS pF^{-1}; $P = 0·057$). Although not reaching statistical significance, this result

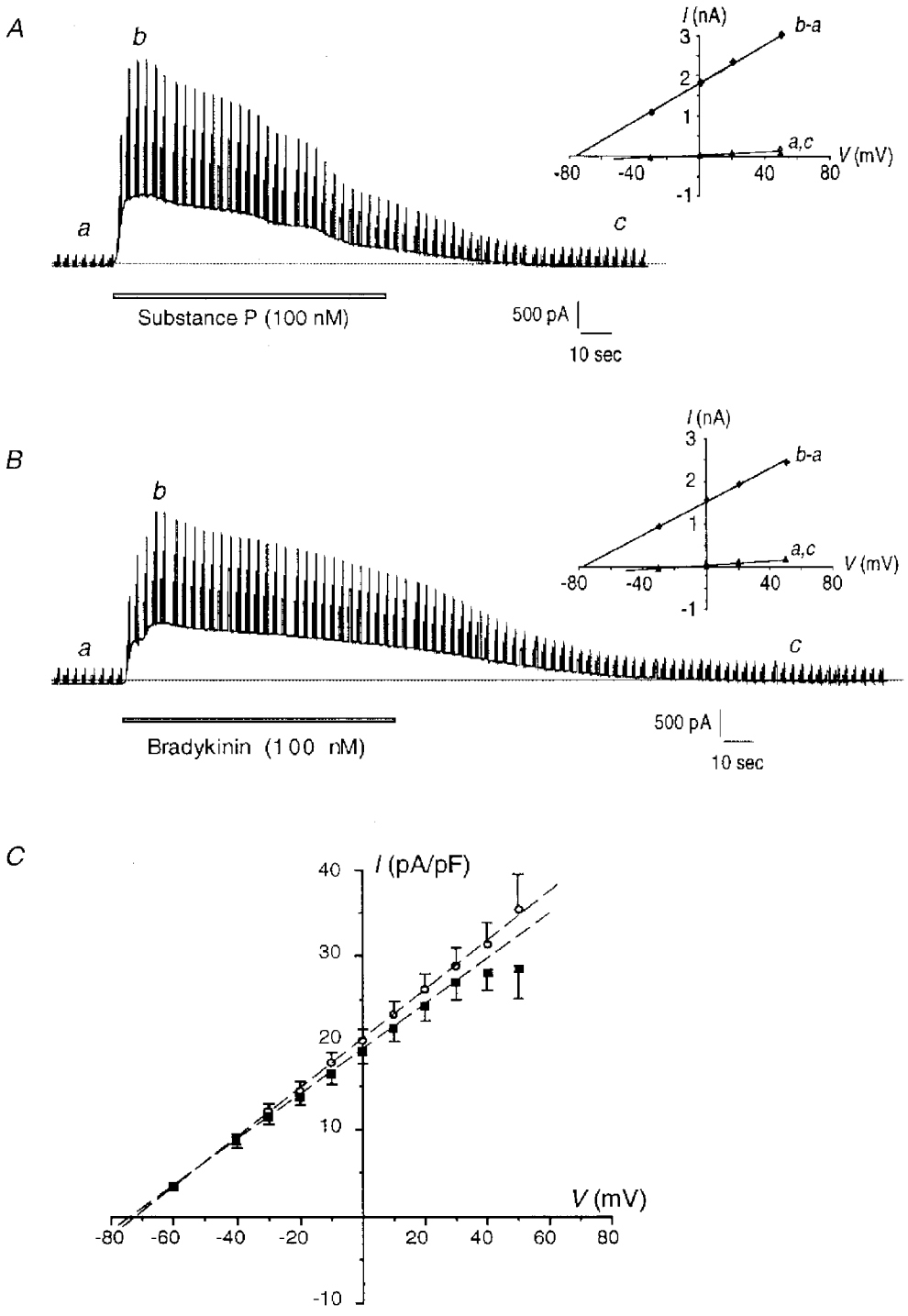

Figure 2. Whole-cell currents induced by substance P and bradykinin in pig coronary artery endothelial cells

A and *B*, outward current elicited by 100 nM substance P (*A*) followed by 100 nM bradykinin (*B*) on the same recorded cell, separated by a 5 min washout period. The holding potential was −30mV, and voltage steps were of 30, 50 and 80 mV above the holding potential. Peptides were applied in the bathing solution for the period indicated by the open bar. Corresponding current–potential curves are shown on the right insets, with *a* being obtained in control condition, *c* after peptide washout and *b* − *a* corresponding to the stimulated current. Dotted lines indicate the zero current. *C*, mean current–potential curves of K^+ current stimulated by substance P (■) and bradykinin (○). Maximal currents are represented, normalized by membrane capacitance. Data were obtained with a standard bathing solution containing 130mM NaCl and 2 mM $CaCl_2$. Data are means ± S.E.M.

strongly suggested that bradykinin somehow inhibited a part of the substance P-induced K_{Ca} current.

Membrane hyperpolarization induced by substance P and bradykinin

The putative role played by SK_{Ca} and the BK_{Ca} channels in the response due to substance P and bradykinin, respectively, was further investigated by measuring the change in membrane potential elicited by both peptides, using intracellular microelectrode recording. Endothelial cells had a resting membrane potential of $-31{\cdot}1 \pm 1{\cdot}6$ mV ($n = 68$). Figure 4*A* shows the electrical response of an endothelial cell following application of substance P and then bradykinin. Typically, the two agonists induced hyperpolarizations of similar amplitude; the membrane potential change was $27{\cdot}0 \pm 1{\cdot}7$ mV ($n = 31$) for substance P and $28{\cdot}1 \pm 1{\cdot}6$ mV ($n = 46$) for bradykinin ($P = 0{\cdot}6$, substance P *vs.* bradykinin, Fig. 4*B*). Similar to data obtained on K^+ current and $[Ca^{2+}]_i$ rise, the response duration evoked by substance P was about 50% shorter, with a half-time of $109{\cdot}7 \pm 11{\cdot}0$ s ($n = 25$), compared with $224{\cdot}8 \pm 34{\cdot}2$ s ($n = 27$; $P < 0{\cdot}05$) with bradykinin. The application of 1 μM apamin strongly reduced the substance P response without affecting the hyperpolarization due to bradykinin, as shown on the original recording in Fig. 4*A*. On average, the substance P-induced hyperpolarization was reduced by 60%, to a value of $10{\cdot}0 \pm 3{\cdot}0$ mV ($n = 7$; $P < 0{\cdot}05$ *vs.* control $27{\cdot}0 \pm 1{\cdot}7$ mV; Fig. 4*B*). Conversely, iberiotoxin (50 nM) significantly reduced the bradykinin-induced hyperpolarization ($19{\cdot}8 \pm 3{\cdot}3$mV; $n = 9$; $P < 0{\cdot}05$ *vs.* control $28{\cdot}1 \pm 1{\cdot}6$ mV), without affecting the response to substance P. Neither apamin nor iberiotoxin affected the resting membrane potential.

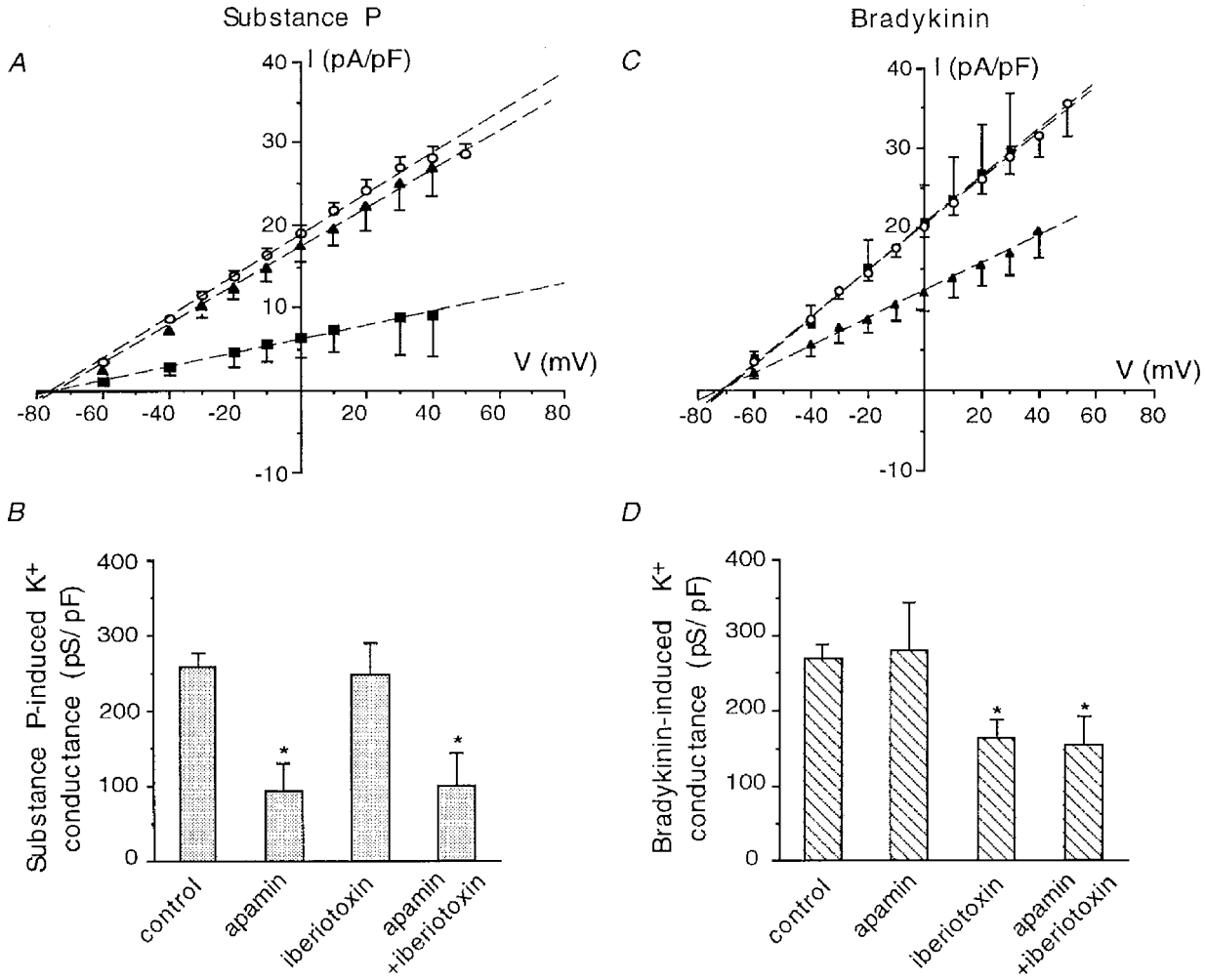

Figure 3. Effect of K_{Ca} channel blockers on the K^+ current activated by substance P (*A* and *B*) and bradykinin (*C* and *D*)

A and *C*, current–potential curves for 100 nM substance P (*A*) and 100 nM bradykinin (*C*) obtained in control conditions (○), and in the presence of 1 μM apamin (■) or 50 nM iberiotoxin (▲). Data were linearly fitted, and are means ± S.E.M. *B* and *D*, maximal K^+ current conductances activated by 100 nM subtance P (*B*) and 100 nM bradykinin (*D*) are represented in control conditions, and in the presence of apamin (1 μM), iberiotoxin (50 nM) or a combination of both inhibitors. Both points and columns represent the mean of 5 experiments in the presence of inhibitors, and a mean of 40–46 experiments in control conditions. Data are means ± S.E.M. *$P < 0{\cdot}05$.

Involvement of cytochrome P450 epoxygenase in the endothelium-dependent relaxation and K^+ current due to substance P and bradykinin

Epoxyeicosatrienoic acids (EETs), produced by cytochrome P450 epoxygenase following endothelial cell stimulation (Harder *et al.* 1995), have been shown to modulate smooth muscle cell K_{Ca} channel activity (Hu & Kim, 1993; Li & Campell, 1997). Furthermore, EETs relax and hyperpolarize smooth muscles, thus leading some authors to propose that they are the putative EDHF (Hecker *et al.* 1994; Campbell *et al.* 1996). We have previously demonstrated that EETs enhance the open state probability of a 285 pS BK_{Ca} channel activated by bradykinin in pig coronary artery endothelial cells (Baron *et al.* 1997). In addition, EETs are produced by porcine coronary artery endothelial cells following stimulation by bradykinin (Hecker *et al.* 1994; Popp *et al.* 1996). In this context, we wondered if bradykinin or substance P could stimulate cytochrome P450 and, consequently, if EETs could explain the difference of K_{Ca} channel activation. To address this question, we looked at the involvement of cytochrome P450-derived compounds as relaxing factors. Concentration–response curves were performed on intact tissues precontracted with 10 μM $PGF_{2\alpha}$, in the presence of 10 μM indomethacin and 100 μM L-NA, to reveal the L-NA/indomethacin-resistant component of the endothelium-dependent relaxation. The application of 17-ODYA (3 μM), a suicide substrate of cytochrome P450, did not influence the substance P response: $EC_{50} = 0{\cdot}27 \pm 0{\cdot}04$ nM ($n = 11$) in control conditions and $0{\cdot}32 \pm 0{\cdot}04$ nM ($n = 8$; $P = 0{\cdot}4$; Fig. 5*A*) in the presence of inhibitor. Conversely, 17-ODYA caused a rightward shift in the concentration–response curve to bradykinin: $EC_{50} = 3{\cdot}1 \pm 0{\cdot}6$ nM ($n = 9$) in control conditions, and $15{\cdot}1 \pm 6{\cdot}1$ nM ($n = 7$; $P < 0{\cdot}05$) in the presence of 17-ODYA; and it reduced the maximal relaxation by about 20% (Fig. 5*B*), thus suggesting that cytochrome P450 was stimulated by bradykinin but not by substance P.

We then tested the effect of 17-ODYA (3 μM) on the bradykinin-induced K^+ current. In the presence of the inhibitor, the response due to bradykinin was reduced to $178{\cdot}9 \pm 36{\cdot}8$ pS pF^{-1} ($n = 11$; $P < 0{\cdot}05$ *vs.* control $268{\cdot}5 \pm 18{\cdot}7$ pS pF^{-1}), corresponding to an inhibition of about 35%. On 12 experiments only one response was more than 2 times higher than mean control value and consequently was not considered. The substance P response was not affected by the application of 17-ODYA ($248{\cdot}7 \pm 63{\cdot}7$ pS pF^{-1}; $n = 7$;

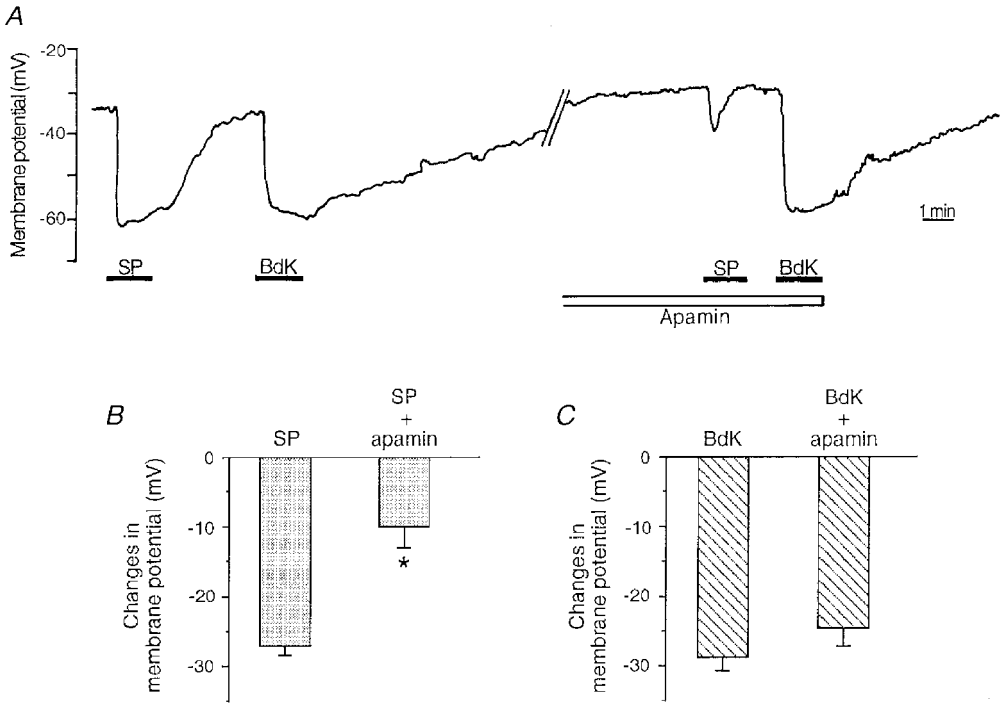

Figure 4. Effect of apamin on the endothelial cell hyperpolarization due to substance P and bradykinin

A, change in membrane potential measured by an intracellular microelectrode, following stimulation by 100 nM substance P (SP) and 100 nM bradykinin (BdK) in control conditions and after application of apamin (1 μM). The entire recording was performed on the same cell; the time between the first bradykinin application and the second substance P application was 26 min. *B* and *C*, mean values of the subtance P- (*B*) and bradykinin-induced (*C*) cell hyperpolarization in control or in the presence of apamin. n ranges from 4 to 7 for each value in the presence of apamin, and from 31 to 37 in controls. Data are means ±S.E.M. $^*P < 0{\cdot}05$.

$P = 0{\cdot}3$ *vs.* control $258{\cdot}7 \pm 17{\cdot}7$ pS pF^{-1}), which gives support to the previous finding that bradykinin but not substance P stimulated the production of EETs.

DISCUSSION

Our present results, obtained on cultured endothelial cells, showed that in the same experimental conditions, cellular responses elicited by substance P and bradykinin were very similar. Substance P and bradykinin evoked large outward currents reaching a conductance at maximal stimulation of around 260 pS pF^{-1}. In the nominal absence of intra- and extracellular Ca^{2+} no currents were observed. This is in agreement with a previous report (Vaca *et al.* 1996) and confirms the central role of $[Ca^{2+}]_i$ in agonist-induced endothelial cell activations. The reversal potential of the kinin-induced outward currents closely corresponds to the K^+ equilibrium potential, which supports the assertion that substance P (Sharma & Davis, 1994) and bradykinin (Colden-Stanfield *et al.* 1990) mainly activate K_{Ca} currents, responsible for the change of membrane potential. Membrane hyperpolarization activated by substance P or bradykinin reached similar amplitudes, although substance P triggered a 30% smaller increase of $[Ca^{2+}]_i$. At this point, the most striking difference was the response duration: K^+ current, membrane hyperpolarization and $[Ca^{2+}]_i$ were about 50% shorter when elicited by substance P. The shorter substance P-induced membrane hyperpolarization has already been observed (Brunet & Bény, 1989). Although it highlighted a significant difference between substance P- and bradykinin-induced endothelial cell activations, it was not further investigated at that time.

Bradykinin stimulated iberiotoxin-sensitive BK_{Ca} current

Large conductance iberiotoxin-sensitive BK_{Ca} channels were activated by bradykinin, whereas they did not seem to be stimulated by substance P. Single channel analysis has shown that bradykinin activates BK_{Ca} channels of rabbit aorta and porcine coronary artery endothelial cells (Rusko *et al.* 1992; Baron *et al.* 1996). BK_{Ca} channel involvement only during bradykinin-induced cellular activation may be explained by considering two parameters. Firstly, bradykinin produced a higher $[Ca^{2+}]_i$ increase compared with substance P (Fig. 1*A*), and BK_{Ca} channels typically required a high Ca^{2+} concentration to be activated (Latorre *et al.* 1989), as was noticed in endothelial cells, with an EC_{50} of about 1 μM Ca^{2+} at +20 mV (Rusko *et al.* 1992; Baron *et al.* 1996). Secondly, EET production by endothelial cells could play a major role in BK_{Ca} channel activation. The present study suggests that bradykinin, as opposed to substance P, activates cytochrome P450 epoxygenase. The application of 17-ODYA, a suicide substrate for cytochrome P450 epoxygenase (Zou *et al.* 1994), caused a rightward shift and reduced the maximal amplitude of the concentration–response curve to bradykinin, but it did not modify the substance P response. As no major non-specific inhibitory effects were observed with 17-ODYA, even at concentrations above 3 μM (Edwards *et al.* 1996), and no inhibition was observed for the substance P response (Fig. 5*A*), we conclude that the shift of the bradykinin concentration–response curve is due to the inhibition of cytochrome P450. Much evidence supports bradykinin as a stimulator of cytochrome P450 epoxygenase in pig coronary

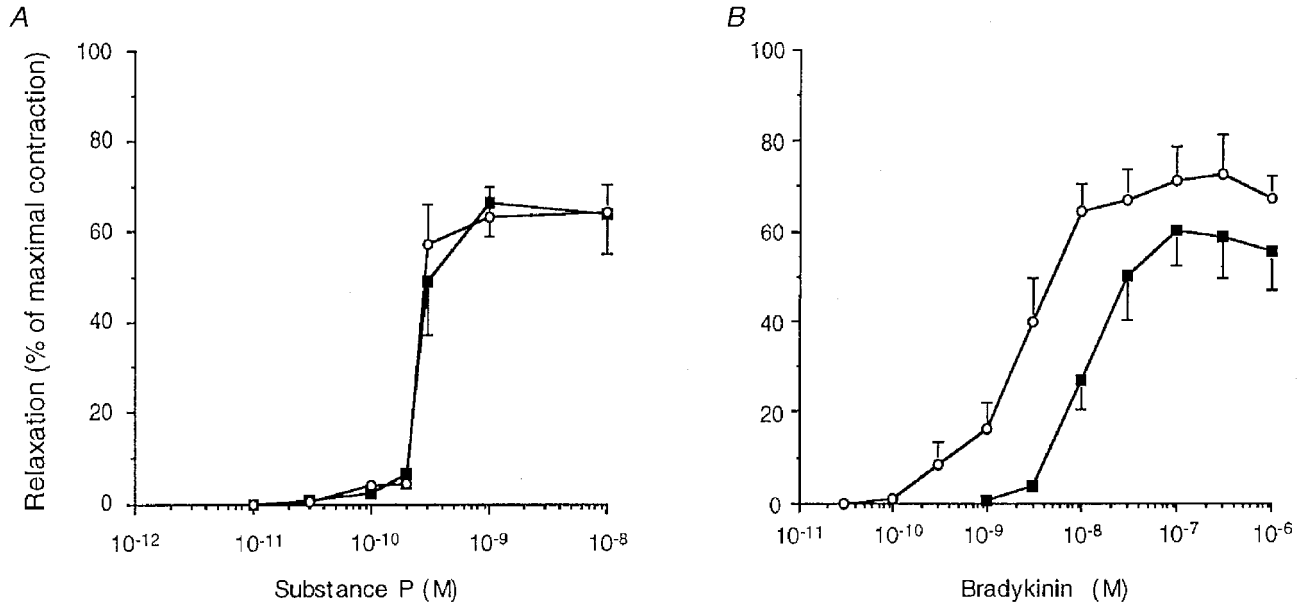

Figure 5. Effect of 17-ODYA on the substance P- (*A*) and bradykinin-induced (*B*) endothelium-dependent relaxation

Concentration–response curves to substance P (*A*) and bradykinin (*B*) on coronary artery strips precontracted with 10 μM $PGF_{2\alpha}$, in the presence of 10 μM indomethacin and 100 μM L-NA. Experiments were done either in the absence (○) or in the presence (■) of 3 μM 17-ODYA. Each point is the mean of 7–11 experiments. Data are means ± S.E.M.

artery, and bovine aorta and coronary artery (Hecker *et al.* 1994; Graier *et al.* 1995; Popp *et al.* 1996). However, few data concern the possible activation of cytochrome P450 epoxygenase by substance P, but Wallerstadt & Bodelsson (1997) suggested that this enzyme was not activated by substance P on human omental artery. Interestingly, we have previously shown that at constant Ca^{2+} concentration, the P450-derived EETs enhanced the open state probability of a 285 pS BK_{Ca} channel activated by bradykinin in pig coronary artery endothelial cells (Baron *et al.* 1997). Therefore, the higher $[Ca^{2+}]_i$ increase and the EET production caused by bradykinin would contribute to BK_{Ca} channel activation. As substance P probably did not induce EET synthesis, and triggered a lower Ca^{2+} increase, the probability of activating BK_{Ca} channels is very low. In addition our results obtained on K^+ current activated in the presence of 17-ODYA confirmed this statement. 17-ODYA reduced the K^+ current evoked by bradykinin to a similar extent to those produced by iberiotoxin (about 35%), suggesting that the activation of the cytochrome P450 is necessary for BK_{Ca} channel stimulation. By contrast, the substance P-induced K^+ current was not affected by the preincubation with 17-ODYA. Thus, in physiological conditions, both an increase in $[Ca^{2+}]_i$ and EET production seem to be required for the stimulation of BK_{Ca} in endothelial cells.

Substance P-stimulated apamin-sensitive SK_{Ca}

Our results demonstrate that SK_{Ca} channels are not involved in the bradykinin-evoked K^+ current, but appear to be involved in the substance P-induced response. Similarly to the effects produced on K^+ current, apamin strongly reduced the substance P-induced hyperpolarization, whereas no significant inhibition was produced on the bradykinin-induced response. In pig coronary artery, Sharma & Davis (1994) showed that substance P stimulated a 23 pS K_{Ca} channel, that corresponds to a SK_{Ca} channel, even though its apamin sensitivity was not determined. Regarding changes in $[Ca^{2+}]_i$ induced by substance P and the high Ca^{2+} sensitivity of the SK_{Ca} channels (Latorre *et al.* 1989), their activation by this peptide is not surprising. However, their absence of involvement during cell stimulation by bradykinin is unexpected, and is not in agreement with previous reports. In pig aorta, bradykinin activated a 9 pS K_{Ca} channel, responsible for most of the K^+ current elicited by bradykinin in these cells (Groschner *et al.* 1992). In bovine aorta, Vaca *et al.* (1996) have shown that noxiustoxin, an inhibitor of SK_{Ca} channels in this tissue (Vaca *et al.* 1993), completely inhibited the hyperpolarization due to bradykinin. In the same tissue, bradykinin stimulated K_{Ca} channels with conductances of 30–40 pS (Sauvé *et al.* 1990; Vaca *et al.* 1992), but their apamin sensitivity was not investigated. Conversely, apamin only slightly reduced, by 15%, the bradykinin-evoked membrane hyperpolarization in guinea-pig coronary artery (Mehrke & Daut, 1990). Thus, depending on the tissue, SK_{Ca} channels are stimulated differently by bradykinin. In pig coronary artery endothelial cells, we cannot exclude the possibility that bradykinin stimulated a particular type of SK_{Ca} channel insensitive to apamin, which accounts for a part of the K^+ current insensitive to iberiotoxin. For instance Marchenko & Sage (1996) have described in rat aorta an 18 pS K_{Ca} channel, a conductance that corresponds to the usual classification for SK_{Ca} channels (Latorre *et al.* 1989), that is insensitive to apamin.

One important question was to determine if the SK_{Ca} channels were not activated by bradykinin, or if they were secondarily inhibited following cell stimulation by bradykinin. If distinct K_{Ca} channels were activated by substance P and bradykinin, and if no inhibition of SK_{Ca} channels occurred, then endothelial cell stimulation by a combination of substance P and bradykinin would have produced a larger activated K^+ current, compared with the application of one peptide alone. Our results demonstrated that this was not the case, as substance P and bradykinin together produced a K^+ current of the same amplitude as that with either peptide applied alone. Furthermore, the K^+ current stimulated by substance P and bradykinin in the presence of iberiotoxin was reduced compared with that stimulated by substance P and iberiotoxin alone. Even if this inhibition did not reach statistical significance, probably reflecting the high heterogeneity of K^+ channels present in cultured endothelial cells, this revealed that bradykinin secondarily inhibited some of the channels usually activated by substance P, likely to be the SK_{Ca} channels. Thus it could explain why when both peptides were applied, the K^+ current was not enhanced. In this condition we suggested that bradykinin stimulated BK_{Ca} channels at the same time as it inhibited SK_{Ca} channels, leading to the total K^+ current being unaltered. The difference in K_{Ca} channel stimulation implies an intracellular signalling pathway different for substance P and bradykinin, which requires further investigation to be elucidated.

Other studies have shown that a combination of K_{Ca} channel blockers potentiates the effect of either inhibitor applied alone. This was mainly studied for the endothelium-dependent hyperpolarization of smooth muscle cells, with the combination of apamin and charybdotoxin (Chen & Cheung, 1997; Petersson *et al.* 1997). Such potentiating effects were not observed in the present study. The combination of iberiotoxin and apamin resulted in a similar inhibition compared with that in the presence of the effective inhibitor alone (iberiotoxin for bradykinin and apamin for substance P). These discrepancies could be due to different preparations used, i.e. cultured endothelial cells (present work) and intact strips with two cell types, endothelial and smooth muscle cells, in the above-mentioned studies.

Regarding the strong inhibition of endothelial cell K^+ current produced by apamin or iberiotoxin, we can probably suppose that vasoactive factor production will be altered (Lückhoff & Busse, 1990). As already stressed by Mombouli & Vanhoutte (1997), particular care has to be taken when

addressing the question of K^+ channels underlying the endothelium-dependent smooth muscle cell hyperpolarization in intact tissue, by applying K^+ channel blockers that can act either at the level of endothelial or smooth muscle cells, or most likely on both cell types.

In conclusion, we have shown that substance P and bradykinin, both endothelium-dependent vasodilators, stimulated endothelial cells in apparently the same way. Looking at the membrane potential and outward K^+ current induced by the two peptides, no major differences were observed, except for the response durations. Nevertheless, different populations of K_{Ca} currents were stimulated by substance P and bradykinin. Substance P mainly evoked a current consistent with the activation of small conductance, apamin-sensitive SK_{Ca} channels, which accounted for about 65% of the total K^+ current. A current consistent with the activation of large conductance iberiotoxin-sensitive BK_{Ca} channels did not appear to be stimulated by this agonist, but contributed about 40% of the total bradykinin-evoked K^+ current. The production of EETs by bradykinin only is likely to explain the differential BK_{Ca} channel stimulations. Apamin-sensitive SK_{Ca} channels were not activated, and possibly were inhibited, by bradykinin, by an as yet undetermined intracellular second messenger. Our observation that a similar electrical response is produced by different pools of channels implies distinct intracellular pathways leading to K_{Ca} current activations.

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