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BRADYKININ B_2- AND 5-HYDROXYTRYPTAMINE (5-HT_2)-RECEPTOR STIMULATED INCREASES IN INTRACELLULAR CALCIUM IN CULTURED GUINEA-PIG AORTIC SMOOTH MUSCLE CELLS

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Abstract—The effect of 5-hydroxytryptamine (5-HT) and bradykinin on intracellular free calcium concentration ($[Ca^{2+}]_i$) has been studied in cultured guinea-pig aortic smooth muscle cells loaded with the Ca^{2+}-sensitive fluorescent dye fura-2. Bradykinin and 5-HT elicited rapid increases in $[Ca^{2+}]_i$ with log EC_{50} values of -7.9 ± 0.05 M (N = 3) and -6.1 ± 0.1 M (N = 4), respectively. Both agonists stimulated the release of intracellular calcium (Ca^{2+} responses still present in the absence of extracellular calcium) and to a lesser extent Ca^{2+} influx (observed when extracellular calcium was re-applied to cells initially stimulated in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA). The B_1-receptor antagonist des-Arg^9,[Leu^8]-bradykinin had no effect on calcium responses elicited 100 nM bradykinin, whereas the B_2-receptor antagonist D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin (10 μM) inhibited the response to 100 nM bradykinin by 43%. The bradykinin B_2-receptor antagonist D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin virtually abolished the response to 10 nM bradykinin (IC_{50} 4 μM). No increase in $[Ca^{2+}]_i$ was observed with the B_1-receptor agonist des-Arg^9-bradykinin (300 nM). The response to 5-HT (100 μM) was abolished by the 5-HT_2 receptor antagonist ketanserin ($IC_{50} = 2 \pm 0.4$ nM; N = 3). These data suggest that in cultured guinea-pig aortic smooth muscle cells bradykinin B_2- and 5-HT_2-receptor activation stimulates the release of Ca^{2+} from intracellular stores and Ca^{2+} influx through Ca^{2+} entry pathways in the plasma membrane.

Key words: bradykinin B_2-receptors; 5-HT_2-receptors; intracellular calcium; guinea-pig aorta; fura-2; vascular smooth muscle

Numerous agonists stimulate vascular smooth muscle contraction by inducing a rise in intracellular free calcium. Agonist stimulated increases in intracellular calcium have been measured in a variety of vascular smooth muscle preparations. For example, histamine H_1- and angiotensin II receptor activation in rat aortic smooth muscle cells stimulate the release of Ca^{2+} from intracellular stores [1–3]. Whereas, Neylon *et al.* [4] measured thrombin-stimulated increases in $[Ca^{2+}]_i$ in single human internal mammary artery cells.

Studies have also shown that 5-HT‡ and bradykinin can elicit vascular smooth muscle contraction. For example, in rat aorta 5-HT, acting via 5-HT_2 receptors, induced smooth muscle contraction [5]. These authors concluded that the initial phase of 5-HT induced muscle contraction involved inositol phospholipid hydrolysis and calcium mobilization, whereas the maintenance of muscle contraction (tonic phase) involved voltage dependent Ca^{2+} channels. Bradykinin B_1-receptor activation causes contraction of rabbit aorta smooth muscle [6].

Vascular smooth cells cultured from guinea-pig aorta have recently been shown to express 5-HT_2 and bradykinin B_2-receptors coupled to inositol phospholipid hydrolysis [7]. Therefore the aims of the present study were: (i) to investigate the characteristics of 5-HT and bradykinin-receptor mediated increases in $[Ca^{2+}]_i$ (i.e. the involvement of intracellular and extracellular Ca^{2+} in the overall response) and (ii) to pharmacologically characterize the Ca^{2+} responses elicited by 5-HT and bradykinin.

MATERIALS AND METHODS

Chemicals. Fura-2/AM and ionomycin were from Calbiochem (Nottingham, U.K.). Histamine, bradykinin, 5-HT, ketanserin, D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, des-Argh^9,[Leu^8]-bradykinin, des-Arg^9-bradykinin, α-smooth muscle actin monoclonal antibody, fluorescein isothiocyanate-conjugated anti-mouse antibody and collagenase (type I) were obtained from the Sigma Chemical Co. (Poole, U.K.). DMEM and FCS were from Northumbria Biologicals (U.K.) and trypsin-EDTA (10 × solution) from Gibco (Paisley, U.K.). All other chemicals were of analytical grade.

Cell culture. Guinea-pig aortic smooth muscle cells were grown from explant cultures. Pieces of guinea-pig aorta were incubated in DMEM containing 1 mg/mL collagenase (type I) for 30 min until the outer

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‡ Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; 5-HT, 5-hydroxytryptamine; FCS, foetal calf serum; DMEM, Dulbecco's modified Eagle's medium.

sheath of connective tissue could be dissected away. Explants of aortic smooth muscle (1 mm^3) were then placed in 75-cm^3 flasks containing DMEM supplemented with 2 mM L-glutamine, 10% (v/v) FCS, penicillin G (200 U/mL), streptomycin (200 μg/mL) and amphotericin B (0.5 μg/mL) and incubated at 37° in humidified air/CO_2 (95:5). Cells were fed with fresh growth medium every 3–4 days and the antibiotic supplements removed from the medium after the initial culture of the explants. At confluence, explants were removed and smooth muscle cells passaged (1/2 split ratio) using trypsin(0.05%)-EDTA (0.02%) solution into 75 cm^3 flasks. Cells for $[Ca^{2+}]_i$ determinations were grown on 24 × 10 mm glass coverslips in 90 mm petri dishes. All experiments were performed on confluent monolayers (cells were used between passages 4 and 10).

Immunohistochemical analysis. Smooth muscle cells were confirmed by immunohistochemistry using α-smooth muscle actin monoclonal antibody. Cells were plated into 8-well glass chamber slides. At or near confluency cells were washed (3 × 5 min in PBS) and fixed using −20° methanol for 15 min. Cells were rinsed again (3 × 5 min in PBS) and incubated for 45 min in 10% horse serum (v/v) in PBS at room temperature. Cells were then incubated for 60 min at room temperature with the α-smooth muscle actin monoclonal antibody (1:40 dilution in 10% horse serum in PBS). Cells were washed (3 × 5 min PBS) and exposed to fluorescein isothiocyanate-conjugated anti-mouse antibody (1:40 dilution in 10% horse serum in PBS) for 60 min at room temperature. Finally, cells were washed (3 × 5 min in PBS and 1 × 5 min water) and mounted in UV free aqueous mountant. Cells were viewed under a Zeiss epifluorescent photomicroscope III using the 487710 filter set.

Measurement of intracellular free calcium. Intracellular free calcium was measured by loading confluent cell monlayers with the calcium-sensitive fluorescent dye fura-2. Individual coverslips were placed in 35-mm petri dishes with 1 mL of physiological buffer (145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES, 2 mM $CaCl_2$, pH 7.45) containing 10% FCS (v/v), 3 μM fura-2/AM and incubated for 30 min at 37°. After this "loading" period the fura-2 containing buffer was replaced with fresh buffer, that was free of fura-2 and FCS but contained 0.1% bovine serum albumin, and left at 37° for a further 15 min. Loaded coverslips were then mounted in a specially designed holder which enabled the coverslip to be positioned across the diagonal of a polymethacrylate cuvette. Each cuvette contained 2.9 mL of physiological buffer (drugs were added to the cuvettes in 100-μL aliquots) and fluorescent measurements were made at 37° using a Perkin Elmer LS 50 spectrometer. The excitation wavelengths were 340 and 380 nm, with emission at 500 nm. The slit-widths were set at 10 nm, with emission at 500 nm. The slit-widths were set at 10 nm for both the excitation and emission wavelengths and the time taken to switch between 340 and 380 nm was 0.8 sec. Intracellular Ca^{2+} was calculated every 1.9 sec from the ratio (R) of 340 nm/380 nm fluorescent values using the equation of Grynkiewicz *et al.* [8].

$$[Ca^{2+}]_i = \frac{(R - R_{min})}{(R_{max} \times R)} \times (S_{380,min}/S_{380,max}) \times K_d$$

where K_d is the affinity of fura-2 for Ca^{2+} (224 nM at 37°) and $S_{380,min}/S_{380,max}$ is the ratio (β value) of the fluorescent values obtained at 380 nm in the absence and presence of saturating $[Ca^{2+}]_i$. The maximum and minimum R values (R_{max} and R_{min}) were determined on separate coverslips under saturating $[Ca^{2+}]_i$ (achieved by increasing the extracellular $[Ca^{2+}]$ to 20 mM followed by 10 μM ionomycin, pH 7.45) and calcium-free (achieved using 8.3 mM EGTA immediately followed by 25 μL of 1.0 M NaOH to compensate for the decrease in pH, in the presence of 10 μM ionomycin) conditions, respectively. Corrections for autofluorescence were made by measuring the fluorescence produced by coverslips that had not been loaded with fura-2. Where Ca^{2+}-free conditions were required experiments were performed in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA.

Data analysis. Agonist and antagonist concentration–response curves were fitted to a logistic equation using the non-linear regression program GraphPAD (ISI). Rises in intracellular free $[Ca^{2+}]$ were evaluated by importing the fluorescence data into the spreadsheet AsEasyAs (TRIUS Inc). Basal $[Ca^{2+}]_i$ levels were determined by calculating the mean of the 10 data points (measured every 1.9 sec) prior to drug addition, whereas the maximum Ca^{2+} signal was deemed to be the largest Ca^{2+} response obtained immediately after drug addition. Due to the fluctuations in basal $[Ca^{2+}]_i$, the mean of the 10 data points measured immediately after drug addition were also calculated to determine whether the measured response was significantly different from basal levels (Mann–Whitney U test). Data are shown as means ± SEM. Statistical analysis was performed using the Student's unpaired t-test. A P value < 0.05 was considered as statistically significant. N in the text refers to the number of separate experiments.

RESULTS

Immunohistochemistry

Staining with α-smooth muscle actin monoclonal antibody confirmed the identity of the guinea-pig aortic smooth muscle cells (see Fig. 1).

Bradykinin stimulated increases in intracellular Ca^{2+}

Bradykinin elicited a rapid and concentration-dependent increase in $[Ca^{2+}]_i$ in cultured guinea-pig aortic smooth muscle cells (log EC_{50} (M) = −7.9 ± 0.05; N = 3; see Fig. 2). In the presence of extracellular Ca^{2+} (2 mM) bradykinin (BK; 1 μM; see Fig. 3a) increased $[Ca^{2+}]_i$ from 58 ± 4 to 1848 ± 210 nM (N = 12) within 20 sec of application, with $[Ca^{2+}]_i$ returning to basal values after approximately 150 sec. The percentage of the maximum bradykinin response measured at 50 and 100 sec after the peak response was 21 ± 4% (N = 12) and 12 ± 2% (N = 12), respectively. Figure 3b shows a profile obtained by stimulating with bradykinin (1 μM) in the absence of extracellular Ca^{2+} (Ca^{2+}-free buffer containing 0.1 mM EGTA). In these experiments bradykinin (1 μM) increased

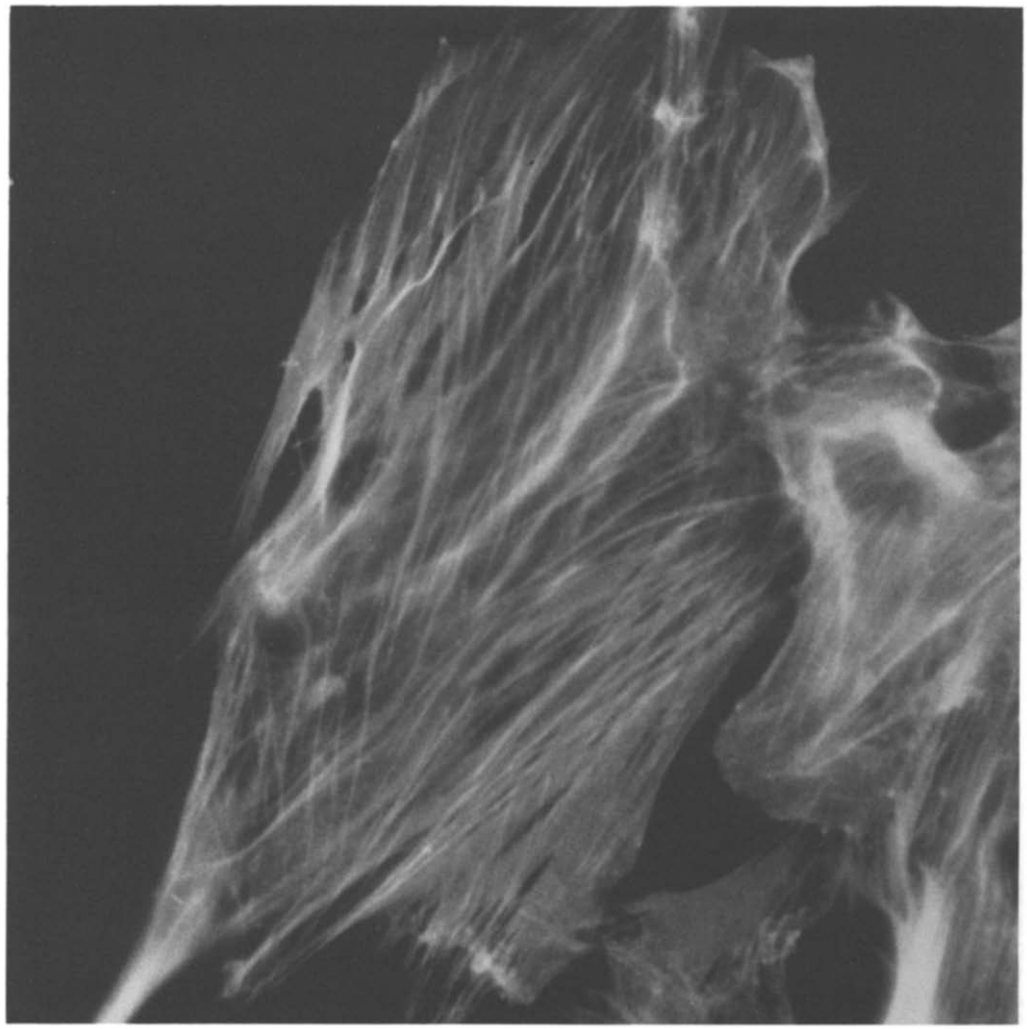

Fig. 1. Immunohistochemical staining of guinea-pig aortic smooth muscle cells with a monoclonal antibody to α-smooth muscle actin.

$[Ca^{2+}]_i$ from 52 ± 3 to 1712 ± 250 nM (N = 6). In the absence of extracellular Ca^{2+} the percentage of the maximum bradykinin response remaining 50 and 100 sec after the peak response was 7 ± 1% (N = 6) and 2 ± 0.5% (N = 6), respectively. However, if Ca^{2+} (2 nM) was re-applied, after the cells were stimulated in nominally Ca^{2+}-free buffer, there was a rise in $[Ca^{2+}]_i$ (58 ± 4 to 134 ± 17 nM; N = 6) indicative of calcium influx (see Fig. 3b). To dismiss the possibility that this rise in $[Ca^{2+}]_i$ is simply a consequence of fura-2 leakage into the extracellular medium, an experiment was performed (results not shown) in which bradykinin was replaced with vehicle. There was no observable increase in $[Ca^{2+}]_i$ during this experiment, indicating the rise in $[Ca^{2+}]_i$ (after re-applying 2 mM $CaCl_2$) shown in Fig. 3b is a result of calcium entry into the cell. These data suggest that bradykinin-receptor activation stimulates release of Ca^{2+} from intracellular stores and Ca^{2+} influx (entry) across the plasma membrane.

The bradykinin B_1-receptor agonist des-Arg^9-

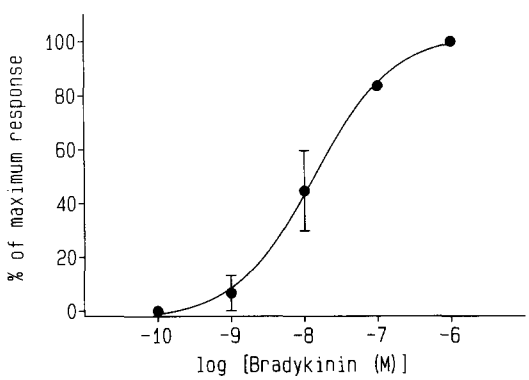

Fig. 2. Concentration–response curve for bradykinin-stimulated increases in $[Ca^{2+}]_i$ in guinea-pig aortic smooth muscle cells. The data is expressed as a percentage of the maximum response to 1 μM bradykinin (expressed as an increase in F_{340}/F_{380} ratio minus the basal fluorescence ratio). The curve was fitted by use of a logistic equation as described in Materials and Methods. Data are means ± SEM of three experiments.

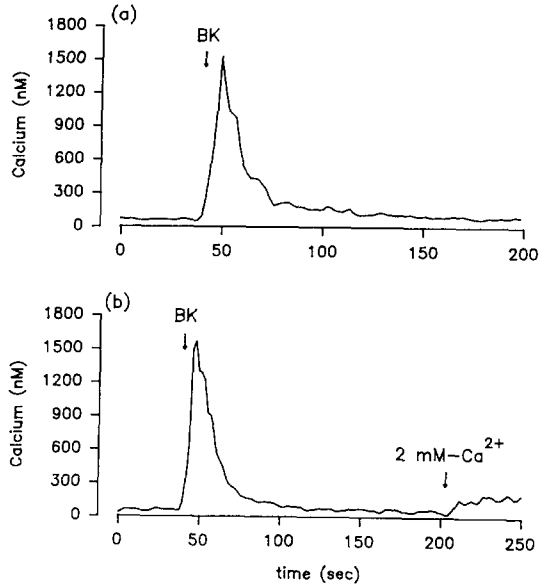

Fig. 3. The effect of bradykinin receptor stimulation on $[Ca^{2+}]_i$ in guinea-pig aortic smooth muscle cells. (a) In the presence of extracellular Ca^{2+} (2 mM); (b) in the presence of nominally Ca^{2+}-free buffer and 0.1 mM EGTA followed by the re-addition of extracellular Ca^{2+} (2 mM). Bradykinin (BK; 1 μM) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in 11 (a) and four (b) other experiments.

bradykinin (300 nM) had no effect on $[Ca^{2+}]_i$ (N = 3; see Fig. 4a). Pretreatment with the B_1-receptor antagonist des-Arg^9,[Leu^8]-bradykinin (10 μM; 2 min) did not attenuate bradykinin (100 nM) stimulated increases in $[Ca^{2+}]_i$ (98 ± 3% of control; N = 3; see Fig. 4b). However, pretreatment with the B_2-receptor antagonist D-Arg[Hyp^3,Thi^{5,8},D.Phe^7]-bradykinin (10 μM; 2 min) attenuated bradykinin (100 nM) stimulated increases in $[Ca^{2+}]_i$ by 43 ± 2.5% [N = 3; $P < 0.05$; $[Ca^{2+}]_i$ increased from 75 ± 12 to 913 ± 45 nM (N = 3) in control cells and from 83 ± 8 to 556 ± 45 nM (N = 3) in B_2-antagonist treated cells). An inhibition concentration curve for the B_2-receptor antagonist D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin in the presence of a constant bradykinin concentration (10 nM) produced an IC_{50} of 4 ± 0.7 μM (N = 4). In the presence of the B_2-antagonist (10 μM) the Ca^{2+} response to 10 nM bradykinin was 15 ± 5% (N = 4; $P < 0.05$) of that obtained in control experiments [$[Ca^{2+}]_i$ increased from 90 ± 9 to 320 ± 35 nM (N = 4) in control cells (Fig. 5a) and from 85 ± 7 to 145 ± 14 nM (N = 4) in B_2-antagonist treated cells (Fig. 5b)]. These data suggest that bradykinin B_2-receptors mediate the mobilization of intracellular Ca^{2+} in cultured guinea-pig aortic smooth muscle cells.

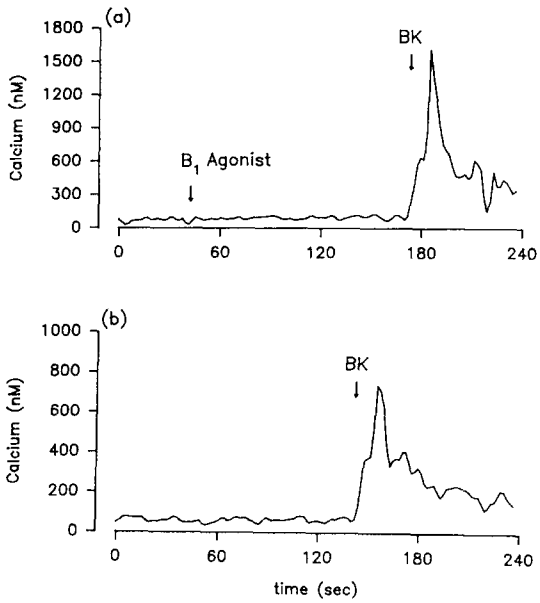

Fig. 4. Effect of (a) the bradykinin B_1-receptor agonist des-Arg^9-bradykinin on $[Ca^{2+}]_i$ and (b) the B_1-receptor antagonist des-Arg^9,[Leu^8]-bradykinin on bradykinin stimulated increases in $[Ca^{2+}]_i$. (a) The bradykinin B_1-receptor agonist des-Arg^9-bradykinin (300 nM) did not increase $[Ca^{2+}]_i$. Bradykinin (1 μM) was applied at the end of the experiment to show cell viability. (b) Pretreatment (2 min) with the B_1-receptor antagonist des-Arg^9,[Leu^8]-bradykinin (10 μM) had no effect on bradykinin stimulated Ca^{2+} responses (100 nM). The B_1-agonist and bradykinin (BK) were added where indicated. Similar results were obtained in two other experiments.

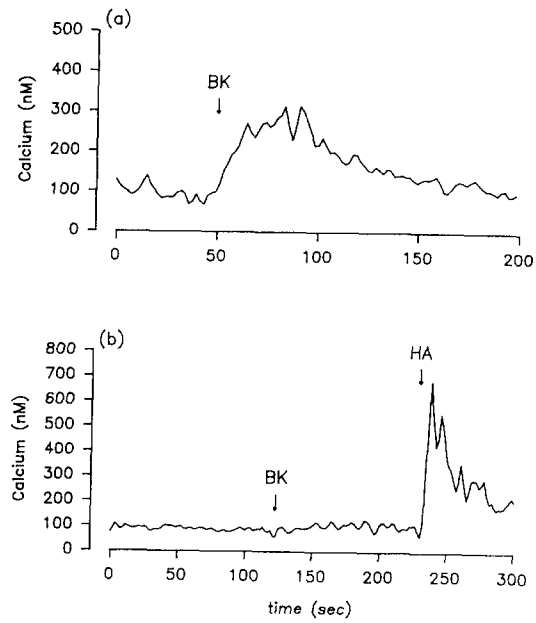

Fig. 5. The effect of the bradykinin B_2-receptor antagonist D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin on bradykinin stimulated increases in $[Ca^{2+}]_i$. (a) Control experiment in the presence of extracellular Ca^{2+} showing the Ca^{2+} response elicited by 10 nM bradykinin. (b) Cells treated for 2 min with 10 μM D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin before the addition of 10 nM bradykinin. Histamine was added at the end of the experiment to confirm the viability of the cells in this experiment. Experiments (a) and (b) were performed on the same batch of cells. Bradykinin (BK; 10 nM) and histamine (HA; 100 μM) were added where indicated. Similar results were obtained in three other experiments.

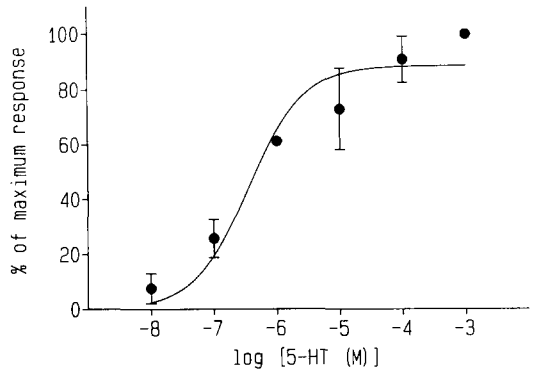

Fig. 6. Concentration–response curve for 5-HT stimulated increases in $[Ca^{2+}]_i$ in guinea-pig aortic smooth muscle cells. The data is expressed as a percentage of the maximum response to 1 μM bradykinin (expressed as an increase in F_{340}/F_{380} ratio minus the basal fluorescence ratio). The curve was fitted by use of a logistic equation as described in Materials and Methods. Data are means ± SEM of four experiments.

5-HT stimulated increases in intracellular Ca^{2+}

Stimulation of 5-HT receptors also increased $[Ca^{2+}]_i$ in cultured guinea-pig aortic smooth muscle cells (log $EC_{50} = -6.1 \pm 0.1$ M (N = 4) see Fig. 6).

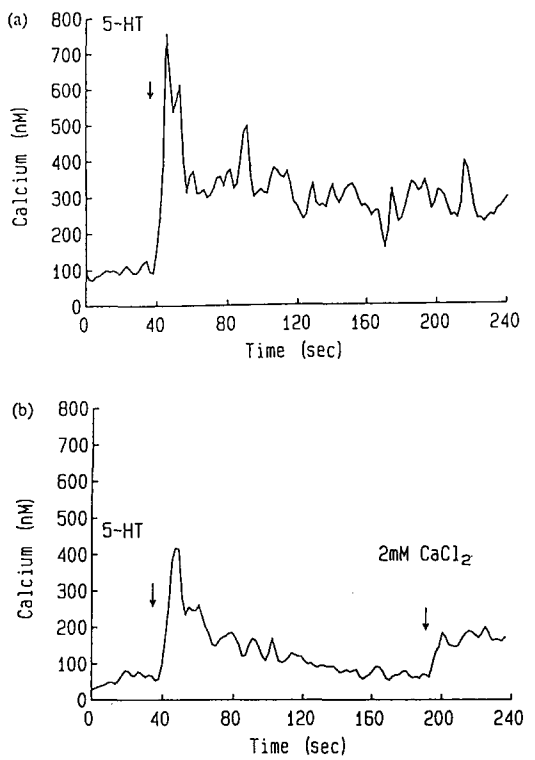

Fig. 7. The effect of 5-HT receptor stimulation on $[Ca^{2+}]_i$. (a) In the presence of extracellular Ca^{2+} (2 mM); (b) in the presence of nominally Ca^{2+}-free buffer and 0.1 mM EGTA followed by the re-addition of extracellular Ca^{2+} (2 mM). 5-HT (100 μM) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in 13 (a) and four (b) other experiments.

In the presence of extracellular Ca^{2+} 5-HT (100 μM) increased $[Ca^{2+}]_i$ from of 80 ± 7 to 615 ± 50 nM (N = 14). The response was fairly well maintained (compared to bradykinin) and declined slowly towards basal levels (Fig. 7a). In the absence of extracellular Ca^{2+} (Ca^{2+}-free buffer containing 0.1 mM EGTA) 5-HT (100 μM) increased $[Ca^{2+}]_i$ from 78 ± 6 to 410 ± 25 nM (N = 5). Removing extracellular Ca^{2+} resulted in a transient response to 5-HT, i.e. $[Ca^{2+}]_i$ returns to basal levels approximately 100 sec after stimulation (Fig. 7b). The attenuation of the maintained phase of the response is clearly demonstrated if the percentage of the maximum response (obtained using 100 μM 5-HT) remaining after 100 sec is compared between experiments performed in Ca^{2+}-free and Ca^{2+} containing buffer. In Ca^{2+}-free buffer (0.1 mM EGTA) the percentage of the maximum 5-HT response remaining at 100 sec is significantly lower (6 ± 2%, N = 5; $P < 0.05$) than that obtained in the presence of extracellular Ca^{2+} (42 ± 7%; N = 5). These data suggest the maintained phase of the 5-HT Ca^{2+} response is dependent upon extracellular Ca^{2+} and may involve Ca^{2+} influx (entry) across the plasma membrane. The addition of extracellular Ca^{2+} (2 mM) after the cells have been stimulated with 100 μM 5-HT in the absence of extracellular Ca^{2+} (to release intracellular Ca^{2+}) produced a second increase in $[Ca^{2+}]_i$ which is indicative of calcium influx (see Fig. 7b). In these experiments the addition of extracellular Ca^{2+} increased $[Ca^{2+}]_i$ from 98 ± 10 to 210 ± 15 nM (N = 5). The class of 5-HT receptor mediating these increases in $[Ca^{2+}]_i$ was examined using the selective 5-HT_2 antagonist ketanserin. Ketanserin (100 nM; 15 min preincubation) completely inhibited the response to 100 μM 5-HT ($IC_{50} = 2 \pm 0.4$ nM; N = 3; see Fig. 8). These data suggest 5-HT mediates increases in $[Ca^{2+}]_i$ via the 5-HT_2-receptor subtype.

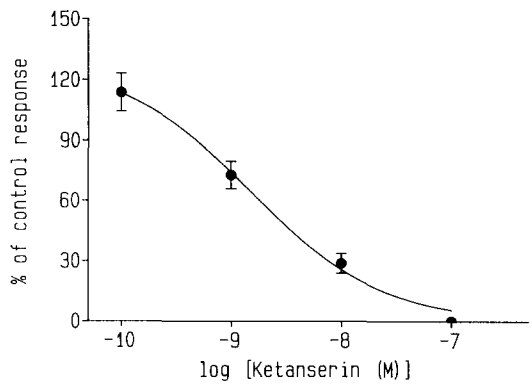

Fig. 8. The effect of the 5-HT_2 receptor antagonist ketanserin on 5-HT stimulated increases in $[Ca^{2+}]_i$. Cells were incubated with the relevant concentration of ketanserin for 15 min prior to stimulation with 5-HT. The data are expressed as a percentage of the response to 100 μM 5-HT (expressed as an increase in F_{340}/F_{380} ratio minus the basal fluorescence ratio). The curve was fitted by use of a logistic equation as described in Materials and Methods. Data are means ± SEM of three experiments.

DISCUSSION

This study demonstrates that bradykinin and 5-HT are able to stimulate increases in $[Ca^{2+}]_i$ in cultured guinea-pig aortic smooth muscle cells. The evidence obtained in this study suggests that the bradykinin-stimulated increase in $[Ca^{2+}]_i$ is mediated by the B_2 receptor subtype since the B_2-receptor antagonist D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin inhibited the response to bradykinin, whereas the B_1-receptor antagonist des-Arg^9,[Leu^8]-bradykinin was without effect. Furthermore the B_1-receptor agonist des-Arg^9-bradykinin [9] did not stimulate an increase in $[Ca^{2+}]_i$. The 5-HT_2 receptor antagonist ketanserin potently inhibited (IC_{50} 2 nM) the 5-HT stimulated increase in $[Ca^{2+}]_i$ suggesting the involvement of the 5-HT_2-receptor subtype. These data are in agreement with the previously reported expression of 5-HT_2 and bradykinin B_2-receptors coupled to inositol phospholipid hydrolysis in guinea-pig aortic smooth muscle cells [7].

The Ca^{2+} responses elicited by 5-HT and bradykinin are comprised of two distinct components: (1) release of intracellular Ca^{2+} (which in view of the inositol phospholipid hydrolysis data is presumably mediated through inositol 1,4,5-trisphosphate [10] and (2) influx of extracellular Ca^{2+} through Ca^{2+} entry pathways in the plasma membrane. Interestingly, the extracellular Ca^{2+} component (Ca^{2+} influx) is most prevalent in 5-HT mediated Ca^{2+} responses. A possible explanation is that 5-HT stimulation activates a voltage-dependent Ca^{2+} channel, similar to that reported in rat aortic smooth muscle cells [5]. However, the mechanism(s) involved in 5-HT and bradykinin stimulated Ca^{2+} influx in guinea-pig aortic smooth muscle cells remains to be unravelled. Several mechanisms have been proposed to explain agonist stimulated Ca^{2+} influx. These include the following: (1) the receptor may be directly linked to a Ca^{2+} channel, an example of which is the ATP-activated Ca^{2+} channel found in rabbit arterial smooth muscle [11]; (2) a second messenger operated Ca^{2+} channel which may be activated by increases in $[Ca^{2+}]_i$ or Ins(1,3,4,5)P_4, as recently reported in bovine aorta endothelial cells [12]; (3) a G-protein coupled Ca^{2+} channel; or (4) depletion of the intracellular Ca^{2+} store stimulates the production of an unknown messenger which activates Ca^{2+} influx [13]. Recently, cytochrome P450 has been implicated in store-dependent Ca^{2+} influx [14].

In summary, cultured guinea-pig aortic smooth muscle cells appear to provide an ideal system in which to study the mechanisms involved in the regulation of agonist-stimulated increases in $[Ca^{2+}]_i$ in vascular smooth muscle.

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