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B1 and B2 Kinin Receptors Mediate Distinct Patterns of Intracellular Ca^{2+} Signaling in Single Cultured Vascular Smooth Muscle Cells

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SUMMARY

Stimulation of B1 and B2 kinin receptors on cultured rabbit superior mesenteric artery smooth muscle cells with des-Arg9-bradykinin (DBK) and bradykinin (BK), respectively, results in significantly different patterns of intracellular Ca^{2+} mobilization. Single-cell fluorescence imaging of Fura-2-loaded cells revealed that although both DBK and BK initially triggered similar rapid increases in cytosolic free Ca^{2+}, the DBK response was biphasic and sustained, whereas the BK response was transient. The DBK response was maintained for ≥20 min with the second phase characterized by an elevated plateau and/or base-line oscillations. The BK response was limited to an initial transient peak with the exception of a few cells, which after a prolonged latency period, exhibited weak but regular base-line oscillations. The initial BK- and DBK-stimulated rises in cytosolic free Ca^{2+} were dependent on the release of Ca^{2+} from intracellular stores that seemed to be common for the two agonists. On the other hand, the continuation of the sustained phase of the DBK response required the influx of extracellular Ca^{2+}, as well as continuous receptor occupancy by the agonist. Stimulation of cells with DBK followed by washing and restimulation with the same agonist within ≤2 min resulted in a second B1 receptor response that was not significantly different from the first response. In contrast, the same protocol with BK yielded a dramatically decreased second B2 receptor response. This attenuation did not seem to be due to a lack of Ca^{2+} in the agonist-sensitive intracellular stores because DBK elicited a full response after BK stimulation. This study shows that in single cultured RSMA smooth muscle cells, agonist stimulation of B1 receptors generates a sustained intracellular Ca^{2+} signal, whereas stimulation of B2 receptors promotes rapid and homologous desensitization, resulting in a transient Ca^{2+} signal. These distinct receptor-specific patterns of Ca^{2+} mobilization imply significantly different roles for B1 and B2 kinin receptors in vascular smooth muscle cells.

Kinins are potent inflammatory peptide mediators that promote contraction and relaxation of numerous vascular and nonvascular smooth muscles (1–3). Two main kinin receptor subtypes have been delineated, and they were named B2 and B1. The B2 receptor mediates the action of BK, whereas the B1 receptor mediates the effect of the carboxypeptidase fragment DBK (1). The family of natural kinin agonists also includes Lys-BK, which is active on both B1 and B2 receptors, and des-Arg10-Lys-BK, which acts through the B1 receptor. B2 receptors are expressed constitutively in many tissues, whereas B1 receptors are expressed in very low numbers or not at all under nonpathological conditions but can be induced after trauma such as *in vitro* incubation or after systemic injections of a bacterial lipopolysaccharide (4–8). Recent cloning has revealed that both receptor subtypes are members of the superfamily of seven-transmembrane-domain, G protein-coupled receptors but exhibit a relatively low degree of homology (9–12).

Considering that BK and DBK are formed sequentially and that many cells, under appropriate conditions, express both B1 and B2 receptors, it is important to determine whether intracellular signals triggered through these two receptor subtypes are redundant or serve distinct physiological functions. Both DBK and BK relax the intact RSMA in an endothelium-dependent manner through interaction with B1 and B2 receptors, respectively (13–16). Both receptor subtypes are also present on cultured smooth muscle cells from RSMA, in which they mediate DBK and BK stimulation of InsP formation and the release of arachidonic acid metabolites (17).

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ABBREVIATIONS: BK, bradykinin; DBK, des-Arg9-bradykinin; DLBK, des-Arg9[Leu8]bradykinin; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; HBSS, Hanks' balanced salt solution; HIFBS, heat-inactivated fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RT, room temperature; InsP, inositol phosphate; MGTA, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; RSMA, rabbit superior mesenteric artery; $InsP_1$, inositol monophosphate; $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Agonist stimulation of InsP formation is a consequence of receptor-mediated stimulation of phospholipase C and hydrolysis of phosphatidylinositol bisphosphate, leading to the formation of the two second messengers $Ins(1,4,5)P_3$ and diacylglycerol (18). $Ins(1,4,5)P_3$ interacts with a specific receptor on the endoplasmic/sarcoplasmic reticulum that functions as a Ca^{2+} release channel to increase cytosolic free Ca^{2+} (19, 20). The patterns of intracellular Ca^{2+} mobilization can vary drastically among agonists, ranging from a monophasic and transient response to a multiphasic sustained response that includes elevated plateaus and oscillations (21, 22). Several theories about the mechanisms and significance of each response have been presented (22). Both sustained and transient Ca^{2+} responses have been described as integral and discrete signals in a variety of cell activities.

We used single-cell imaging of Fura-2-loaded cells to characterize the nature of the intracellular signaling pathways activated by agonist stimulation of B1 and B2 receptors in cultured RSMA smooth muscle cells and to evaluate their potential roles in vascular smooth muscle physiology. In this study, we show for the first time that agonist stimulation of these two kinin receptor subtypes in vascular smooth muscle cells results in markedly different patterns of Ca^{2+} mobilization, implying distinct intracellular signaling roles for the B1 and B2 receptors. We found that this difference in the Ca^{2+} signal is, at least in part, a consequence of homologous desensitization of the B2 receptor pathway.

Experimental Procedures

Materials. New Zealand White rabbits (2–3 kg) were obtained from a local supplier. DMEM, phosphate-buffered saline, HBSS, and Leibovitz's L-15 medium were obtained from GIBCO (Gaithersburg, MD); FBS from Hyclone Laboratories (Logan, UT); BK, DBK, DLBK, bovine serum albumin, and Type III trypsin from bovine pancreas from Sigma Chemical Co. (St. Louis, MO); Lys-BK (kallidin) from Sigma and Peninsula Laboratories (Belmont, CA); HOE-140 from Bachem (Torrance, CA); thapsigargin, ionomycin, and MGTA from Calbiochem (San Diego, CA); Fura-2/AM from Molecular Probes (Eugene, OR); dimethylsulfoxide from Aldrich (Milwaukee, WI); and collagenase (CLS I) from Worthington Biochemical Corp. (Freehold, NJ).

Cell isolation and culture. Smooth muscle cells were isolated from RSMA and cultured as described by Tropea *et al.* (17) with a few modifications. Briefly, after excision of the artery and removal of the adventitial layer through dissection, the tissue was incubated in 0.25% collagenase in HBSS for 20 min at 37° and then washed, minced, and incubated in 0.1% collagenase in growth medium (DMEM/10% HIFBS) for 4 hr at 37°. The resulting deaggregated cells were plated in growth medium, and the attached smooth muscle cells were gently washed after 24 hr. New cell preparations were routinely frozen at approximately passage 5, and thawed cells were used between passages 5 and 15. For Fura-2 experiments, the cells were plated ~1:3 onto glass coverslips that had been pretreated through incubation in growth medium. The plated cells were allowed to grow for ~48 hr, and experiments were performed at ~50–60% confluency.

Fura-2 loading and experimental procedure. Cells grown on glass coverslips were incubated in loading buffer (Leibovitz's L-15 medium supplemented with 15 mM HEPES, pH 7.4, 5 mM D-glucose, and 1 mg/ml bovine serum albumin) at 37° for 30 min. After exchange of the loading buffer, the cells were incubated in 5 μM Fura-2/AM (in 0.5% dimethylsulfoxide) at RT for 60 min followed by an additional 30 min at RT in the absence of Fura-2/AM. The loaded cells were stored in fresh loading buffer at 4° for 15–150 min. Just before each experiment, a coverslip was washed in RT HBSS, pH 7.4 (1.3 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.8 mM $MgCl_2$, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM D-glucose), mounted in a Leiden coverslip holder, and placed on a temperature-controlled microincubator stage (Medical Systems Corp.). The cells were allowed to equilibrate in 1 ml of HBSS to 37° before data acquisition was initiated. Compounds and washes were added manually with a pipette and removed under vacuum with the use of a stage-mounted flat-tipped microaspirator. Washes were accomplished through five exchanges of assay buffer over various periods of time as indicated in the figure legends. In experiments with EGTA, care was taken to maintain the HBSS at pH 7.4.

On consideration of previously established EC_{50} values for DBK (0.08 μM) and BK (0.001 μM) in these cells (17), 1.0 μM DBK and 0.1 μM BK were used in many of the studies of the B1 and B2 receptor-mediated Ca^{2+} responses, respectively. Because lower concentrations of each agonist (0.01 and 0.1 μM DBK, 0.001 and 0.01 μM BK), which would submaximally activate the receptors, produced the same characteristic patterns of Ca^{2+} responses, specific concentrations were chosen to optimize conditions for the pharmacological and desensitization experiments. In pharmacological experiments, a 100-fold excess of DLBK (10 μM) was used to compete DBK (0.1 μM), whereas a 10-fold excess of HOE140 (1 μM) was used to compete BK (0.1 μM). In desensitization experiments, we used 0.01 μM and 0.001 μM BK and 0.1 μM DBK. These lower concentrations were chosen to be able to efficiently remove the agonists during the washes after the initial agonist stimulation.

Data acquisition and analysis. The digital imaging fluorescence microscopy system included a Zeiss axiovert inverted microscope configured for Ca^{2+} ratio imaging equipped with a xenon lamp and quartz objectives, a Hamamatsu Intensified CCD camera, and Image 1/FL software with pseudocolor display and on-screen graphic representation capabilities (Universal Imaging Corp., West Chester, PA). During data acquisition, Fura-2 was alternately excited at 340 nm, for the Ca^{2+}-bound signal, and 380 nm, for the Ca^{2+}-unbound signal, and the emissions were monitored at 510 nm. The cytosolic free Ca^{2+} signal, determined by the ratio of the fluorescent emissions of matched 340-nm/380-nm excitations (23), is presented in the figures as F_{340}/F_{380}. After acquisition and processing, the data were imported to the scientific graphics program FigureP (Biosoft, Ferguson, MO) for analysis and represented as a representative individual cell or as the average of the cells in a field, as noted in the figure legends.

Estimation of the basal cytosolic free Ca^{2+} level in RSMA smooth muscle cells. Experiments to estimate the basal level of cytosolic free Ca^{2+} were performed based on the *in situ* method described by Thomas and Delaville (24) and yielded an average of 150 ± 11 nM (standard error, 30 cells). After determining the 340-nm/380-nm and 380-nm minimum and maximum values in the nominal absence of free Ca^{2+} and in the presence of saturating Ca^{2+}, the basal level was calculated using the K_D value of 2.24×10^{-7} M (25).

Statistical analysis. In Fig. 1, the F_{340}/F_{380} value for each time point was expressed as the average ± standard error. In Figs. 6B, 7C, and 8, B–D, the peak F_{340}/F_{380} value for a response was calculated by subtracting the average of the values for each individual cell immediately before the agonist addition from the average of the values for the peaks for each individual cell. The peak F_{340}/F_{380} values for the second and third responses were compared with the first responses and analyzed for statistically significant difference by the paired Student's t test. A value of $p < 0.05$ was considered statistically significant.

Results

Agonist stimulation of B1 and B2 receptors increases cytosolic free Ca^{2+}. The basal cytosolic free Ca^{2+} concentration in cultured RSMA smooth muscle cells was estimated to be 150 ± 11 nM (30 cells). Analysis of individual cells (45 cells) from control time

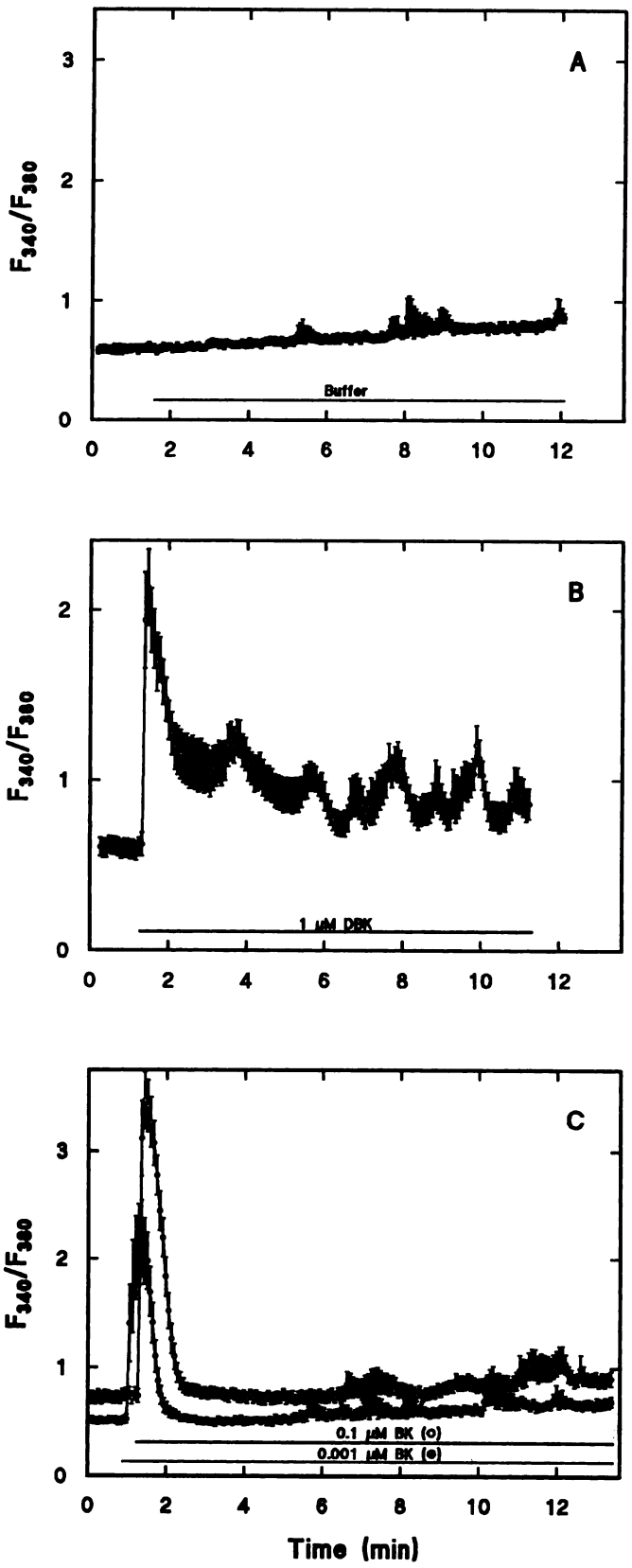

Fig. 1. DBK and BK stimulate increases in cytosolic free Ca^{2+} in cultured RSMA smooth muscle cells. During the times indicated (*bars*), the cells were exposed to buffer (A), 1 μM DBK (B), or 0.1 μM BK (C). Traces are average ± standard error of cells in representative fields of 15 cells (A), 13 cells (B), and 16 cells (C). See Table 1 for related data and statistical analysis.

courses (4) revealed that during incubation periods of 12 min, 18% of the cells spontaneously generated a single Ca^{2+} spike and, occasionally, multiple spikes, which in an average trace (15 cells) appear as irregular fluctuations of the base-line (Fig. 1A). Agonist stimulation with DBK resulted in a rapid elevation of cytosolic free Ca^{2+} (Fig. 1B). The average signal from a field of cells (13 cells) shows that after the initial peak, the DBK-stimulated response declined to a sustained second phase of elevated Ca^{2+} that fluctuated irregularly around a plateau that was significantly above base-line (Fig. 1B). Traces of individual cells (Fig. 2, A–C, and Table 1) revealed that in 51% of the cells, the sustained phase consisted exclusively of base-line oscillations (Fig. 2A). The frequency of oscillations varied from cell to cell (mean = 0.6 peaks/min) but was constant within each cell. In 47% of the cells, the sustained phase of elevated Ca^{2+} was characterized by a plateau, and the level was similar from cell to cell (Fig. 2B). However, over a period of 10 min of DBK exposure, in half of these cells, the sustained phase spontaneously converted to base-line oscillations (Fig. 2C). Analysis of a field of cells (16 cells) revealed that stimulation with BK also resulted in a rapid rise in the cytosolic free Ca^{2+} level (Fig. 1C). In contrast to DBK, the BK response pattern was transient, with the Ca^{2+} level rapidly returning to and remaining at a level that was not significantly different from the basal level for ~8 min. This response pattern was not concentration dependent because 0.001 μM BK elicited the same pattern as 0.1 μM BK (Fig. 1C). Comparison of individual cells within the same field showed that the profile of the base-line after a BK-stimulated Ca^{2+} transient was not significantly different from that in a cell that did not respond to BK (compare Fig. 2, D and E). Although the shape of the BK-stimulated Ca^{2+} peak in some individual cells included a tail or, rarely, a shoulder, the majority of the peaks were quite sharp. With the exception of spontaneous activity described above, experiments with prolonged exposure to BK showed that after the initial transient peak, the Ca^{2+} level in most cells continued at basal level throughout the remaining incubation period (≥20 min) (Fig. 2E). However, in a minority of the cells (34%), regular but low-frequency oscillations were observed but only after a considerable latency period (8.7 ± 0.3 min) (Fig. 2F), The onset of this occasional activity was always delayed and never contiguous with the initial transient peak.

Fig. 3, A and B, shows that individual cells responded to both BK and DBK. Inclusion of 100-fold excess of DLBK (10 μM), a specific B1 receptor antagonist, completely inhibited the response to 0.1 μM DBK but did not perturb the response to 0.1 μM BK (Fig. 3C). In the presence of 1 μM HOE140, a specific B2 receptor antagonist, the response to 0.1 μM BK was completely inhibited, whereas the response to 0.1 μM DBK was not inhibited (Fig. 3D). The addition of 1.0 μM HOE140 after the onset of the delayed oscillations that appeared in some cells after prolonged BK exposure (Fig. 2F) also completely blocked this Ca^{2+} release (data not shown). These results clearly show that DBK stimulates intracellular Ca^{2+} mobilization in cultured RSMA smooth muscle cells through a B1 receptor, whereas BK stimulates this response through a B2 receptor, and that the two receptor subtypes are localized on the same cell.

We previously reported that in cultured RSMA smooth muscle cells, low concentrations (EC_{50} = 0.0006 μM) of the kinin agonist Lys-BK stimulate InsP formation only through B2 receptors, whereas the response to higher concentrations (EC_{50} = 0.05 μM) of this agonist includes B1 receptors (17). As shown in Fig. 4A, after the addition of 0.001 μM Lys-BK, a concentration sufficiently low to stimulate InsP formation exclusively through B2 receptors, individual cells exhibited a transient pattern of intracellular Ca^{2+} mobilization. The same response was observed with 0.01 μM Lys-BK. The addition of 0.1 μM Lys-BK, however, resulted in a Ca^{2+} response that was biphasic and oscillating, and a further addition of Lys-BK at 1 μM increased the frequency, but not the amplitude, of the oscillations. In the presence of 10 μM DLBK, the response to 0.1 μM Lys-BK was transient, which is characteristic of the BK B2 receptor response in these cells (Fig. 4B). On the other hand, in the presence of 1 μM

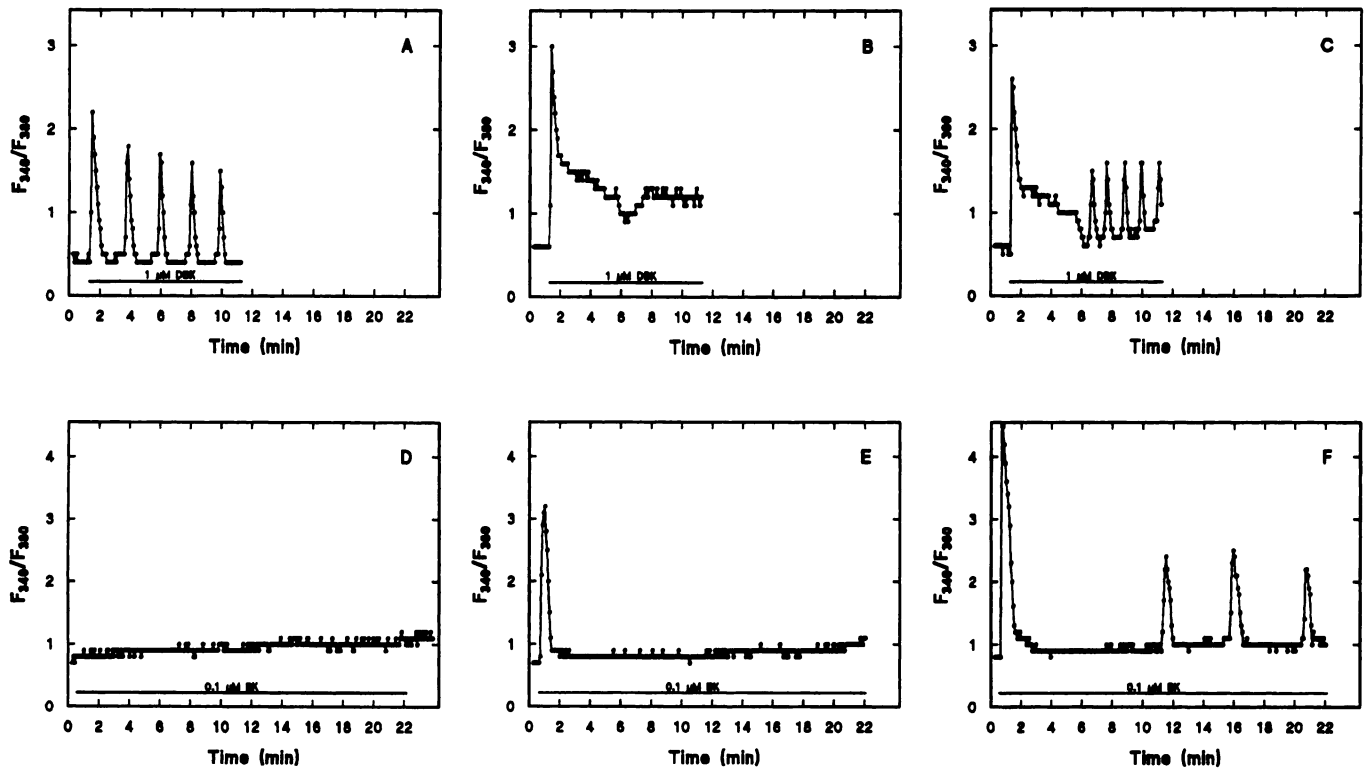

Fig. 2. Individual cells respond with multiple patterns of biphasic increases in cytosolic free Ca^{2+}. During the times indicated (*bars*), the cells were exposed to 1 μM DBK (A–C) for ~12 min or 0.1 μM BK (D–F) for ~22 min. Traces are of individual cells from representative fields of 13 cells (A–C) and 12 cells (D–F). See Table 1 for related data and statistical analysis.

TABLE 1

DBK- and BK-stimulated intracellular Ca^{2+} mobilization in single cultured RSMA smooth muscle cells

Agonist	Total no. of cells (no. of expts.)	No. of cells responding with initial Ca^{2+} transient (% of total)	Sustained Ca^{2+} phase[a]		
			Oscillations only	Plateau ± oscillations	No sustained phase
				%	
DBK (1 μM)	128 (9)	117 (91)	51	47	2
BK (0.1 μM)	89 (7)	70 (79)[b]	0	0	0

[a] A response was categorized as sustained when an initial peak was immediately followed by a continuing Ca^{2+} signal, either in the form of a plateau significantly elevated above the baseline or with the regular cycling of Ca^{2+} originating at the baseline (oscillations). Percent values are based on the number of responding cells.
[b] After a prolonged latency period (8.7 ± 0.3 min), and consequently not continuous with the initial peak, regular but very low frequency oscillations were observed in 34% of BK-stimulated cells.

HOE140, the response to 0.1 μM Lys-BK displayed all of the characteristics that are typical of the DBK B1 receptor response in these cells (Fig. 4C). These results strongly suggest that Lys-BK stimulates intracellular Ca^{2+} mobilization in these cells directly through both B1 and B2 receptors. However, an alternative explanation for the B1 receptor-like effects of Lys-BK is the degradation of this agonist to the potent B1 receptor agonist des-Arg10-Lys-BK by cellular carboxypeptidases during the course of an experiment. This possible explanation was evaluated by pretreatment of cells with 10 μM MGTA, a carboxypeptidase inhibitor. The response to 1 μM Lys-BK in the presence of MGTA remained (data not shown). Considering that little if any degradation of Lys-BK is likely to occur in the time frame between the addition of and the response to this agonist (≤1 sec), our results strongly argue that Lys-BK acts directly on B1 receptors as well as B2 receptors in these cells.

B1 and B2 receptor agonists mobilize Ca^{2+} from intracellular stores. Cells were pretreated with 2 mM EGTA for 2 min to chelate extracellular Ca^{2+}. Fig. 5, A and B, shows that in single representative cells, EGTA had no apparent effect on the initial increase in cytosolic free Ca^{2+} in response to either BK (0.1 μM) or DBK (0.1 μM), indicating that the Ca^{2+} stores supplying the initial responses are intracellular. In contrast, continuation of the sustained phase of the DBK response was entirely dependent on extracellular Ca^{2+}. Under these conditions, the second phase consisted of a maximum of one Ca^{2+} spike. In Fig. 5C, which shows the analysis of two individual representative cells, the addition of EGTA during the sustained response to 1 μM DBK resulted instantaneously in a drop in the cytosolic free Ca^{2+} from plateaus to basal levels and termination of the oscillations. Again, one Ca^{2+} spike was subsequently observed after both plateaus and oscillations. Thus, the second phase of the response to DBK, with either 0.1 or 1 μM, requires the influx of extracellular Ca^{2+} to remain sustained. Nifedipine (0.1 μM) had no effect on the sustained phase of the DBK response, indicating that L-type voltage-sensitive plasma membrane Ca^{2+} channels were not involved in this event (data not shown).

Several types of agonist-sensitive intracellular Ca^{2+} stores have been identified in cells. One group is sensitive to and can be emptied by the Ca^{2+}-ATPase inhibitor thapsigargin and is generally considered to include stores that are $Ins(1,4,5)P_3$ sensitive (26–28). Another group includes the caffeine- and ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release channel (29). As shown by the average trace of a field of cells (Fig. 6A), the addition of 1 μM thapsigargin to RSMA smooth muscle cells resulted in an increase in the cytosolic free Ca^{2+}. The initial increase was biphasic, followed by a slow decline to a plateau

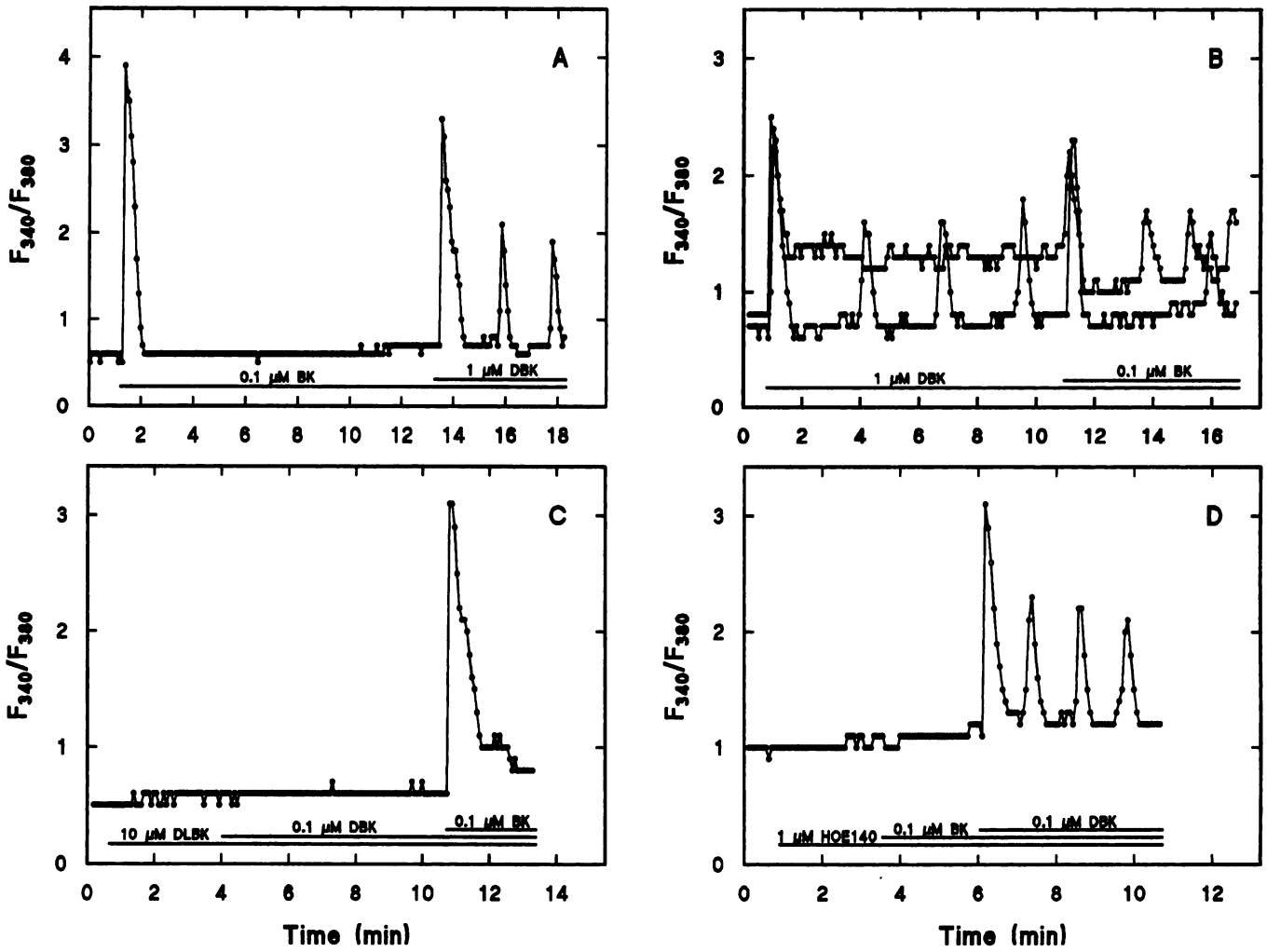

Fig. 3. DBK and BK stimulate increases in cytosolic free Ca^{2+} through B1 and B2 kinin receptors, respectively. During the times indicated (*bars*), the cells were exposed to various kinin agonists and antagonists: 0.1 μM BK followed by 1 μM DBK (A), 1 μM DBK followed by 0.1 μM BK (B), 10 μM DLBK followed first by 0.1 μM DBK then by 0.1 μM BK (C), or 1 μM HOE140 followed first by 0.1 μM BK then by 0.1 μM DBK (D). The traces are of individual cells from fields of 16 cells (A), 17 cells (B), 8 cells (C), and 12 cells (D) and represent typical responses. B, 2 cells are traced. Each experiment was performed two to four times with 7–17 cells/experiment.

level that was consistently above basal. In the presence of EGTA, the thapsigargin response was attenuated and monophasic and declined to the basal level. Therefore, in these cells, the depletion of the thapsigargin-sensitive stores stimulates refilling by influx of extracellular Ca^{2+} as has been described in several other cell types (30). After thapsigargin treatment, with or without EGTA pretreatment, neither BK nor DBK was able to stimulate an increase in cytosolic free Ca^{2+} (Fig. 6A). The addition of 10 mM caffeine to cultured RSMA smooth muscle cells had no effect on the cytosolic free Ca^{2+} or on the BK and DBK responses (data not shown). In addition, pretreatment with 10 μM ryanodine did not alter the BK or DBK response (data not shown). Therefore, caffeine- and ryanodine-sensitive Ca^{2+} stores do not serve as a source of Ca^{2+} for the BK and DBK responses and may not be present in these cells.

If DBK and BK mobilize common intracellular Ca^{2+} stores in these cells, the initial responses to high concentrations of each agonist should not be additive. In the experiment shown in Fig. 6B, 1 μM DBK was added first to determine the control level for an agonist-stimulated Ca^{2+} response in the field. DBK rather than BK was chosen as the control agonist because of the magnitude of BK-promoted desensitization of the B2 receptor signal (see below). After the cells were washed for ~1 min, 1 μM DBK plus 0.1 μM BK were then added to the cells, and, as shown in Fig. 6B, the second response was not significantly different from the first. Because the initial response to the agonists in combination was the same as the response to one of the agonists alone, these results provide further support that these two agonists mobilize common intracellular Ca^{2+} stores. These stores are sensitive to thapsigargin but insensitive to ryanodine/caffeine and provide Ca^{2+} for both the initial and the sustained responses.

Differential regulation of B1 and B2 receptor agonist-stimulated increases in cytosolic free Ca^{2+}. Fig. 7A shows that in individual cells in which the sustained phase of the response to 0.1 μM DBK was characterized by either a plateau or base-line oscillations, the addition of an excess of DLBK during the sustained phase to displace receptor-bound DBK terminated both types of sustained responses almost instantaneously. Analysis of individual cells revealed that the DBK response was sustained at every concentration tested (Fig. 7B). The effect of a cumulative increase in the concentration of DBK was primarily that of an increase in the frequency of the oscillations, sometimes accompanied by a small increase in the initial response (Fig. 7B). The same effect was observed when the concentration of Lys-BK was increased above the threshold for B1 receptor stimulation (Fig. 4A). Taken together, these results show that like the initial response, the B1 agonist-stimulated sustained phase is dependent on agonist occupancy of the B1 receptor. Fur-

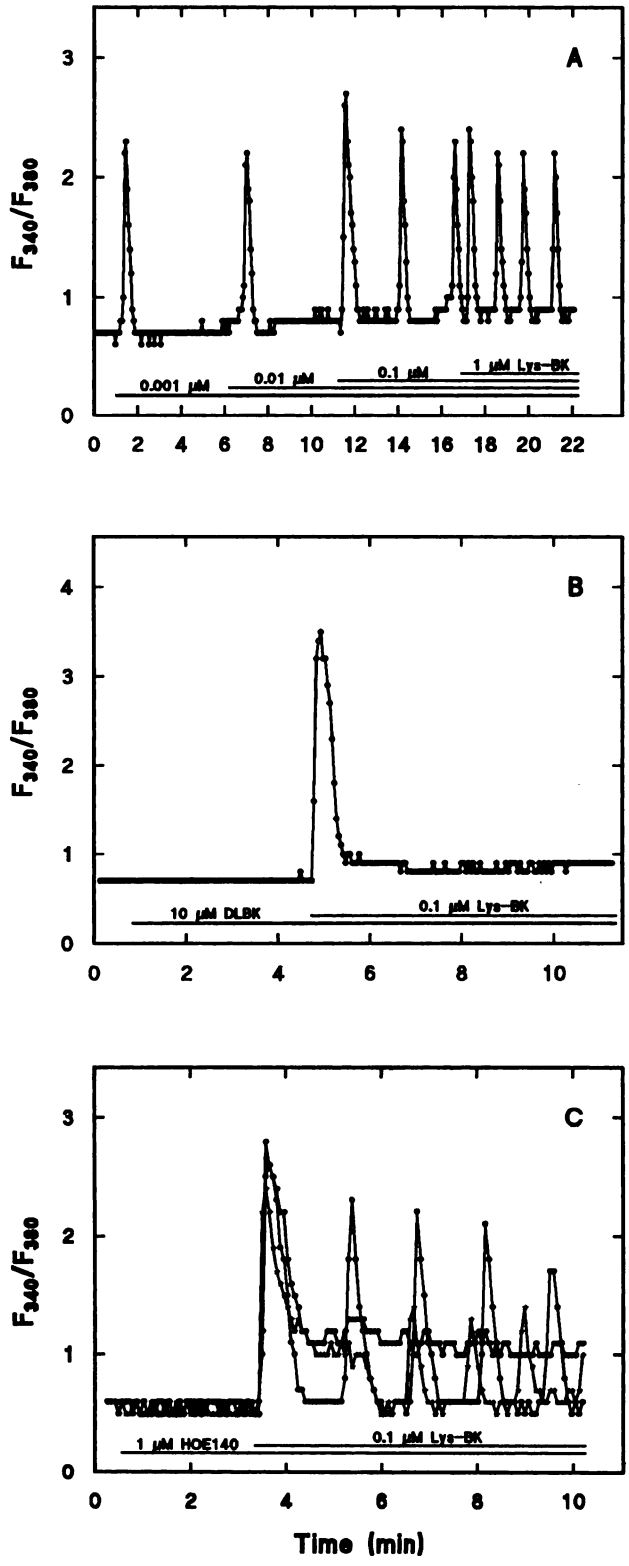

Fig. 4. Lys-BK stimulates an increase in cytosolic free Ca^{2+} through both B1 and B2 kinin receptors. During the times indicated (*bars*), the cells were exposed to Lys-BK and various kinin antagonists: sequential additions of 0.001, 0.01, 0.1, and 1 μM Lys-BK (A); 10 μM DLBK followed by 0.1 μM Lys-BK (B); and 1 μM HOE140 followed by 0.1 μM Lys-BK (C). The traces are of individual cells from fields of 15 cells (A), 12 cells (B), and 13 cells (C) and represent typical responses. C, 3 cells are traced. Each experiment was performed two or three times with 9–18 cells/experiment.

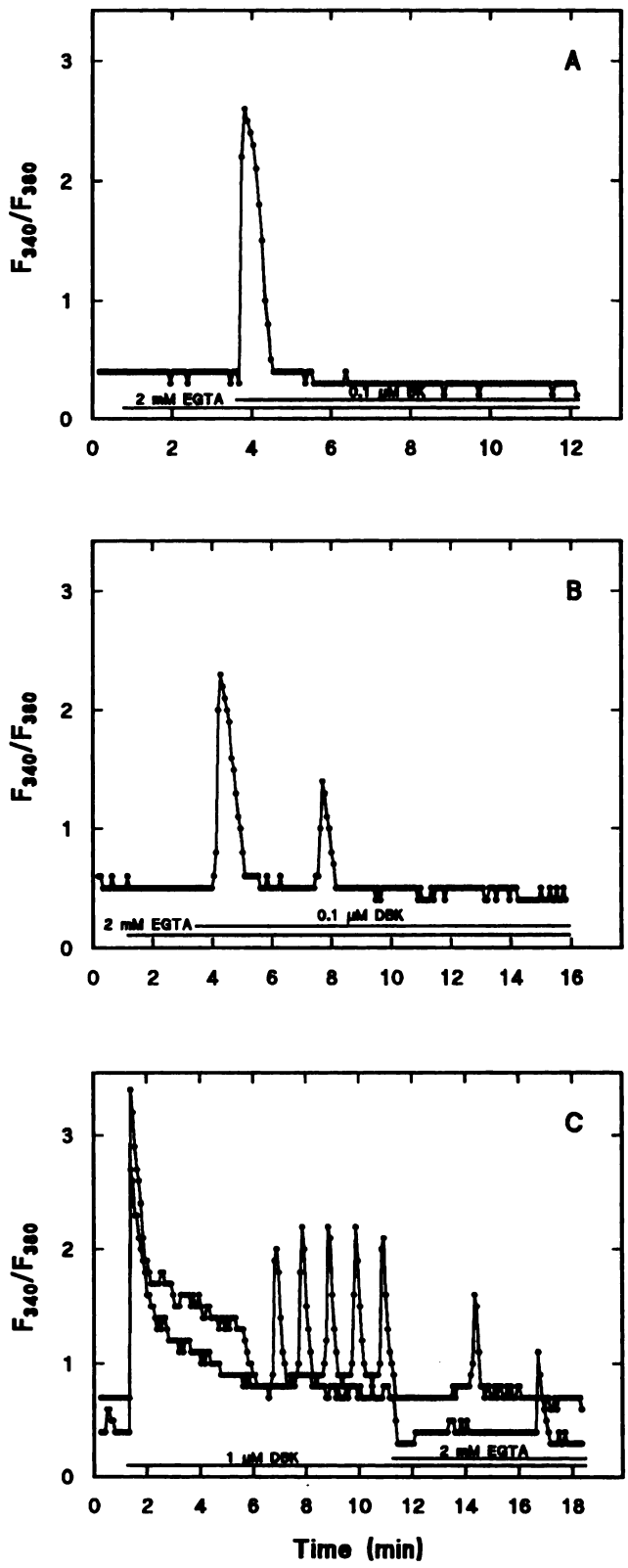

Fig. 5. Extracellular Ca^{2+} plays an important role in sustained increases in cytosolic free Ca^{2+}. During the times indicated (*bars*), extracellular Ca^{2+} was chelated before or after the addition of various kinin agonists: 2 mM EGTA followed by 0.1 μM BK (A), 2 mM EGTA followed by 0.1 μM DBK (B), and 1 μM DBK followed by 2 mM EGTA (C). Traces are of individual cells from fields of 18 cells (A), 9 cells (B), and 13 cells (C) and represent typical responses. C, 2 cells are traced. Each experiment was performed two or three times with 8–13 cells/experiment.

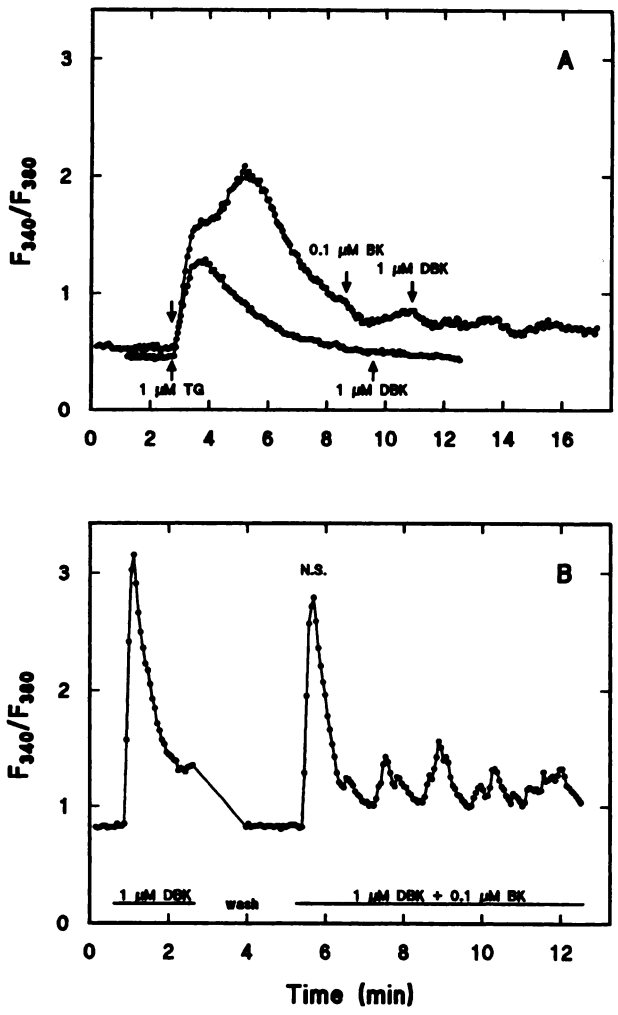

Fig. 6. DBK and BK mobilize Ca^{2+} from common thapsigargin-sensitive intracellular Ca^{2+} stores. A, Cells were incubated in the absence (○) or presence (●) of 2 mM EGTA. Then, 1 μM thapsigargin, 0.1 μM BK, and/or 1 μM DBK was added at the times indicated (*arrows*). B, Cells were exposed to 1 μM DBK, washed, and then re-exposed to 1 μM DBK plus 0.1 μM BK, as indicated (*bars*). The response to DBK alone serves as the control for the response to DBK plus BK. Traces are averages of cells from fields of 8 cells (A, ○), 10 cells (A, ●), and 11 cells (B). Each experiment was performed two times with similar results, with 6–12 cells/experiment. For statistical analysis, see Experimental Procedures. *N.S.*, not significantly different from peak F_{340}/F_{380} value of the response to DBK alone.

thermore, the B1 receptor remains responsive to successive increases in the concentration of the agonist. To test for agonist-induced desensitization of the B1 receptor signal, or the lack thereof, cells were stimulated with 0.1 μM DBK, washed free of the agonist for ~1 min, and then restimulated with 0.1 μM DBK. As shown in Fig. 7C, the second and third initial responses of a field of cells to this agonist were 79 ± 12% and 75 ± 10% of the first response, respectively. The second response was not significantly different from the first, whereas the third response was significantly different ($p < 0.05$). Clearly, agonist occupancy of the B1 receptor induces only very limited desensitization of the B1 receptor signal.

Fig. 8A shows a representative cell in which the BK response was transient at every concentration tested. Furthermore, the response decreased with each cumulative increase in the BK concentration, suggesting that the responsiveness of the B2 receptor signaling pathway rapidly attenuates during continued agonist stimulation. After stimulation of cells with 0.01 μM BK and washing for ~1 min, restimulation with the same concentration of BK resulted in an average response from the field of cells that was only 15 ± 5% of the first response (Fig. 8B). Attenuation was also observed with 0.001 μM BK (data not shown). The response recovered slightly but remained significantly attenuated after washing for 5 min (Fig. 8C) and 10 min (Fig. 8D). Thus, agonist occupancy of the B2 receptor seems to result in a dramatic desensitization of the Ca^{2+} response in these cells. As demonstrated by the representative single cell in Fig. 9A, although BK-treated cells were essentially unresponsive to the second challenge with the same concentration of BK, they partially responded to a third challenge with a 10-fold higher concentration of BK. Thus, the decreased responsiveness can be partially overcome by increasing the BK concentration, showing that Ca^{2+} stores are accessible under the described conditions. Thapsigargin (1 μM) was able to mobilize a significant amount of Ca^{2+} from a similar BK-stimulated cell after washing for only 1 min (Fig. 9B). A thapsigargin response of the same magnitude was observed in a BK-unresponsive cell in the same field (Fig. 9B). Consequently, as thapsigargin can release Ca^{2+} in these cells shortly after a BK-stimulated release of Ca^{2+}, these results suggest that the attenuated response to the second BK challenge is not due to depletion of intracellular Ca^{2+} stores. Like thapsigargin, DBK (1 μM) also stimulated Ca^{2+} mobilization in a BK-stimulated cell (Fig. 9C). This DBK response seemed to be maximal as a response of the same magnitude was seen in a BK-unresponsive cell in the same field (Fig. 9C). Thus, considering that BK and DBK seem to mobilize Ca^{2+} from common stores that are thapsigargin sensitive, these results suggest that the limited ability of these cells to respond to a second BK challenge is probably not due to a lack of accessible intracellular Ca^{2+} but rather to desensitization of the B2 receptor signal. Furthermore, this desensitization seems to be homologous because it seems to be restricted to the BK B2 receptor response.

B2 receptor agonists suppress the B1 receptor agonist-induced sustained phase of intracellular Ca^{2+} mobilization. Two related observations indicate that agonist stimulation of B2 receptors result in the suppression of elevated levels of intracellular free Ca^{2+}. First, as shown in Fig. 3B (●), in a cell exhibiting a DBK-maintained plateau, the BK-induced Ca^{2+} transient was followed by an immediate drop in the Ca^{2+} level and the subsequent appearance of oscillations. If the latter effect is B2 receptor agonist specific, then plateau-type sustained Ca^{2+} responses should not occur after stimulation of cells with Lys-BK at concentrations sufficiently high to activate both B1 and B2 receptors. Fig. 4A shows representative responses to different concentrations of Lys-BK and demonstrates the complete absence of any plateau responses. On the other hand, plateaus did occur after Lys-BK stimulation when the cells had been pretreated with HOE140 to block B2 receptors (Fig. 4C, ● and ⋆). Second, Fig. 3B (○) also shows that in a cell exhibiting DBK-driven base-line oscillations, the BK-induced Ca^{2+} transient was followed by a temporary delay in the resumption of the oscillations. Together, these results show that B2 agonists seem to suppress elevated levels of intracellular free Ca^{2+}.

Discussion

In a previous study (17), we found that populations of cultured RSMA smooth muscle cells constitutively express B1 and B2 kinin receptors that are coupled to stimulation of InsP production and arachidonic acid release. In this study, we continued to investigate kinin receptor-mediated intracellular signaling pathways by examining intracellular Ca^{2+} mobilization in single cells. Our results show that the patterns of intracellular Ca^{2+} signals elicited by agonist stimulation of B1 and B2 receptors in cultured vascular smooth muscle cells are significantly different: B1 receptor agonists generate a signal that is sustained, although in time possibly subject to very limited desensitization, whereas B2 receptor

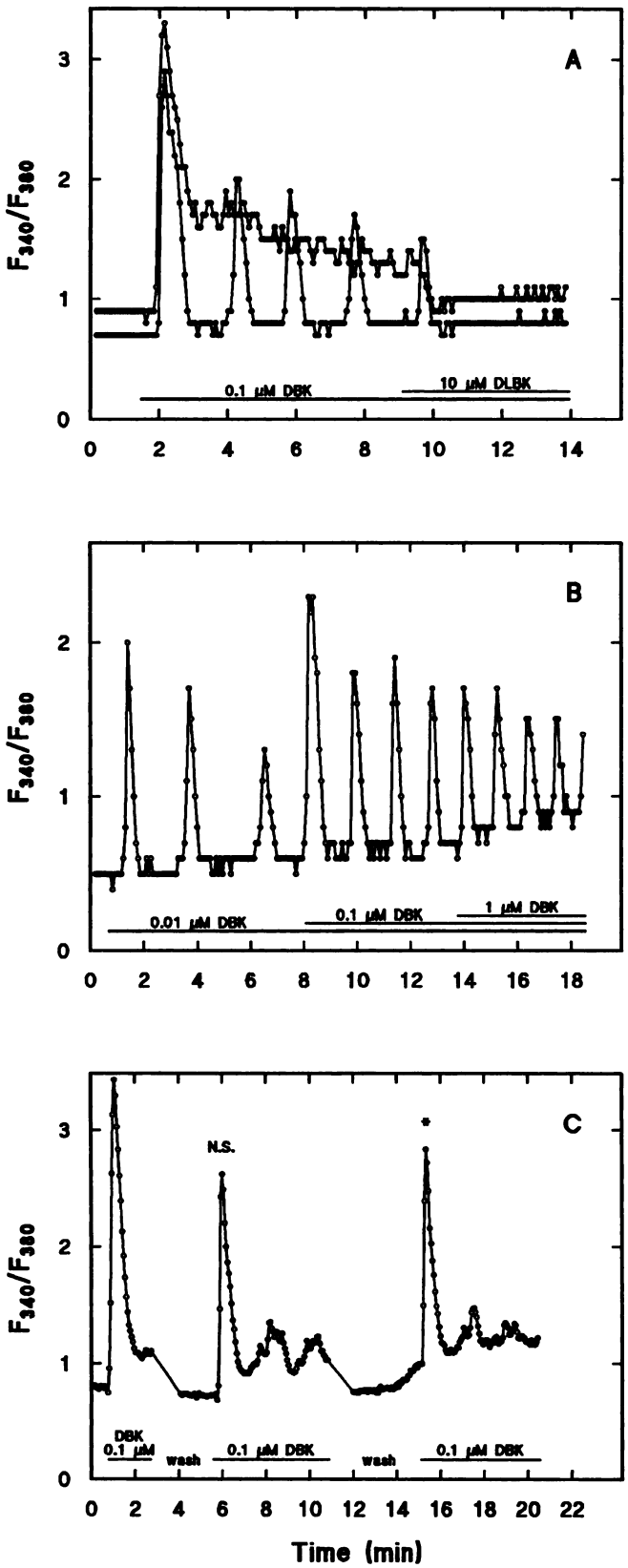

Fig. 7. The B1 receptor-mediated sustained increase in cytosolic free Ca^{2+} is dependent on agonist occupancy and sensitive to agonist concentration, and the response is subject only to limited desensitization. During the times indicated (*bars*), the cells were exposed to DBK and/or DLBK: 0.1 μM DBK followed by 10 μM DLBK (A), sequential additions of 0.01, 0.1, and 1 μM DBK (B), and 0.1 μM DBK, followed by washing and re-exposure to 0.1 μM DBK that was repeated one time(C). A, Traces of two individual cells from a field of 12 cells that represent typical responses. B, Individual representative cell from a field of 17 cells. C, Average trace of the cells from a representative field of 14 cells. *, $p < 0.01$, significantly different from peak F_{340}/F_{380} value of the first response. *N.S.*, not significantly different from peak F_{340}/F_{380} value of the first response. Each experiment was performed two times with 14–21 cells/experiment.

agonists generate a signal that is transient and highly sensitive to rapid and homologous desensitization.

Single-cell imaging of these cells showed that DBK and BK stimulate mobilization of intracellular Ca^{2+} in the same cells with pharmacological profiles indicative of B1 and B2 receptors, confirming that these two receptor subtypes are colocalized on individual cells. The kinin agonist Lys-BK stimulates intracellular Ca^{2+} mobilization through both receptors, albeit with higher potency through the B2 receptor, which agrees with results obtained in our previous study in which we monitored InsP production and arachidonic acid release in populations of these cells (17). In contrast, in pharmacological studies with rabbit aorta, the B1 receptor-mediated contractile response to Lys-BK was reported to be caused by the carboxypeptidase fragment des-Arg10-Lys-BK (31). However, recent radioligand binding studies indicate that Lys-BK exhibits affinities for B1 receptors in membranes from COS-7 cells transfected with human B1 receptors and in IMR-90 cells that are very close to the concentration necessary to elicit a B1 receptor-specific response in the RSMA smooth muscle cells (12). Attempts to measure kinin receptor radioligand binding on intact RSMA smooth muscle cells have been unsuccessful due to low receptor numbers.

The total amounts of InsP formed in RSMA smooth muscle cells after stimulation of the two receptors for 10 min were very similar. On the other hand, the relative levels of each InsP isomer and the kinetics of the responses were not similar (17). BK stimulation of B2 receptors resulted in rapid increases in the levels of $Ins(1,4,5)P_3$, inositol bisphosphate, and $InsP_1$. In the presence of Li^+, which blocks dephosphorylation of $InsP_1$, the increases in the former two products were transient, whereas $InsP_1$ reached a plateau. In contrast, under the same conditions, DBK stimulation of B1 receptors resulted in rapid but much lower increases in the levels of $Ins(1,4,5)P_3$ and inositol bisphosphate that remained elevated, whereas the level of $InsP_1$ continued to increase. The relatively lower maximum value of the $Ins(1,4,5)P_3$ level in response to B1 receptor stimulation has since been confirmed by other investigators (32, 33). These results show that although both receptors are coupled to a phosphatidylinositol bisphosphate-specific phospholipase C, the difference in the kinetics of the responses and the relative levels of $Ins(1,4,5)P_3$ formed suggest that the mechanics and/or regulation of the coupling of B1 and B2 receptors to phospholipase C is different. This difference could be due to the involvement of different G proteins, phospholipase C isozymes, and/or factors that regulate receptor/effector coupling.

A difference in the coupling of B1 and B2 receptors to the inositol phospholipid/Ca^{2+} pathway was clearly confirmed in this study. As with DBK-stimulated InsP formation, DBK-stimulated Ca^{2+} mobilization was also sustained. The initial peak of Ca^{2+} mobilization in response to this agonist was followed immediately by a sustained phase that lasted for

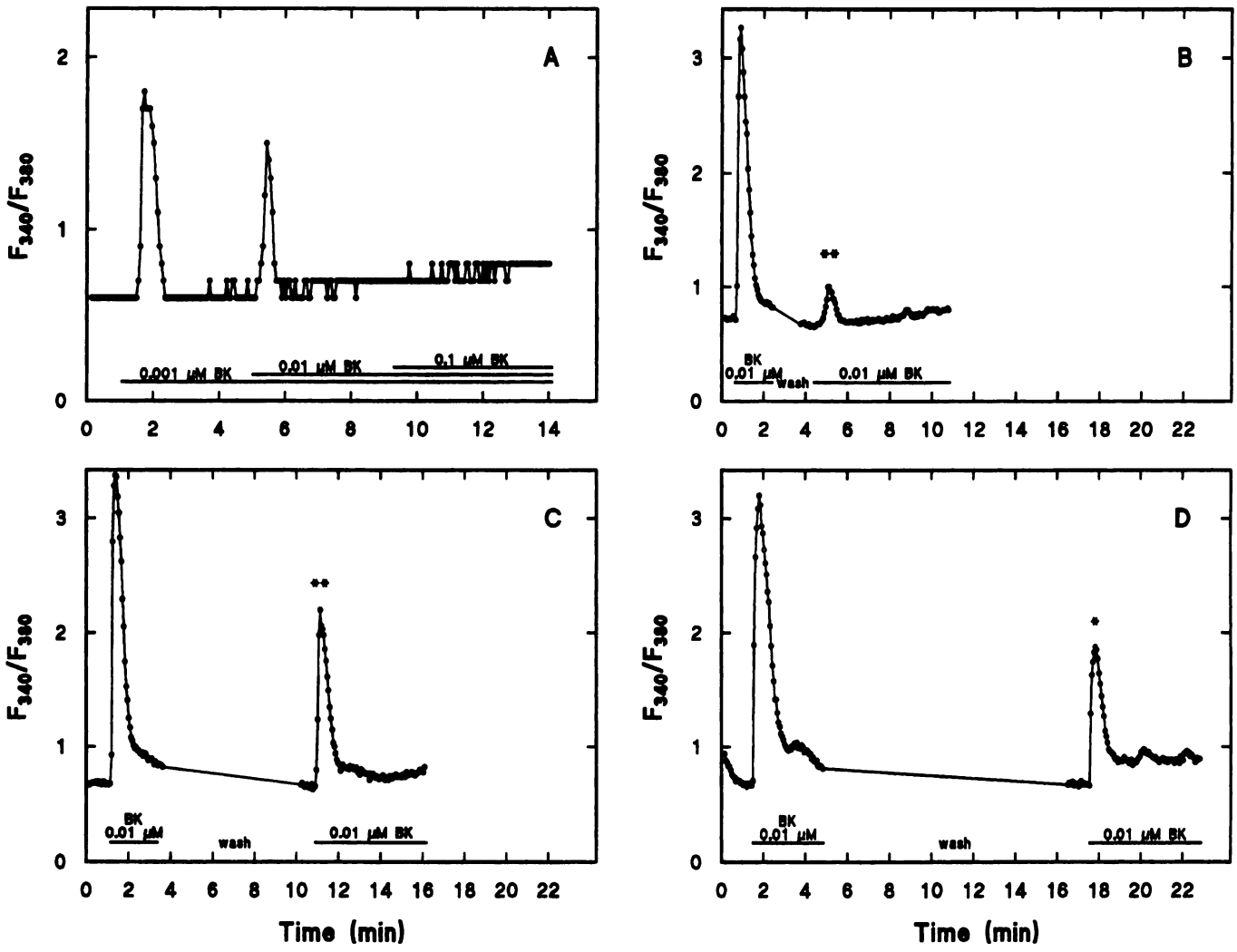

Fig. 8. The B2 receptor-mediated increase in cytosolic free Ca^{2+} is subject to pronounced desensitization. During the times indicated (*bars*), the cells were exposed to sequential additions of 0.001, 0.01, and 0.1 μM BK (A) or 0.01 μM BK, washed for ~1 min (B), ~5 min (C), or ~10 min (D), followed by re-exposure to 0.01 μM BK. A, Individual representative cell from a field of 13 cells. B–D, Average traces of cells from representative fields of 14 cells, 15 cells, and 14 cells, respectively. In D, the F_{340}/F_{380} values immediately after both the first and the second BK-stimulated transient were higher than those before the BK addition. As indicated by the values before the first BK addition, this field of cells displayed significant spontaneous BK-independent oscillatory activity. *, $p < 0.01$, **, $p < 0.001$, significantly different from peak F_{340}/F_{380} value of the first response. Each experiment was performed two times with 12–15 cells/experiment.

≥20 min. This phase was characterized by a plateau of elevated Ca^{2+} and/or base-line Ca^{2+} oscillations. On the other hand, BK-stimulated Ca^{2+} mobilization, which correlates closely with BK-stimulated InsP formation, triggered a transient response pattern, although after prolonged BK exposure, some weak oscillatory activity was observed in a few cells. A transient Ca^{2+} response to BK has been observed in a number of other cells, including, for example, N1E-115 (34, 35) and SH-SY5Y (36) neuroblastoma cells. In contrast, only very few studies have investigated DBK-induced Ca^{2+} responses. DBK induced a sustained plateau of elevated Ca^{2+} in single bovine tracheal smooth muscle cells (32) and bovine pulmonary artery endothelial cells (33). Interestingly, the BK response in the latter cells was sustained at submaximal levels of receptor activation, whereas the response was transient at a maximal level of receptor activation (33).

The initial Ca^{2+} responses to BK and DBK seemed to be insensitive to chelation of extracellular Ca^{2+} with EGTA, indicating that the primary source of Ca^{2+} for these responses is intracellular. The initial responses were not additive, and pretreatment with thapsigargin to deplete intracellular Ca^{2+} stores, including $Ins(1,4,5)P_3$-sensitive stores, prevented both BK and DBK from mobilizing Ca^{2+}. Caffeine was unable to release any intracellular Ca^{2+}, and neither caffeine nor ryanodine significantly perturbed either the BK or the DBK response. These results strongly indicate that the initial BK- and DBK-stimulated Ca^{2+} responses originate from common intracellular stores. Considering that both BK and DBK rapidly stimulate formation of $Ins(1,4,5)P_3$ in these cells (17), these results suggest that both agonists mobilize Ca^{2+} from stores that are sensitive to $Ins(1,4,5)P_3$.

In contrast, continuation of the sustained phase of the DBK response was abolished by the addition of EGTA, indicating that this phase was dependent on the influx of extracellular Ca^{2+}. Nifedipine had no effect on the sustained phase of the DBK response, clearly indicating that L-type voltage-sensitive plasma membrane Ca^{2+} channels are not involved in this event. In essentially all EGTA-treated cells,

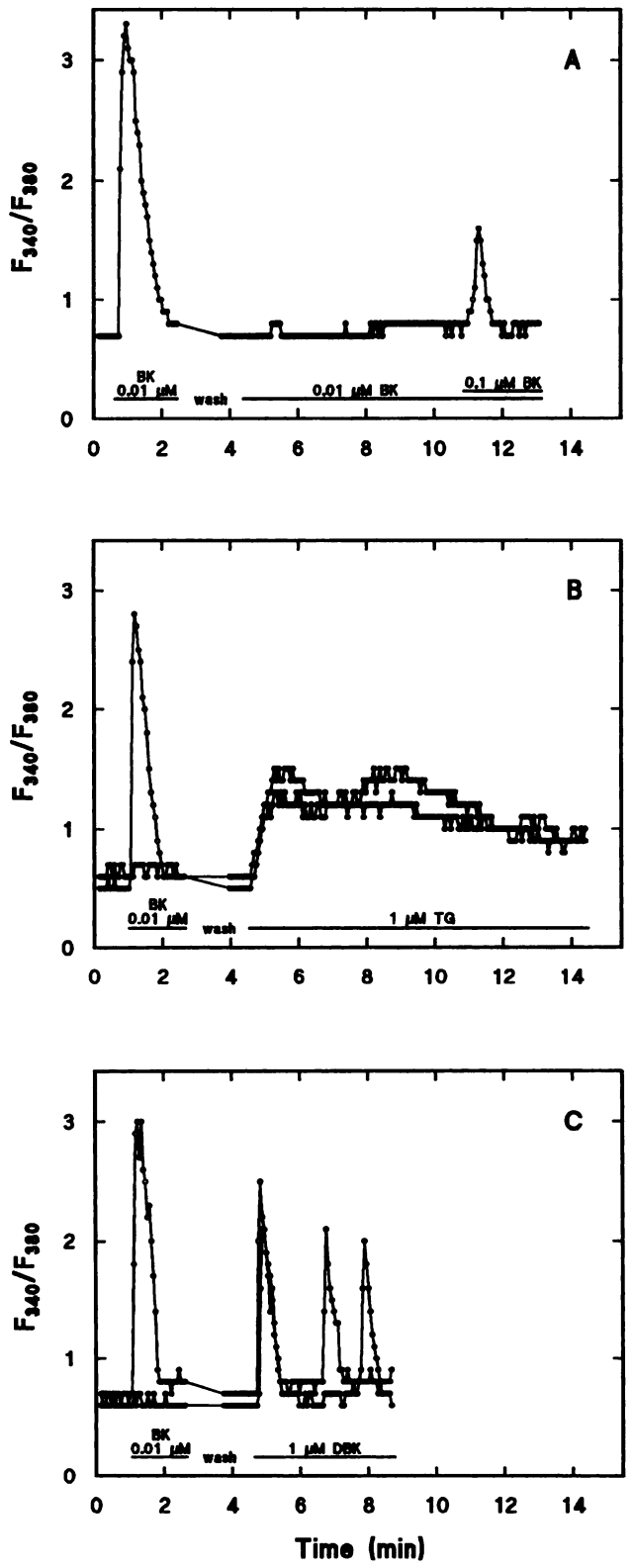

Fig. 9. The desensitization of the B2 receptor-mediated increase in cytosolic free Ca^{2+} is homologous. The cells were exposed to agonists and/or thapsigargin during the times indicated (*bars*). BK (0.01 μM) was followed by washing and 0.01 μM BK and then 0.1 μM BK (A), 1 μM thapsigargin (B), or 1 μM DBK (C). Traces show individual representative cells from fields of 14 cells (A), 16 cells (B), and 14 cells (C). B and C include traces of individual cells that were BK unresponsive and served as the controls for the BK-responsive cells in the fields. Each experiment was performed two or three times with 8–16 cells/experiment.

the initial peak of the DBK response was followed by one Ca^{2+} spike. The same result was obtained when EGTA was added during the sustained phase. Consequently, the truncated sustained phase indicates that the sustained phase must be supported by intracellular stores that need to be refilled by influx of extracellular Ca^{2+} to continue to release Ca^{2+}. The appearance of one additional Ca^{2+} spike indicates that these stores may to a limited extent be refilled also by resequestration of cytosolic Ca^{2+}. One set of observations suggests that the intracellular sources of Ca^{2+} for the initial response and for the sustained response are accessed through different mechanisms of release. In cumulative dose-response curves with either DBK or Lys-BK, the addition of increasing concentrations of the same agonist or BK during ongoing oscillations triggered an initial Ca^{2+} response that occurred instantaneously regardless of the oscillatory frequency. These results argue that the initial response can occur independently of the sustained response. One possible scenario is that the initiation of the sustained response depends on the prior triggering of refilling of stores that occurs in response to the decrease in the Ca^{2+} content in the store after the initial response, in accordance with the capacitative model originally described by Putney *et al.* (37). However, because the sustained response is critically dependent on continued agonist occupancy of the receptor, the continuation of the sustained response still requires the action of a receptor second messenger such as $Ins(1,4,5)P_3$.

In cells in which the B1 receptor-mediated sustained phase was initially characterized by a plateau, the Ca^{2+} level often spontaneously dropped to base-line and the sustained phase was continued as oscillations. The same phenomenon could be induced by the addition of BK, suggesting that all of these cells are inherently capable of oscillating in response to B1 receptor stimulation even though they may initially exhibit plateaus. This observation leads to the question of whether the two types of sustained phases occur at the same time, with base-line oscillations simply "hidden" under the plateau. An argument against this possibility is that the peak of the oscillations often reached a level that was higher than that of the plateau. In such a case, it would be expected that the top portions of the hidden base-line oscillations would extend above the plateau, giving the appearance of slow sinusoidal oscillations. Such oscillations were never observed in these cells, suggesting that the two types of sustained phases do not occur simultaneously.

Several oscillator models have been proposed and it is difficult to say which best describes the B1 receptor-mediated base-line oscillations, but $Ins(1,4,5)P_3$ is believed to play a primary role in maintaining many forms of this type of activity (18, 22). The close correlation between the levels of $Ins(1,4,5)P_3$ and Ca^{2+} release suggests that this also is the case in RSMA smooth muscle cells. In terms of oscillatory frequency, one possibility is that this rate is exclusively dependent on the rate by which the stores refill. However, based on observations in cumulative dose-response experiments, the average frequency of the oscillations appears to be slower than the rate of refilling. Another possibility, which requires that oscillations do not occur during plateaus of elevated Ca^{2+}, is that the elevated free cytosolic Ca^{2+} reversibly inhibits the release of Ca^{2+} during the oscillations. Thus, each individual spike would be triggered by $Ins(1,4,5)P_3$ and subsequently terminated by the following elevation of free

Ca^{2+}. As the cytosolic free Ca^{2+} concentration drops, inhibition of release would be relieved and $Ins(1,4,5)P_3$ would again be able to trigger Ca^{2+} release. Consequently, the elevated Ca^{2+} inhibits further Ca^{2+} release only temporarily. In favor of this theory is the observation that cytosolic free Ca^{2+} potentiates $Ins(1,4,5)P_3$-mediated Ca^{2+} release at $<\sim 300$ nM while inhibiting it at higher concentrations in some cells (38–40). Feedback inhibition of Ca^{2+} release by Ca^{2+} has been proposed to be the underlying mechanism for agonist-induced Ca^{2+} oscillations in pancreatic acinar cells (41), although in RSMA smooth muscle cells, the main driving force for the initiation of each Ca^{2+} spike would still be the formation of $Ins(1,4,5)P_3$. The occurrence of short-lived plateaus in a number of these cells may be the result of an imbalance in the feedback regulation of Ca^{2+} and/or Ca^{2+} efflux.

Although the characteristic response pattern of the B2 receptor-mediated Ca^{2+} mobilization after agonist stimulation was transient, long term incubations revealed that after a prolonged latency period some individual cells were capable of producing oscillations of low frequency. The physiological relevance of such delayed and weak oscillatory Ca^{2+} signals is unclear, but the response was B2 receptor specific and therefore may be of interest from a mechanistic point of view. These results show that although the B2 receptor-mediated signaling pathway seems to contain the necessary components to support sustained Ca^{2+} release in the form of oscillations, the onset of oscillations is apparently subject to suppression by a mechanism or mechanisms that attenuate with time. Such suppression may be a direct B2 receptor signal and/or an indirect consequence of B2 receptor regulation by desensitization. Several observations indicate that the B2 receptor suppresses sustained Ca^{2+} release through both of these mechanisms. During DBK-maintained plateaus, the BK-induced Ca^{2+} transient was always followed by an immediate and sharp drop in the Ca^{2+} level to basal levels and the DBK response was continued as base-line oscillations. Furthermore, during DBK-driven oscillations, the BK-induced Ca^{2+} transient was always followed by a significant but temporary delay in the resumption of the oscillations. Again, this delay does not seem to be due to inadequately refilled Ca^{2+} stores because the addition of DBK immediately following a BK-induced Ca^{2+} transient always resulted in an increase in intracellular free Ca^{2+}. This suppressive effect was also apparent in responses to Lys-BK in which plateau-type sustained phases were never observed at agonist concentrations high enough to stimulate both B1 and B2 receptors. On the other hand, in the presence of HOE140 to block B2 receptors, Lys-BK did induce Ca^{2+} plateaus, indicating that the suppression clearly involved B2 receptors. Thus, it seems that stimulation of the B2 receptor may activate a mechanism or mechanisms that suppress sustained Ca^{2+} mobilization in these cells, perhaps at the point of Ca^{2+} release or by assisting Ca^{2+} efflux.

Clear evidence was obtained that the B1 and B2 receptor responses are differentially regulated by desensitization in these cells. Successive additions of increasing concentrations of BK showed that the B2 receptor signal attenuates. Furthermore, stimulation of cells with BK followed by washing and restimulation with the same agonist resulted in a second B2 receptor response that was dramatically decreased. In contrast, the same protocols with DBK yielded a second B1 receptor response that was not significantly different from the first response, although the response to the third stimulation was slightly attenuated. It should be noted that attenuation of responsiveness to some agonists may not be due to desensitization of the receptor signal but rather may be accounted for by depletion of intracellular Ca^{2+} stores, as reported recently in SH-SY5Y neuroblastoma cells (36). There also were no differences between BK-responsive and -unresponsive cells in thapsigargin- or DBK-stimulated increases in intracellular free Ca^{2+} after BK exposure, indicating that depletion of Ca^{2+} stores does not seem to explain the BK-promoted attenuation in RSMA smooth muscle cells. Thus, our results strongly suggest that the agonist-induced attenuation of the B2 receptor signal is due to rapid homologous desensitization. On the other hand, the B1 receptor signal in the RSMA smooth muscle cells is subject to very limited desensitization, which seems to be slow in onset. Two recent studies directly addressed whether B1 and B2 receptor-mediated increases in cytosolic free Ca^{2+} are differentially regulated by desensitization (33, 42). In rat mesangial cells, both DBK and BK were reported to induce heterologous desensitization in cell populations (42), and in a single-cell imaging study with bovine pulmonary artery endothelial cells that was published while our work was in progress, BK was reported to induce homologous desensitization, whereas no desensitization was observed in response to DBK (33). Because neither study excluded depletion of Ca^{2+} stores as a possible reason for attenuation of responsiveness, it is unclear whether the attenuation of the signals observed in mesangial or endothelial cells represents receptor signal desensitization of the same nature as we observed in vascular smooth muscle cells. Interestingly, in the latter study (33), as well as in a study with single N1E-115 murine neuroblastoma cells (35), maximal activation of the B2 receptor by BK was shown to elicit a transient Ca^{2+} response, whereas submaximal activation also included a sustained phase. These results indicate that the EC_{50} value for BK-promoted desensitization is higher than that for BK-promoted Ca^{2+} mobilization. Our results are different in that submaximal and maximal activation of the B2 receptor on RSMA smooth muscle cells with 0.001 μM and 0.1 μM BK, respectively, elicited response patterns that were virtually identical. Thus, the EC_{50} values for BK in stimulating desensitization and Ca^{2+} mobilization in these cells seem to be similar, suggesting that these events may be more closely coupled than in N1E-115 neuroblastoma cells and bovine pulmonary artery endothelial cells.

BK is released in response to injury and trauma and has been proposed to serve a role in the ensuing inflammatory response. This peptide has a relatively short half-life, serving as a substrate for both monocarboxypeptidases and dicarboxypeptidases. Although the product of the latter enzymes is the apparently biologically inactive fragment BK(1–7), the former enzymes produce the active fragment BK(1–8) or DBK. Consequently, in the presence of both B1 and B2 receptors, the action of DBK becomes an important component of the overall kinin signal. The results presented in this report show that stimulation of B2 and B1 receptors on cultured smooth muscle cells from the RSMA by BK and DBK, respectively, results in a rapid mobilization of intracellular Ca^{2+}. Clearly different patterns of Ca^{2+} mobilization were observed, arguing that the actions of these two receptor subtypes are not redundant but that they have distinct phys-

iological roles in the cell. The difference between the two agonist signals is at least in part due to the magnitude of the agonist-induced desensitization of the B2 receptor-mediated signal, which is homologous in nature. In addition, BK may directly stimulate regulatory mechanisms that inhibit release of Ca^{2+} from intracellular stores and/or perhaps assist in the rapid removal of excess cytosolic free Ca^{2+}. The results reported here may signify important physiological implications for kinin actions in general because in the absence of continued BK release and carboxypeptidase activity, kinins would produce a transient signal in these cells, whereas in their presence, kinins would produce a sustained intracellular signal.

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