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RECEPTOR-ACTIVATED CALCIUM INFLUX IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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SUMMARY

1. Fluorescence measurements of intracellular calcium concentrations ($[Ca^{2+}]_i$) were made on cultured human airway smooth muscle cells using the dye Fura-2. The response to either histamine (100 μM) or bradykinin (1 μM) was biphasic, with a transient increase in $[Ca^{2+}]_i$ followed by a sustained $[Ca^{2+}]_i$ increase lasting many minutes. The average steady-state (plateau) $[Ca^{2+}]_i$ following agonist activation was 267 ± 5 nM, whereas the average basal $[Ca^{2+}]_i$ was 148 ± 4 nM.

2. The sustained rise in $[Ca^{2+}]_i$ required the continued presence of either histamine or bradykinin and was dependent on extracellular Ca^{2+}. The magnitude of the transient rise in $[Ca^{2+}]_i$ was not dependent on extracellular Ca^{2+}. Sustained, receptor-activated rises in $[Ca^{2+}]_i$ were rapidly abolished by chelation of extracellular Ca^{2+}, or addition of non-permeant polyvalent cations, whereas these agents had minor effects in the absence of agonist. These data indicate that the sustained increase in $[Ca^{2+}]_i$ was dependent on receptor-activated Ca^{2+} influx.

3. Receptor-activated Ca^{2+} influx was not affected by treatment with organic Ca^{2+} channel antagonists (nifedipine (10 μM), nisoldipine (10 μM) or diltiazem (10 μM)) or agonists (Bay K 8644 (500 nM to 10 μM) or Bay R 5417 (500 nM)). The magnitude of the sustained rise was also not affected by pre-treatment with ouabain (100 μM) indicating little involvement of Na^+–Ca^{2+} exchange in the influx mechanism.

4. Receptor-activated Ca^{2+} influx could be completely inhibited by several polyvalent cations (Co^{2+}, Mn^{2+}, Ni^{2+}, Cd^{2+} or La^{3+}). Quantitative estimates of the potency of block were obtained for Ni^{2+} and La^{3+}. These measurements indicate that the pK_i for Ni^{2+} was 3·6 and for La^{3+} was 3·5.

5. Both Mn^{2+} and Co^{2+} ions caused a time-dependent quench of intracellular Fura-2; however, permeation of neither ion was increased following receptor activation, indicating that the influx pathway is not permeable to these cations.

6. Fura-2 was used to monitor the rate of Ba^{2+} entry into airway smooth muscle cells by monitoring the Ca^{2+}–Fura-2 and Ba^{2+}–Fura-2 isosbestic points as well as the 340 and 380 nm signals. Cell activation did not increase the rate of Ba^{2+} entry indicating that the Ca^{2+} influx pathway was poorly permeant to Ba^{2+} ions. Ba^{2+}

(2 mM) was able to inhibit Ca^{2+} entry as shown by its effects on the Ba^{2+}-independent, Ca^{2+}-dependent wavelength (371 nm).

7. The voltage dependence of Ca^{2+} influx was examined before and after agonist-induced activation. The effect of KCl-induced depolarization prior to cell activation was to cause a slight increase in $[Ca^{2+}]_i$. However, after activation of the influx pathway, KCl caused a concentration-dependent reduction in $[Ca^{2+}]_i$ indicating that the rate of influx was dependent on the electrochemical gradient for Ca^{2+}.

8. These results indicate that agonist exposure activates calcium influx through a pathway that has pharmacological and biophysical characteristics that are distinct from voltage-dependent calcium channels.

INTRODUCTION

Current evidence indicates that a rise in $[Ca^{2+}]_i$ is an integral component of agonist-induced smooth muscle contraction (Kamm & Stull, 1985; Somlyo, 1985; Yagi, Becker & Fay, 1988; Itoh, Ikebe, Kargacin, Hartshorne, Kemp & Fay, 1989). Despite the importance of the regulation of $[Ca^{2+}]_i$ to the process of smooth muscle contraction, many fundamental details regarding the way in which contractile agonists elevate $[Ca^{2+}]_i$ remain incompletely understood, particularly in airway smooth muscle. Inositol trisphosphate-induced Ca^{2+} release from intracellular stores has been well documented in airway smooth muscle, and this mechanism underlies the initial transient increase in $[Ca^{2+}]_i$ caused by contractile agonists in airway smooth muscle (Baron, Cunningham, Strauss & Coburn, 1984; Hashimoto, Hirata & Ito, 1985; Chilvers, Challis, Barnes & Nahorski, 1989; Murray, Bennett, Fluharty & Kotlikoff, 1989). A less well understood aspect of airway smooth muscle effector-coupling mechanisms is the maintenance of tonic force generation by contractile agonists. Tonic force generation in smooth muscle appears to be associated with sustained elevations in $[Ca^{2+}]_i$ and myosin phosphorylation over basal levels (Morgan & Morgan, 1982; Kamm & Stull, 1985; Himpens, Matthijs, Somlyo, Butler & Somlyo, 1988; Taylor & Stull, 1988; Gerthoffer, Murphy & Gunst, 1989), but the mechanism by which sustained levels of elevated $[Ca^{2+}]_i$ are achieved in airway smooth muscle has not yet been demonstrated. In vascular smooth muscle, Ca^{2+} influx through voltage-dependent Ca^{2+} channels is an important contractile mechanism (Nelson, Standen, Brayden & Worley, 1988) and one which has been successfully exploited clinically with dihydropyridine Ca^{2+} channel blockers. Although agonist-induced contractions in airway smooth muscle have been shown to be partially dependent on extracellular Ca^{2+} (Farley & Miles, 1978), the ability of dihydropyridines to attenuate these contractions has been variable (Kirkpatrick, 1975; Cerrina, Advenier, Renier, Floch & Duroux, 1983; Ahmed, Foster & Small, 1985). This raises the possibility that a sustained rise in $[Ca^{2+}]_i$ represents a receptor-activated influx pathway that is distinct from voltage-dependent Ca^{2+} channels, as suggested by previous authors (Bolton, 1979; van Breeman, Aaronson & Loutzenhiser, 1979). While dihydropyridine-sensitive voltage-dependent Ca^{2+} channels have been documented in airway smooth muscle at the whole cell (Kotlikoff, 1988; Marthan, Martin, Amédée & Mironneau, 1989) and single-channel level (Worley & Kotlikoff, 1990), the role of these channels in agonist-induced increases of cytosolic Ca^{2+} in airway smooth muscle remains unclear, since agonist-induced

contractions are not completely inhibited by concentrations of dihydropyridines sufficient to completely block Ca^{2+} currents, and since contractions occur with relatively modest depolarizations (Farley & Miles, 1977; Coburn, 1979). Receptor-activated influx mechanisms with properties distinct from voltage-dependent Ca^{2+} channels have been described in a number of cell types (see Penner, Matthews & Neher, 1988; Hallam & Rink, 1989; van Breeman & Saida, 1989), and a receptor-operated channel has been demonstrated in smooth muscle (Benham, Bolton, Byrne & Large, 1987*a*; Benham & Tsien, 1987; Benham, 1989). Although receptor-activated Ca^{2+} influx may be an important mechanism in raising $[Ca^{2+}]_i$ in some cells, no direct evidence has been advanced to support the presence of such pathways in airway smooth muscle.

In this report, we demonstrate that two important contractile agonists, histamine and bradykinin, evoke a biphasic Ca^{2+} response in human airway smooth muscle cells. These agonists produce a prominent sustained rise in cytosolic Ca^{2+}, which depends on extracellular Ca^{2+} and represents sustained receptor-activated Ca^{2+} influx. This receptor-activated influx of calcium is not blocked by dihydropyridine calcium channel blockers and is relatively insensitive to blockade by polyvalent cations. Using Fura-2 as a probe of influx of other ions, we have demonstrated that the influx pathway is not a non-specific cation influx pathway. Rather, this pathway appears to be highly calcium specific, even with respect to barium. In contrast to voltage-dependent calcium channels, influx of calcium through this pathway is attenuated by KCl-induced depolarization. Our findings suggest that Ca^{2+} influx in human airway smooth muscle cells may represent a distinct pathway for Ca^{2+} entry. This pathway is likely to be of considerable importance in the control of airway smooth muscle tone.

METHODS

Cell isolation and culture

Human tracheobronchial segments were obtained at autopsy under a protocol approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of trachea and main-stem bronchus was removed and the smooth muscle dissected, enzymatically dissociated, and plated at a density of $1{\cdot}0 \times 10^4$ cells cm^{-2} in Ham's F-12 media supplemented with 10% fetal bovine serum, gentamicin (50 μg ml^{-1}) and amphotericin B (2·5 μg ml^{-1}), as previously described (Panettieri, Murray, DePalo, Yadvish & Kotlikoff, 1989). Cells were grown to confluence (14–21 days) and subpassaged by lifting with 0·5% trypsin–1 mM-EDTA solution. For fluorimetric experiments, cells were plated on 1 cm^2 ACLAR (Allied Signal Corporation) plastic cover-slips. All experiments were performed on confluent cells (passage 3–9). These cells have previously been characterized by immunocytochemistry and immunoblot analysis using the CGA7 monoclonal antibody for smooth muscle-specific isoforms of actin (Enzo Biochemistry) (Panettieri *et al.* 1989). NPC 567 was a gift of Dr S. Farmer (NOVA). All other chemicals were obtained from standard sources (Sigma, Calbiochem).

Measurement of cytosolic calcium

Intracellular Ca^{2+} measurements were obtained using the fluorescent indicator Fura-2 in its cell permeant acetomethoxyester form (Fura-2 AM; Calbiochem). Confluent monolayers were loaded at 37 °C in a Phenol Red-free medium M-199 containing 25 mM-HEPES, 15 mM-glucose, 1 mg ml^{-1} bovine serum albumin, and 2·5 μM-Fura-2 AM, at pH 7·38. Monolayers were incubated in this solution for approximately 30 min and then rinsed with an identical solution except for the absence of Fura-2 AM and BSA. Loaded monolayers were stored at 37 °C in the dark until they were used (typically 30–60 min). Fluorescence measurements were made by placing the Fura-2-loaded cover-slips diagonally into a thermostatically controlled (37 °C) stirred quartz cuvette in a four-channel

spectrofluorometer (Biomedical Instruments Group, University of PA), with a 75 watt xenon lamp coupled to an air turbine-driven filter wheel. The four excitation filters used were 340DF10, 380DF10, 360BP and 371BP (Omega Optical, Brattleboro, VT, USA) to monitor the two Ca^{2+}-sensitive wavelengths, and the Ca^{2+}–Fura-2 and Ba^{2+}–Fura-2 isosbestic wavelengths respectively.

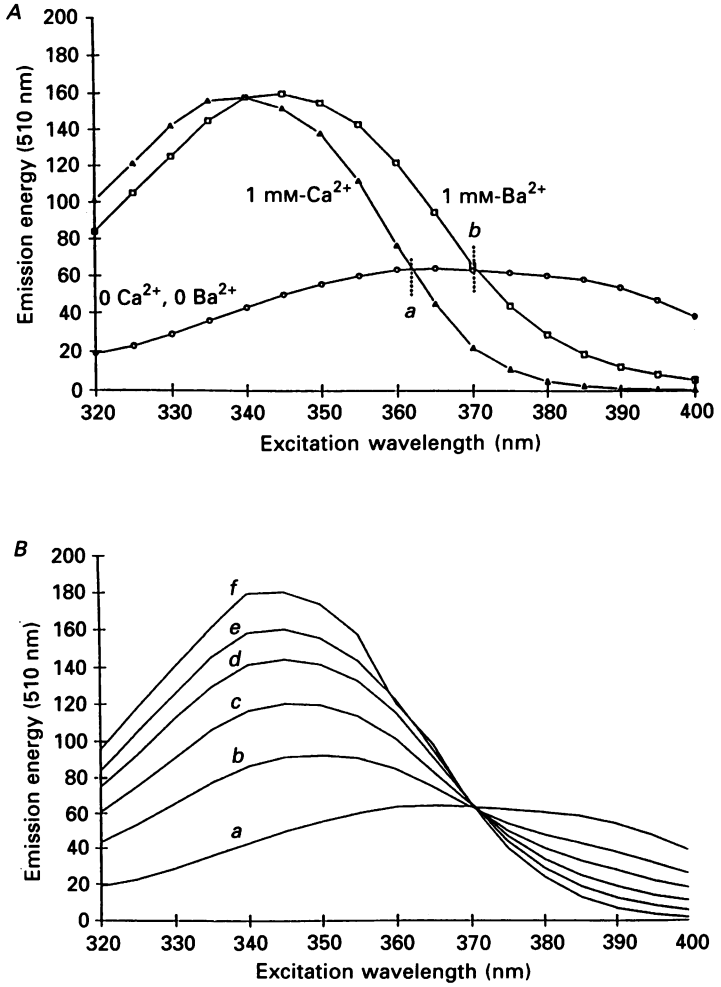

Fig. 1. Interaction of barium with Fura-2. The fluorescent properties of the Ba^{2+}–Fura-2 complex were investigated in solution using the free acid form of Fura-2. *A*, the excitation spectrum for a saturating barium–Fura-2 mixture (1 mM-Ba^{2+}) is similar to that for a Ca^{2+}–Fura-2 mixture, except that it is shifted to longer wavelengths, and the total fluorescence intensity at 340 nm is similar with either ion. *B*, the excitation spectrum of Fura-2 at different barium concentrations ($a = 0$; $f = 1$ mM) reveals a marked barium sensitivity of the dye, and a unique Ba^{2+}–Fura-2 isosbestic point (the barium-insensitive wavelength). The isosbestic point for the Ba^{2+}–Fura-2 complex was determined to be 371 nm (point *b* in *A*), as compared to 360 nm for the Ca^{2+}–Fura-2 complex (point *a* in *A*).

The Ca^{2+} and Ba^{2+} isosbestic wavelengths were determined empirically in solution using a Perkin Elmer LS-5 spectrofluorimeter. Emission energy was monitored at 510 nm using an interference filter (Omega Optical) and a photomultiplier tube. The signal was demodulated into four separate

signals, digitized and acquired by a DEC PDP 1123+ with storage on magnetic disc and videotape (Instrutech, PCM). All fluorescence signals were filtered to a bandwidth of 10 Hz. The background fluorescence was determined by measuring fluorescence at each wavelength in confluent monolayers of unloaded cells at equivalent system gains. Background fluorescence was subtracted from each buffer and the Ca^{2+} concentration calculated using the equation of Grynkiewicz, Poenie & Tsien (1985):

$$[Ca^{2+}] = \frac{(R - R_{min})(S_{f380})}{(R_{max} - R)(S_{b380})} K_d,$$

where R is the fluorescence ratio, R_{min} and R_{max} its values at zero and saturating $[Ca^{2+}]$, respectively, and S is the proportionality coefficient of the amount of free dye (S_f) and Ca^{2+}-bound dye (S_b) measured at 380 nm. K_d was assumed to be 226 nM. R_{min}, R_{max} and S_{f380}/S_{b380} were determined using Ca^{2+}, EGTA and ionomycin, as previously described (Panettieri *et al.* 1989). The values obtained were $R_{max} = 17{\cdot}7$, $R_{min} = 0{\cdot}60$ and $S_{f380}/S_{b380} = 15{\cdot}4$.

Use of Fura-2 as a probe of barium entry

In order to use Fura-2 as a probe of Ba^{2+} entry or to determine its effects on Ca^{2+} influx, we first investigated the interaction of Ba^{2+} with Fura-2 in solution. Figure 1*A* shows that the excitation spectrum for a saturating Ba^{2+}–Fura-2 mixture (1 mM-Ba^{2+}) is similar to that for a Ca^{2+}–Fura-2 mixture, except that it is shifted to longer wavelengths. The total fluorescence intensity at 340 nm is similar with either ion. The excitation spectrum of Fura-2 at different Ba^{2+} concentrations (Fig. 1*B*) demonstrated that Fura-2 is quite sensitive to Ba^{2+} ions, and that the Ba^{2+}–Fura-2 interaction has its own unique isosbestic point (the $[Ba^{2+}]$-insensitive wavelength). The isosbestic point for the Ba^{2+}–Fura-2 complex was determined to be 371 nm, and that for the Ca^{2+}–Fura-2 complex 360 nm, as described by others (Grynkiewicz *et al.* 1985). These two isosbestic points are compared on Fig. 1*A* (point *a vs.* point *b*).

Statistics

All data are expressed as the means (±the standard error of the mean). Statistical comparisons were made using Student's *t* test or with one-way analysis of variance when multiple comparisons were made with a single control (Dunnett's test); a P value of less than 0·05 was considered significant. Dose–response curves for cation-induced inhibition of sustained Ca^{2+} influx were fit to a generalized Boltzmann distribution of the form:

$$\text{percentage inhibition} = 1 + e^{((C - K_i)/k)}$$

where C is the concentration of inhibitor, K_i the inhibitory constant, and k the slope factor of the relationship.

RESULTS

The intracellular calcium response of human airway smooth muscle cells to contractile agonists

Histamine (100–200 μM) or bradykinin (1–3 μM) application to human airway smooth muscle cells caused a biphasic increase in $[Ca^{2+}]_i$. These concentrations of histamine and bradykinin were chosen to ensure maximal Ca^{2+} responses in order to minimize experimental variability. Figure 2 shows representative Ca^{2+} transients in response to histamine (Fig. 2*A*) and bradykinin (Fig. 2*B*). Agonist exposure produced a rapid, transient increase in cytosolic Ca^{2+}, followed by a steady-state $[Ca^{2+}]_i$ that was substantially higher than resting level. The magnitude of the peak of the Ca^{2+} transient was 714±15 nM for histamine ($n = 31$) and 740±23 nM ($n = 19$) for bradykinin; these values were not significantly different. The average basal $[Ca^{2+}]_i$ was 148±4 nM ($n = 50$), whereas the average sustained $[Ca^{2+}]_i$ (measured during a flat period or 45–60 s after agonist delivery) was 271±7 ($n = 31$) for histamine and 257±7 ($n = 19$) for bradykinin. As with the peak of the Ca^{2+} transient,

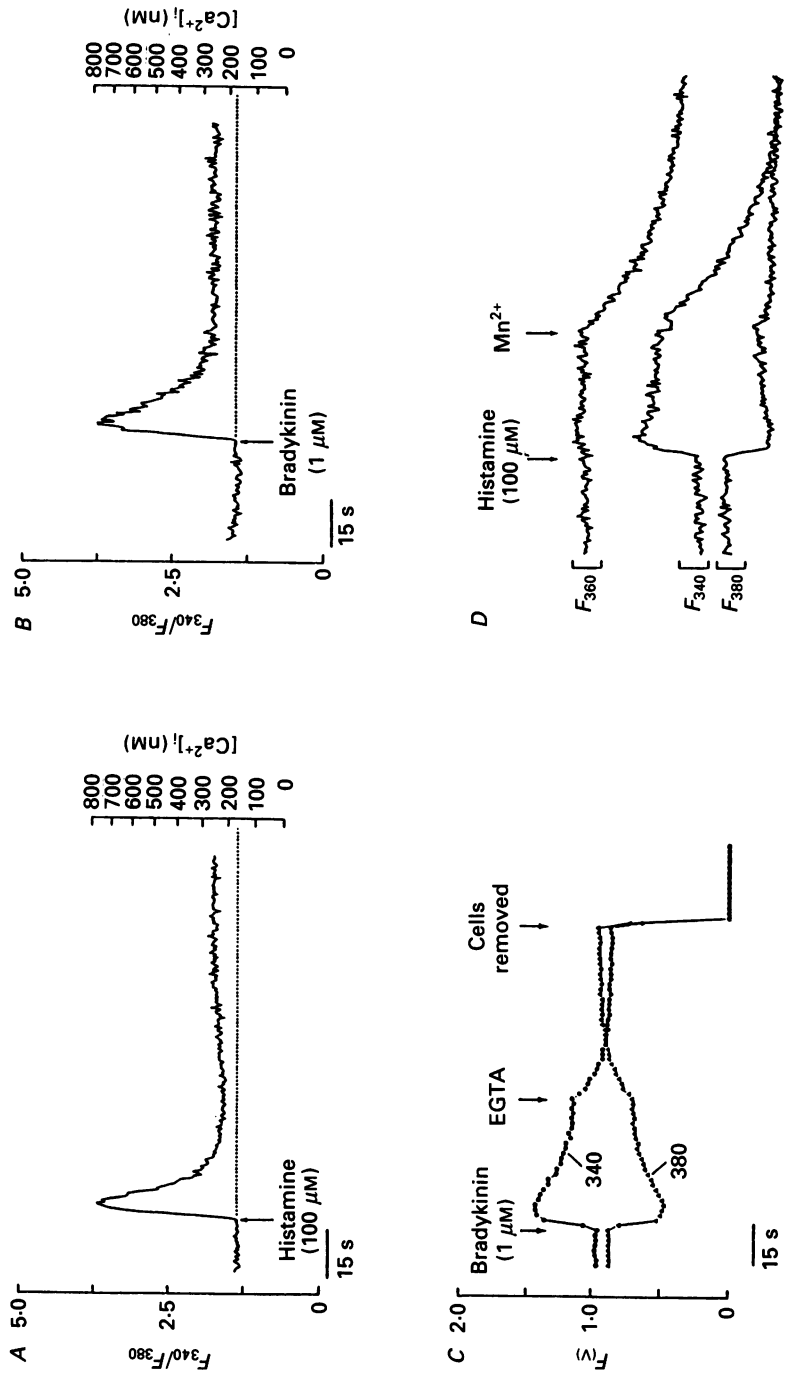

Fig. 2. The calcium response to contractile agonists is biphasic, and not perturbed by the presence of extracellular Fura-2. Addition of agonist to Fura-2-loaded cultured human airway smooth muscle cells evokes a typical biphasic calcium response. *A*, 100 μM-histamine. *B*, 1 μM-bradykinin. During the sustained phase, chelation of calcium by addition of EGTA (3 mM) resulted in symmetric changes in the 340 and 380 nm fluorescences, indicating a decrease in $[Ca^{2+}]_i$ (*C*). Removal of the cells from the cuvette resulted in a fall in both 340 (●) and 380 (▲) nm fluorescence signals to near zero, indicating the absence of significant extracellular Fura-2. When Mn^{2+} (100 μM-$MnCl_2$) was added during the sustained phase (*D*), a time-dependent decrease in fluorescence occurred as the Mn^{2+} gained access to the intracellular Fura-2. Neither *C* nor *D* shows a sudden step decrease in the Fura-2 signal, which would be expected if significant extracellular Fura-2 were present.

the difference between the sustained rise induced by histamine and bradykinin was not significant. The average sustained increase in $[Ca^{2+}]_i$ in fifty experiments was 267 ± 5 nM which was significantly higher than the basal value ($P < 0.001$) (Table 1). The half-maximal concentrations for histamine-induced Ca^{2+} transients was 30 μM, and for bradykinin was 100 nM (data not shown). In general, the responses of human

TABLE 1. Summary of calcium influx data

		$[Ca^{2+}]_i$ (nM)		
Condition	n	Basal	Peak	Sustained
Histamine (2 mM)	31	146 ± 6	714 ± 15	271 ± 7
Bradykinin (2 mM)	19	151 ± 6	740 ± 23	257 ± 7
Hist or BK (controls)	50	148 ± 4	724 ± 13	267 ± 5‡
Low ECF Ca^{2+}	10	121 ± 7†	690 ± 25	105 ± 6**
High ECF Ca^{2+}	4	156 ± 11	880 ± 24*	398 ± 9**
Calcium antagonist	9	143 ± 9	749 ± 23	259 ± 7
Calcium agonist	6	139 ± 6	699 ± 38	231 ± 14
Ouabain treated	5	151 ± 12	728 ± 20	262 ± 8

Values represent means ± S.E.M. *, Significantly different from controls ($P < 0.05$). **, Significantly different from controls ($P < 0.01$). †, Basal (low calcium) significantly different from controls ($P < 0.01$). ‡, Sustained control significantly different from basal ($P < 0.001$).

airway smooth muscle cells to maximally effective doses of histamine or bradykinin were indistinguishable. In order to exclude the possibility that extracellular Fura-2 contributed to the sustained increase in Ca^{2+}-dependent fluorescence, experiments were performed in which the cell monolayer was removed from the cuvette during the sustained phase. Figure 2*C* shows a representative experiment with the 340 and 380 fluorescence signals (volts) displayed individually. After removing the monolayer from the cuvette there was no measurable residual fluorescence, indicating that extracellular Fura-2 (bath) did not contribute to the fluorescence signals. Further, the decrease in the 340 nm fluorescence that occurred following addition of EGTA was not a sudden step function as is observed when extracellular Fura-2 is present. Addition of Mn^{2+} during a sustained phase (Fig. 2*D*) did not cause an abrupt quench in fluorescence which would be seen with extracellular Fura-2, but rather showed a slow, exponential decrease in fluorescence as Mn^{2+} entered the cells and quenched the dye.

Sustained calcium influx is receptor mediated

The sustained rise in $[Ca^{2+}]_i$ persisted until receptor occupancy was terminated by removing the agonist or addition of a receptor antagonist. Figure 3*A* shows the effect of adding the H_1 blocker mepyramine during the sustained phase, with a resultant decrease in $[Ca^{2+}]_i$. When the agonist was removed by washing with agonist-free buffer, $[Ca^{2+}]_i$ also returned to baseline (Fig. 3*B* with bradykinin; also see Fig. 4*E*). In addition, the sustained phase induced by bradykinin could be partially inhibited by adding the bradykinin antagonist NPC 567 (data not shown). Thus, the sustained rise in $[Ca^{2+}]_i$ caused by either agonist is a reversible process and requires the continued presence of the agonist.

The sustained phase is dependent on extracellular calcium

The effects of extracellular Ca^{2+} on agonist-induced rises in $[Ca^{2+}]_i$ was investigated in a series of experiments. The sustained increase in cytosolic Ca^{2+} induced by either agonist could be abolished by addition of 3 mM-EGTA to the extracellular buffer, as

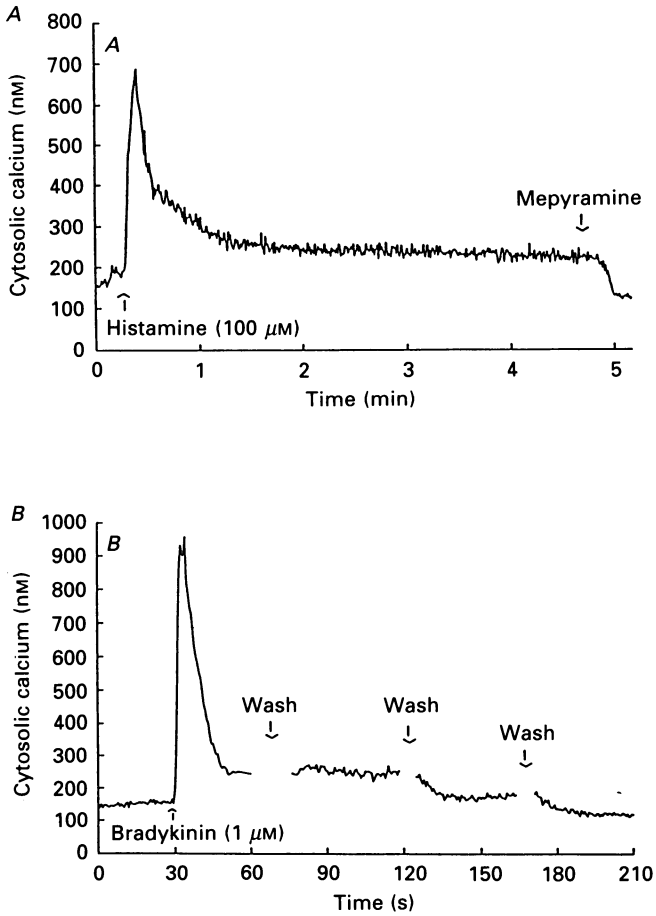

Fig. 3. The sustained calcium response is receptor mediated. *A*, addition of mepyramine (50 μM), a H_1 antagonist, abolished the histamine (100 μM) induced sustained increase in $[Ca^{2+}]_i$, even after a particularly long sustained phase. *B*, removal of bradykinin by successive washes also caused a decrease in $[Ca^{2+}]_i$ to basal values. Thus, continued interaction between agonist and receptor is required for the maintenance of a sustained rise in $[Ca^{2+}]_i$.

shown in Fig. 2*C* and also in Fig. 6*A*. Varying the extracellular Ca^{2+} concentration just prior to cell activation had a large effect on the magnitude of the sustained increase in $[Ca^{2+}]_i$, but a minor effect on the peak $[Ca^{2+}]_i$. Figure 4*A* shows a control transient in the presence of 2 mM-Ca^{2+}. When cells were resuspended in nominally Ca^{2+}-free HEPES-buffered saline solution just prior to activation, a normal Ca^{2+}

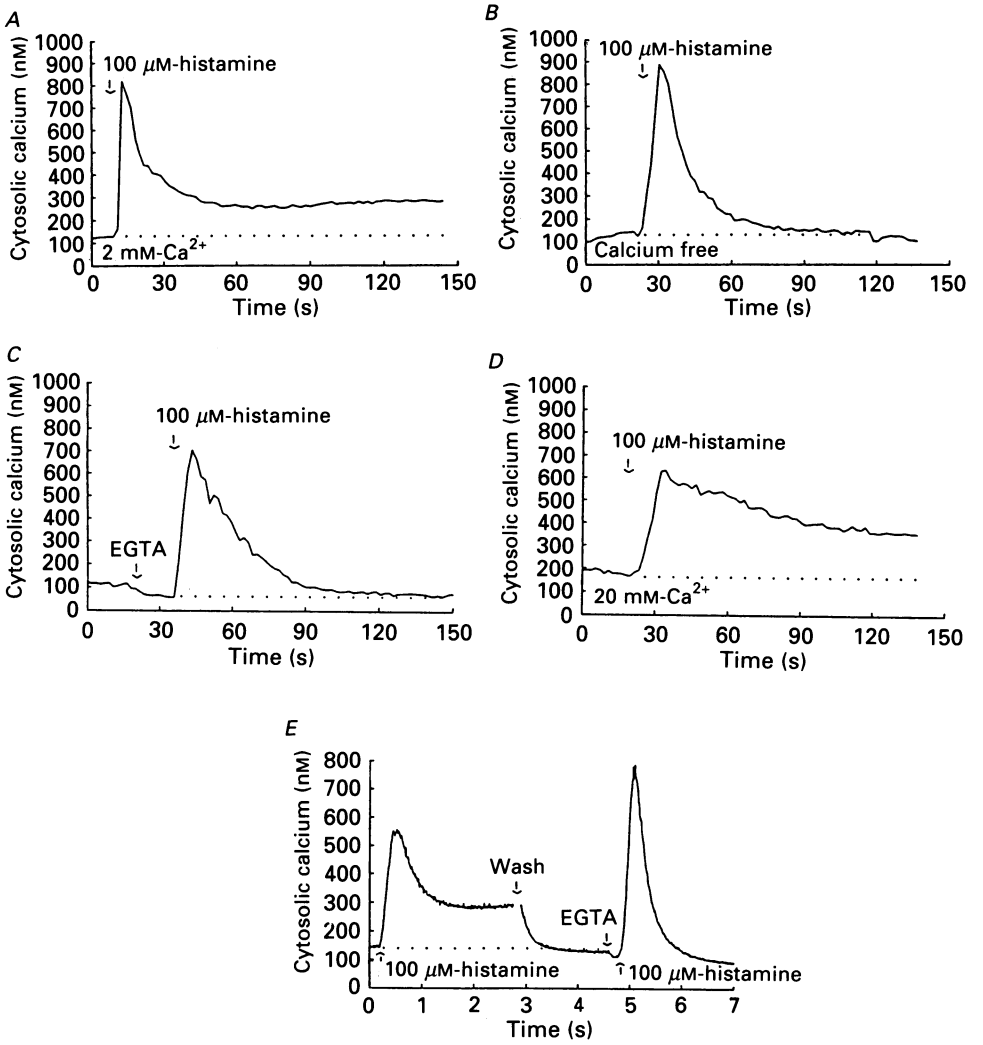

Fig. 4. The sustained calcium response depends on extracellular calcium. *A*, a control transient with a normal sustained phase is seen in the presence of 2 mM-calcium. *B*, a preserved transient with a reduced sustained phase is observed in calcium-free HEPES-buffered saline solution. *C*, results similar to those of *B* were obtained when extracellular calcium was reduced by adding an excess of EGTA just prior to activation (2 mM-Ca^{2+} plus 3 mM-EGTA). *D*, after addition of 20 mM-Ca^{2+}, activation by histamine resulted in a slightly increased transient phase and a greatly increased sustained phase. *E*, following a first calcium transient (2 mM-Ca^{2+}), the sustained phase was abolished by removing the agonist (wash). After addition of EGTA (3 mM), a second transient was induced that did not show a sustained phase.

transient was observed but the sustained rise was attenuated (Fig. 4*B*). Prior treatment with 3 mM-EGTA produced similar effects (Fig. 4*C*). The effect of lowering extracellular Ca^{2+} was to reduce the sustained $[Ca^{2+}]_i$ from 267 ± 5 nM ($n = 50$) to 105 ± 6 nM ($n = 10$). Pre-treatment with EGTA was generally associated with a

demonstrable decrease in $[Ca^{2+}]_i$, which was typically about 40 nM. Removing extracellular Ca^{2+} had no effect on the magnitude of the initial transient. When agonists were added after the addition of 20 mM-Ca^{2+} (Fig. 4*D*), the magnitude of the sustained phase was increased from a mean of 267 ± 5 nM (control; $n = 50$) to a mean of 398 ± 9 nM ($n = 4$). Sustained $[Ca^{2+}]_i$ was significantly lower ($P < 0.01$) in the absence of extracellular Ca^{2+}, and significantly higher ($P < 0.01$) in the presence of high extracellular Ca^{2+}. Increased extracellular Ca^{2+} also significantly increased the peak $[Ca^{2+}]_i$ (from 724 ± 13 (controls) to 880 ± 24 nM; $P < 0.05$), indicating that early influx was capable of augmenting the magnitude of the Ca^{2+} transient. Figure 4*E* shows an experiment in which the same cells were stimulated twice, first in the presence and then in the absence of Ca^{2+}. Following removal of the agonist, $[Ca^{2+}]_i$ returned to baseline; addition of EGTA (3 mM) then produced a decrease in $[Ca^{2+}]_i$. A second addition of agonist resulted in a large Ca^{2+} transient but unlike the previous response, no sustained increase in $[Ca^{2+}]_i$ was observed. The finding that a second transient was larger than the first was variable, and the mechanism of this phenomenon has not yet been investigated. The results from a series of these experiments are summarized in Table 1. In summary, the sustained phase of increased $[Ca^{2+}]_i$ following agonist addition is highly dependent on extracellular Ca^{2+}, indicating an influx mechanism, whereas the initial transient is not significantly effected by removal of extracellular Ca^{2+}, indicating that intracellular release contributes to this value.

Blockade of calcium influx by polyvalent cations

Substantial information is available with respect to the blocking affinities of specific cations for voltage-dependent Ca^{2+} channels and differential blocking affinities have been used to discriminate between different Ca^{2+} channel subtypes (Fox, Nowycky & Tsien, 1987; Narahashi, Tsunoo & Yoshii, 1987). We therefore examined the potency of block by Ni^{2+} and La^{3+}, which did not directly effect Fura-2 fluorescence, on receptor-activated Ca^{2+} influx.

The blockade of sustained Ca^{2+} influx by divalent cations was determined by adding various concentrations of the ion after establishing a sustained agonist-induced Ca^{2+} increase, and determining the effect on $[Ca^{2+}]_i$ (Fig. 5). Both Ni^{2+} and La^{3+} ions were able to abolish the sustained Ca^{2+} influx but required near stoichiometric concentrations to do so. Threshold effects were noted at about 50–100 μM, with full inhibition observed at 1–2 mM. Concentration–effect curves were obtained by serial additions of the cation during the sustained phase and measurement of the percentage inhibition of $[Ca^{2+}]_i$. Data were obtained in duplicate for Ni^{2+} and La^{2+} and fitted to a sigmoidal curve. As shown (insets), the potency of Ni^{2+} and La^{3+} block were quite similar with pK_is of 3·6 and 3·5 respectively. Co^{2+}, Mn^{2+} and Cd^{2+} also inhibited sustained influx, but were more difficult to quantify due to their prominent time-dependent effects on Fura-2 fluorescence; inhibition of the sustained phase was not detectable at concentrations below 50–100 μM, however. Neither Ni^{2+} nor La^{3+} caused a significant change in the fluorescence intensity as determined by measurement of the 360 nm signal.

Effect of organic calcium channel antagonists and agonists

In order to determine whether organic Ca^{2+} channel blockers had any effect on the magnitude of peak or sustained $[Ca^{2+}]_i$, cells were pre-incubated with nisoldipine, nifedipine, diltiazem, or the Ca^{2+} channel agonist Bay K 8644 (or its active

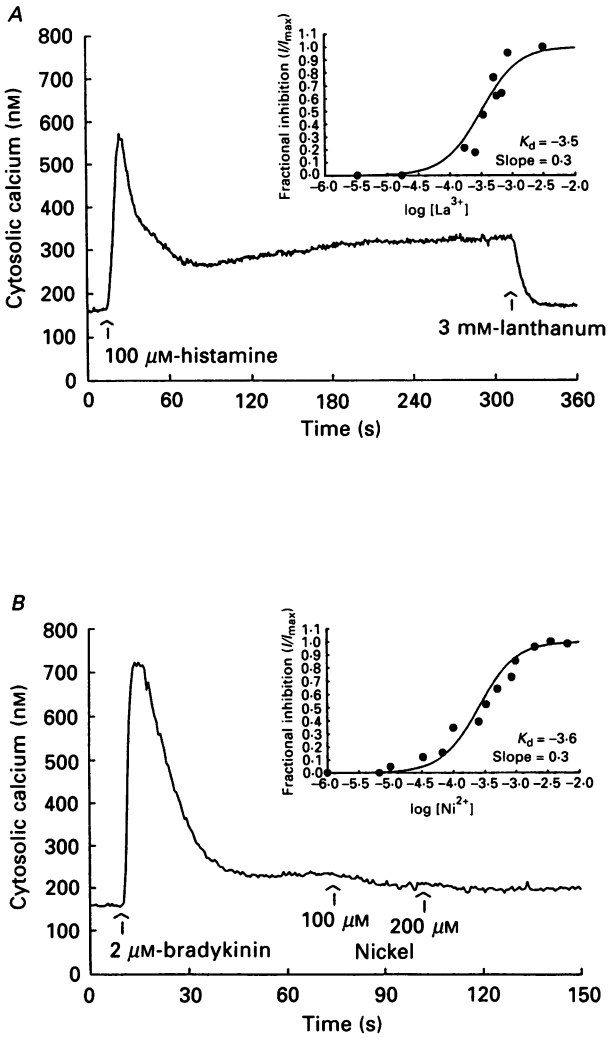

Fig. 5. Calcium influx is blocked by polyvalent cations. Addition of either lanthanum (*A*) or nickel (*B*) during the sustained phase caused a reduction in $[Ca^{2+}]_i$. This effect was concentration dependent, and was well fitted by a Boltzmann distribution (see text). The −log [cation] at which 50% inhibition of the sustained $[Ca^{2+}]_i$ occurred (pK_i) was obtained by least-squares fit. Values for pK_i were quite similar for La^{3+} and Ni^{2+} (inset, *A* and inset, *B*). The best-fit slope factors (k) were identical for both cations (0·3).

enantiomer Bay R 5417). In fifteen experiments, no effects of Ca^{2+} agonists or antagonists were observed on either phase of the Ca^{2+} transient (Table 1). A representative tracing for cells pre-incubated with nisoldipine (10 μM) is shown in Fig. 6*A*. Figure 6*B* shows the effect of the Ca^{2+} channel agonist Bay R 5417 (500 nM),

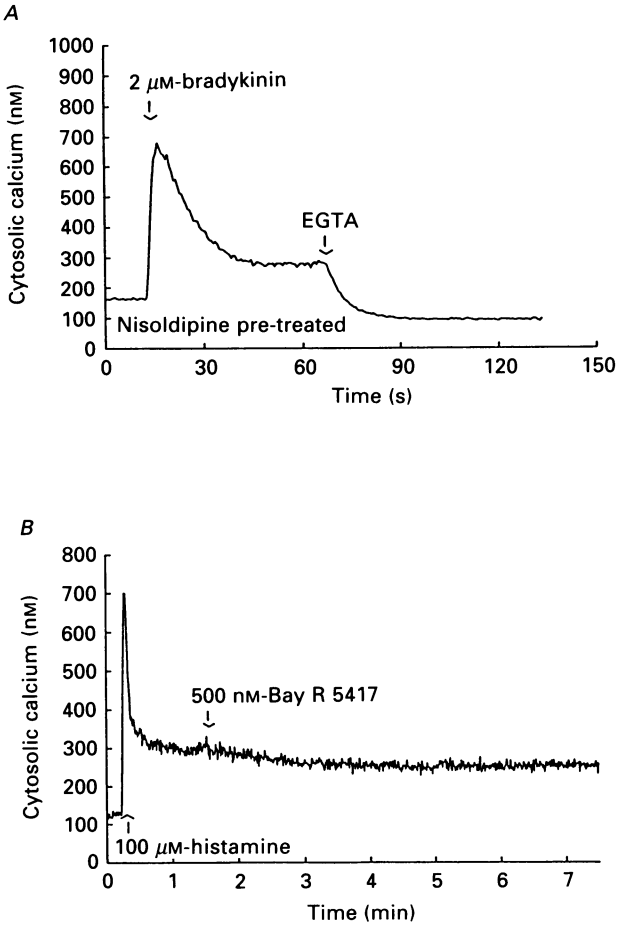

Fig. 6. The sustained rise in $[Ca^{2+}]_i$ is not sensitive to organic calcium channel blockers. Cells were incubated for 30 min with nisoldipine (10 μM, *A*). To prevent inactivation of light-sensitive dihydropyridine before activation by agonist, cells were not exposed to light during pre-treatment and exposed to UV excitation wavelengths for less than 20 s before agonist addition. Pre-treatment with Ca^{2+} channel antagonists did not abolish sustained Ca^{2+} influx, and there was no significant difference in sustained $[Ca^{2+}]_i$ between control and pre-incubated cells (Table 1). When the calcium channel agonist Bay R 5417 was added during the sustained phase (*B*), an increase in $[Ca^{2+}]_i$ was not observed.

applied during a histamine-induced sustained phase. The $[Ca^{2+}]_i$ was not observed to increase over the next 8 min. Pre-treatment with the racemic mixture, Bay K 8644 (up to 10 μM for 30 min), also had no effect on the Ca^{2+} transient. These data are

summarized in Table 1. Thus the influx pathway appears not to be sensitive to organic Ca^{2+} channel antagonists or agonists.

Dependence of influx pathway on sodium–calcium exchange

In order to investigate the possibility that Na^+–Ca^{2+} exchange may play a role in the maintenance of the sustained rise in $[Ca^{2+}]_i$ induced by agonists, attempts were made to stimulate this pathway, with the expectation that such manoeuvres might augment the magnitude of the sustained rise in $[Ca^{2+}]_i$. Similar approaches have been used in Fura-2-loaded smooth muscle cells by others (Pritchard & Ashley, 1987). In order to sodium load cells, cover-slips were incubated with ouabain (100 μM) for 30 min and the response to agonists compared to control cells. In five experiments, ouabain had no effect on the magnitude of the sustained phase (Table 1). In addition, when the cells were treated with ouabain while $[Ca^{2+}]_i$ was continuously monitored, no change in the 340/380 ratio was observed. Ouabain had no demonstrable effect on basal $[Ca^{2+}]_i$ or on the magnitude of the peak of the Ca^{2+} transient, as shown in Table 1. These data indicate that stimulation of Na^+–Ca^{2+} exchange by sodium loading does not augment basal or stimulated $[Ca^{2+}]_i$ and thus that Na^+–Ca^{2+} exchange is unlikely to contribute to the sustained elevation in $[Ca^{2+}]_i$ observed following agonist addition.

Cation specificity of the receptor-activated calcium influx pathway

Receptor-activated Ca^{2+} influx in several types of cells appears to result from activation of a non-specific cation permeability (Hallam & Rink, 1985; Andersson, Dahlgren & Pozzan, 1986; Hallam, Jacob & Merritt, 1988; Merritt & Hallam, 1988; Sage, Merritt, Hallam & Rink, 1989). We performed similar experiments by adding Mn^{2+} ions to the extracellular buffer, and comparing the rate of fluorescence quench (at the Ca^{2+}–Fura-2 isosbestic wavelength) before and after the addition of an agonist. Figure 7*A* shows a representative experiment in which 100 μM-Mn^{2+} was added prior to addition of agonist. Examination of the individual traces (340, 360 and 380) reveal that although all three fluorescence signals decayed over time, an agonist-induced Ca^{2+} transient was still observed in the Ca^{2+}-sensitive wavelengths (340 and 380) following histamine addition. Despite evidence of cellular activation, no increase in the rate of quench was observed at the Ca^{2+}-insensitive wavelength (360 nm), indicating that agonists capable of causing a sustained rise in Ca^{2+} do not increase the rate of Mn^{2+} entry. Application of 10 μM-ionomycin, however, markedly increased the quench rate. Similar results were obtained with 100 μM-Co^{2+} ions which also caused a time-dependent quench of the Fura-2 fluorescence; Co^{2+} influx was not accelerated by histamine (Fig. 7*B*). In a total of twelve experiments, no increase in the quench rate was observed with either bradykinin or histamine. These data indicate that although agonist-induced Ca^{2+} influx occurs in this preparation, the influx pathway is not permeant to Mn^{2+} or Co^{2+}.

Effects of agonists on barium influx, and of barium on calcium influx

In light of the demonstrated selectivity of the receptor-activated influx pathway, experiments were designed to examine the permeability of Ba^{2+}, an ion which readily permeates smooth muscle voltage-dependent Ca^{2+} channels (Worley, Deitmer &

Nelson, 1986; Benham, Haas & Tsien, 1987*b*; Worley & Kotlikoff, 1990). We exploited the unique Ba^{2+}–Fura-2 fluorescent properties in order to determine the effect of Ba^{2+} on receptor-activated Ca^{2+} influx. Addition of Ba^{2+} (2 mM) to resting cells (Fig. 8*A*) caused a slow increase in fluorescence at both 340 and 360 nm,

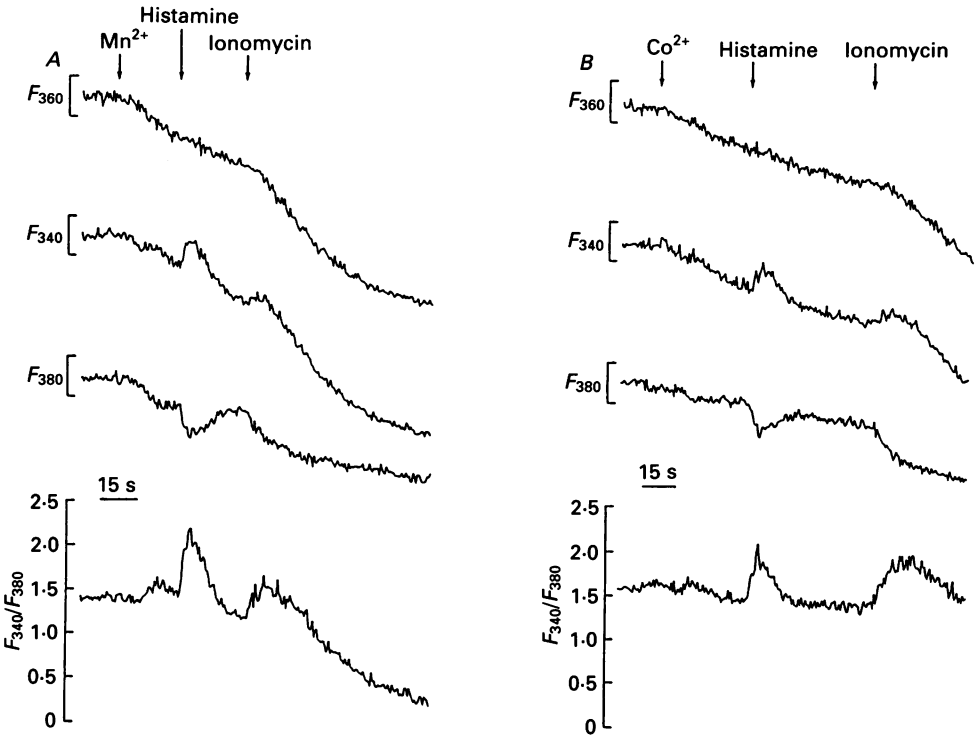

Fig. 7. Activation of calcium influx is not associated with accelerated entry of divalent cations. Divalent cation influx was examined by adding Mn^{2+} ions to the extracellular buffer and measuring the time-dependent quench of the fluorescence before and after addition of agonist. *A* shows a representative experiment in which 100 μM-Mn^{2+} was added. Examination of the individual traces (340, 360 and 380) reveals that although all three fluorescence signals decayed over time, an agonist-induced calcium transient was still observed in the calcium-sensitive wavelengths (340 and 380). Despite evidence of cellular activation, no increase in the rate of quench was observed at the calcium-insensitive wavelength (360 nm), indicating that agonists capable of causing a sustained rise in calcium do not increase Mn^{2+} entry. Application of ionomycin, an ionophore that promotes Mn^{2+} permeation, markedly increased the quench rate. *B*, similar results were obtained with Co^{2+} ions, which also caused a time-dependent quench of the Fura-2 fluorescence that was not accelerated by either histamine or bradykinin.

indicating that Ba^{2+} was entering the cells and increasing the fluorescence at the Ca^{2+}-dependent (340 nm) and Ca^{2+}-independent (360 nm) wavelengths. The Ba^{2+}-independent wavelength (371 nm), which behaves like the 380 trace with respect to $[Ca^{2+}]_i$, rises upon Ba^{2+} addition, indicating that the cytosolic Ca^{2+} is actually decreasing during the time when the *apparent* Ca^{2+} fluorescence (as determined by

the traditional 340/380 ratio) increased. Thus Ba^{2+} slowly permeated the cell, causing both a rise in the 360 nm fluorescence and in the 340/380 ratio. Addition of histamine did not increase the rate of Ba^{2+} entry, since the rate of rise of the 360 signal did not increase. The agonist did trigger an increase in $[Ca^{2+}]_i$ as can be seen by the abrupt drop in the fluorescence at 371 nm.

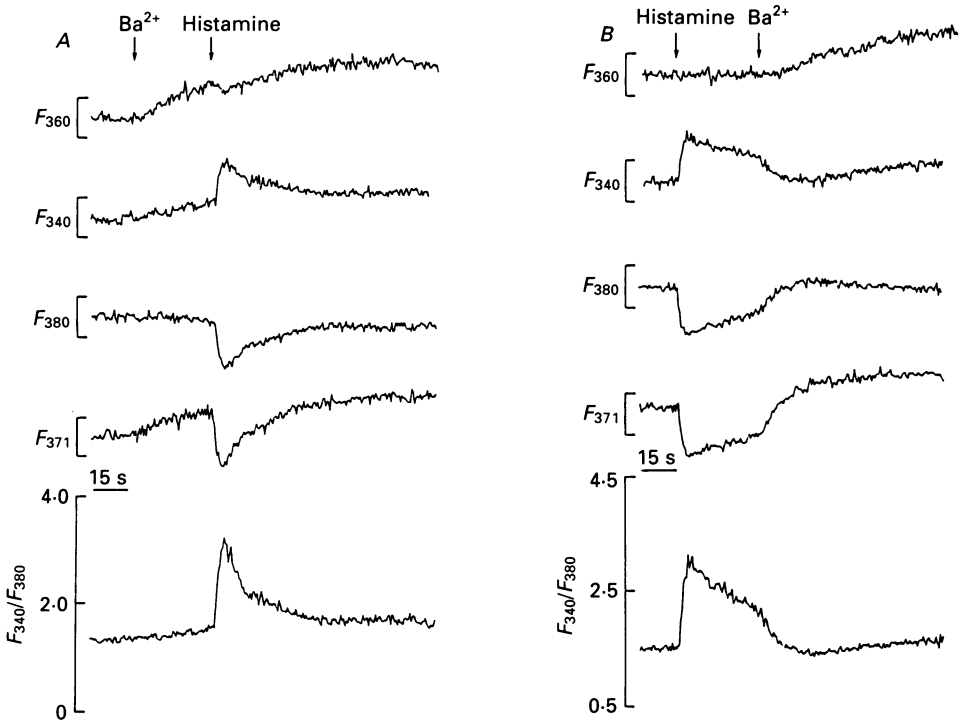

Fig. 8. Effects of barium before and after activation of influx. *A*, addition of Ba^{2+} (2 mM) to unstimulated cells caused a slow increase in fluorescence at 340 and 360 indicating that barium was entering the cells and increasing the fluorescence at the calcium-dependent (340 nm) and also at the calcium-independent (360 nm) wavelengths. Fluorescence at the barium-independent wavelength (371 nm), which behaves like the 380 trace with respect to $[Ca^{2+}]_i$, increased, however, indicating that the cytosolic calcium was actually decreasing during the time when the *apparent* calcium-dependent fluorescence (as determined by the traditional 340/380 ratio) increased. Addition of agonist did not increase the rate of rise of the 360 signal, indicating that the agonist did not increase the rate of barium entry. *B*, addition of Ba^{2+} (2 mM) after activation of influx resulted in an initial drop in the *apparent* $[Ca^{2+}]_i$ (as determined from the 340/380 ratio). Analysis of the 371 signal (which is barium independent) shows that $[Ca^{2+}]_i$ decreased following Ba^{2+} addition. Barium entry was monotonic as demonstrated by the rise in the 360 signal (calcium-independent wavelength) and the secondary rise in 340/380 ratio was not actually due to an increase in $[Ca^{2+}]_i$, but to continued Ba^{2+} entry, since the 371 signal still decreased. These results indicate that, similar to other polyvalent cations, the addition of barium inhibits calcium entry during sustained influx.

To determine the effect of Ba^{2+} on receptor-activated Ca^{2+} influx, Ba^{2+} was added after activating Ca^{2+} influx with histamine (Fig. 8*B*). Addition of Ba^{2+} resulted in a slow rise in the 360 nm fluorescence, indicating Ba^{2+} entry similar to that observed in Fig. 8*A*. This was associated with a fall in the 340/380 ratio as $[Ca^{2+}]_i$ fell following

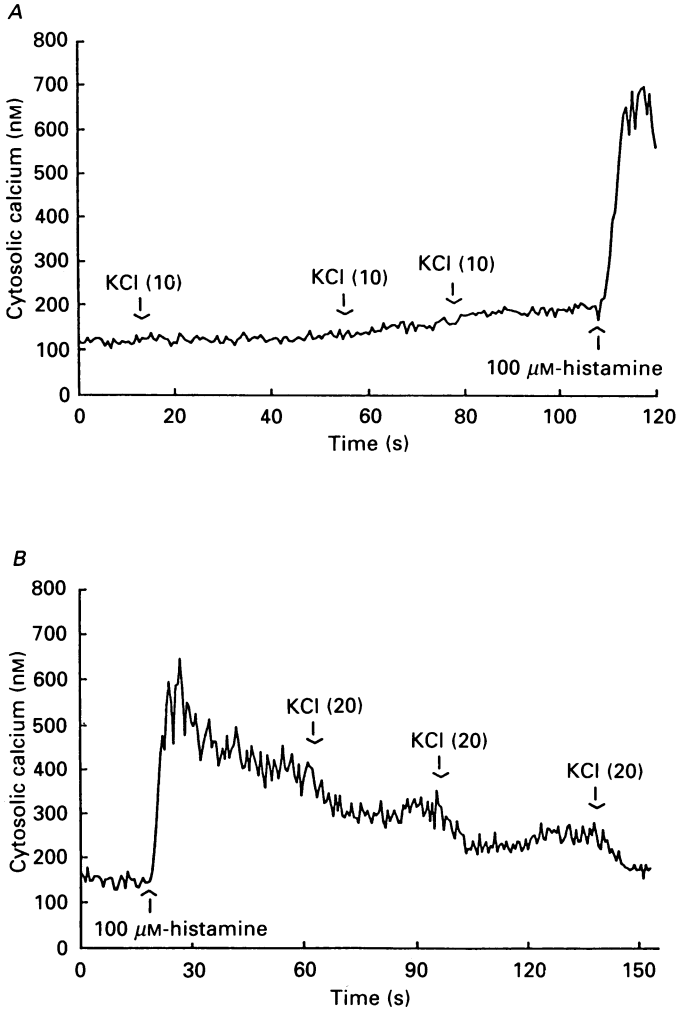

Fig. 9. Effect of KCl on $[Ca^{2+}]_i$ before and after activation of influx. Depolarizing concentrations of K^+ were added before and after cell activation with an agonist. *A*, when serial additions of KCl were added before cell activation, the effect was to slightly increase $[Ca^{2+}]_i$. *B*, serial additions of KCl applied after activation of influx resulted in a decrease in $[Ca^{2+}]_i$. Threshold effects both before or after the transient were observed at 10–20 mM-KCl. In addition, when agonist was applied in the presence of high K^+, a reduced sustained elevation in $[Ca^{2+}]_i$ resulted.

Ba^{2+} addition. Barium entry was monotonic as demonstrated by the 360 nm signal, and the secondary rise in the 340/380 ratio following Ba^{2+} addition was therefore not due to an increase in $[Ca^{2+}]_i$, but to continued Ba^{2+} entry. As was shown in Fig. 8*A*,

the effect of Ba^{2+} was to cause a reduction in $[Ca^{2+}]_i$, which is demonstrated by the opposite slopes of the 371 and 380 nm signals. These results, which are representative of ten experiments, indicate that, similar to other polyvalent cations, the addition of Ba^{2+} inhibits Ca^{2+} entry during sustained influx and that the receptor-activated Ca^{2+} influx pathway is relatively impermeant to Ba^{2+} ions.

Voltage dependence of receptor-activated calcium influx

In order to investigate the voltage dependence of sustained Ca^{2+} influx in human airway smooth muscle cells, we added depolarizing concentrations of K^+ before and after cell activation with an agonist (Fig. 9). When serial additions of KCl were made before cell activation (Fig. 9*A*; representative of eleven experiments), the effect was to slightly increase $[Ca^{2+}]_i$ although the effect was small. When serial additions of KCl were made during the sustained phase, the effect of KCl was to decrease the $[Ca^{2+}]_i$ (Fig. 9*B*; representative of eight experiments). Threshold effects both before or after the transient were observed at about 20 mM-KCl. In four experiments, a single large addition of K^+ (to 55 mM) completely abolished the sustained phase, returning $[Ca^{2+}]_i$ to basal levels. In addition, when cells were exposed to agonist in the presence of high K^+, the sustained rise in $[Ca^{2+}]_i$ was reduced. The effect was not due to increases in extracellular osmolality since serial additions of 20 mM-sucrose (up to 80 mM-sucrose) had no effect on $[Ca^{2+}]_i$. An identical effect of KCl-induced reduction in calcium influx was observed when the experiments were performed using isosmotic additions of KCl (molar substitution of KCl for NaCl). Thus K^+-induced depolarization caused a slight increase in $[Ca^{2+}]_i$ under resting conditions, but a large decrease once the sustained influx pathway had been activated. These results indicate that the open probability of the influx pathway is not increased with depolarization, and are consistent with a reduced Ca^{2+} influx when the electrochemical gradient for Ca^{2+} entry is reduced.

DISCUSSION

Histamine and bradykinin evoked a biphasic increase in $[Ca^{2+}]_i$ in Fura-2-loaded human airway smooth muscle cells. This was a consistent finding in cultured cells examined from the third to the ninth passage. The responses to maximally effective doses of histamine and bradykinin were virtually identical. The initial phase of the $[Ca^{2+}]_i$ response consisted of a rapid, 4- to 5-fold increase in cytosolic Ca^{2+} from an average basal level of approximately 150 nM. The magnitude of this initial rise was independent of extracellular Ca^{2+} concentration and probably represents inositol trisphosphate-mediated Ca^{2+} release. This response was generally larger than that which we previously described in cultured canine airway smooth muscle cells (Kotlikoff, Murray & Reynolds, 1987).

Following the initial transient rise in $[Ca^{2+}]_i$, the response consisted of a sustained elevation in cytosolic Ca^{2+}. This sustained elevation persisted for many minutes or until it was terminated by removal of extracellular Ca^{2+}, removal of the agonist, addition of an antagonist, or addition of an influx blocker. Although we made no attempt to define the maximal duration of the sustained phase, it was frequently shown to persist for more than 10 min. Similar biphasic responses have been recorded

in acutely dissociated canine airway smooth muscle cells (data not shown), indicating that the sustained response is not a unique property of our tissue culture preparation. Sustained increases in $[Ca^{2+}]_i$ following addition of agonists have also been observed in a number of cell preparations (Merritt & Rink, 1987; Penner *et al.* 1988), and in intact strips of smooth muscle (Himpens *et al.* 1988; Gerthoffer *et al.* 1989).

In contrast to the initial transient rise in $[Ca^{2+}]_i$, the magnitude of the sustained rise was highly dependent on extracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, a sustained phase was not observed, whereas elevated extracellular Ca^{2+} augmented the sustained response. However, the sustained rise in $[Ca^{2+}]_i$ was not sensitive to organic Ca^{2+} channel antagonists or agonists, given either before or after activation of Ca^{2+} influx. Furthermore, changes in the resting Ca^{2+} concentration were not observed following incubation with any of the Ca^{2+} channel agonists or antagonists.

Although Ca^{2+} influx could not be inhibited by organic Ca^{2+} channel blockers, it could be blocked with inorganic ions such as Ni^{2+}, La^{3+}, Co^{2+}, Cd^{2+} and Mn^{2+}. Because several of these (Mn^{2+}, Cd^{2+}, Co^{2+}) had prominent direct effects on Fura-2 fluorescence, we were only able to obtain quantitative concentration–effect relationships for Ni^{2+} and La^{3+}. Both Ni^{2+} and La^{3+} could completely abolish the sustained rise in $[Ca^{2+}]_i$; however, maximal effects were obtained only at millimolar concentrations. Both ions had a half-maximal effect of approximately 300 μM in the presence of 2 mM-Ca^{2+}, whereas none of the ions tested had a demonstrable effect on the sustained phase at concentrations below 50 μM. Conversely, similar concentrations of Cd^{2+}, Ni^{2+} or La^{3+} can completely block voltage-dependent Ca^{2+} channels (Fox *et al.* 1987; Narahashi *et al.* 1987; Hagiwara, Irisawa & Kameyama, 1988). Thus receptor-activated Ca^{2+} influx in human airway smooth muscle cells appears to be insensitive to blockade by concentrations of polyvalent cations sufficient to block voltage-dependent Ca^{2+} channels.

In several cell types, receptor-activated Ca^{2+} influx results in influx of other cations. Benham *et al.* (1987*a*) described an ATP-activated Ca^{2+} influx pathway in smooth muscle which was non-specific with respect to mono- and divalent cations. Calcium influx pathways which are permeant to Mn^{2+} have been demonstrated using Fura-2, in platelets (Hallam & Rink, 1985), endothelial cells (Hallam *et al.* 1988) and neutrophils (Andersson *et al.* 1986), although a negative result was reported in parotid acinar cells (Merritt & Hallam, 1988). Agonist activation did not increase the permeation rate of either Mn^{2+} or Co^{2+} in human airway smooth muscle cells although 10 μM-ionomycin provided a positive control. Thus the receptor-activated influx pathway in human airway smooth muscle cells appears not to be a non-specific cation permeability.

Since voltage-dependent Ca^{2+} channels in airway smooth muscle cells are Ba^{2+} permeant (Kotlikoff, 1988; Marthan *et al.* 1989; Worley & Kotlikoff, 1990), we reasoned that if agonist-induced Ca^{2+} influxes were mediated by voltage-dependent Ca^{2+} channels, the influx pathway should have similar characteristics. Fura-2 fluorescence can be used to measure Ba^{2+} influx since the dye demonstrates a Ba^{2+}-dependent change in fluorescence at both 340 and 380 nm, and under saturating conditions, has a total fluorescence energy equal to that achieved with Ca^{2+}. By obtaining solution spectra for Fura-2 with increasing concentrations of Ba^{2+}, we were able to define the isosbestic point for the Ba^{2+}–Fura-2 complex. Our value of 371 nm

is considerably shifted from the isosbestic point for the Ca^{2+}–Fura-2 complex (360 nm) and compares well to spectral data recently published by others (Schilling, Rajan & Strobl-Jager, 1989; Yamaguchi, Green, Kleeman & Muallem, 1989). We used the Ba^{2+}–Fura-2 and Ca^{2+}–Fura-2 isosbestic wavelength measurements to determine the effects of agonists on the rate of Ba^{2+} entry as well as the effect of Ba^{2+} on receptor-activated Ca^{2+} entry. We were unable to demonstrate that agonists could cause an increase in the rate of rise of the 360 nm fluorescence signal (the Ca^{2+}-independent wavelength), indicating that measurable agonist-triggered Ba^{2+} entry did not occur. Thus, if Ba^{2+} permeates this influx pathway, it does so at a very low rate compared to Ca^{2+}. It is unlikely that this effect is caused by differential affinities of Fura-2 for Ba^{2+} *vs.* Ca^{2+} since published estimates of the affinities are within the same order of magnitude (K_d = 226 nM for Ca^{2+} and 780 nM for Ba^{2+}; Schilling *et al.* 1989). This approach has been used to demonstrate triggered Ba^{2+} entry with 'L-type' channels (Schilling *et al.* 1989; Yamaguchi *et al.* 1989).

The effect of Ba^{2+}, added after activation of the influx pathway, was to decrease the rate of Ca^{2+} entry. This effect was similar to that seen with other polyvalent cations and raises the possibility of a direct inhibition by Ba^{2+} on receptor-activated Ca^{2+} entry. We cannot exclude the possibility that Ba^{2+}, which can inhibit K^+ channels (Hagiwara & Byerly, 1981), caused depolarization of the membrane and a lowering of the electrochemical gradient for Ca^{2+} entry as was observed with KCl. Such an effect of Ba^{2+} on Fura-2-detected Ca^{2+} entry has been reported by others (Yamaguchi *et al.* 1989).

If the influx pathway represented a Ca^{2+} flux carried by agonist-activated, voltage-dependent Ca^{2+} channels, the expected effect of K^+-induced depolarization would be to increase influx. Membrane depolarization of unstimulated cells caused a slight increase in Ca^{2+}, presumably by activation of voltage-dependent Ca^{2+} channels. However, following receptor activation, additions of 20–80 mM-K^+ caused a graded reduction in the magnitude of the sustained receptor-activated influx. This decline in Ca^{2+} influx suggests that: (1) voltage-dependent Ca^{2+} channels do not underlie the response, (2) influx is sensitive to the electrochemical gradient, and (3) the influx mechanism is not stimulated by depolarization. Similar effects of depolarization on Ca^{2+} influx have been reported in mast cells (see Penner *et al.* 1988).

In summary, we have described a biphasic Ca^{2+} response of human airway smooth muscle cells to the contractile agonists histamine and bradykinin. The response consists of an initial transient increase which is independent of extracellular Ca^{2+} and probably represents inositol trisphosphate-induced release from sarcoplasmic reticulum stores. The second phase, which lasts for many minutes, is dependent on extracellular Ca^{2+} and represents sustained Ca^{2+} influx. The biophysical and pharmacological characteristics of this influx pathway are distinct from those of voltage-dependent Ca^{2+} channels, since it is dihydropyridine insensitive, relatively insensitive to blockade by polyvalent cations, Ba^{2+} impermeant, and not augmented by depolarization.

The influx pathway we have described in airway smooth muscle appears to differ from several other non-voltage-dependent Ca^{2+} channel calcium influx pathways which have been described. Such pathways have frequently been cation non-specific (Hallam & Rink, 1985; Benham *et al.* 1987*a*; Hallam *et al.* 1988), or more sensitive

to blockade by polyvalent cations (Colden-Stanfield, Schilling, Ritchie, Eskin, Navarro & Kunze, 1987; Wallnöfer, Cauvin, Lategan & Rüegg, 1989). Since relatively modest elevations in $[Ca^{2+}]_i$ may be associated with substantial force generation by smooth muscle (DeFeo & Morgan, 1985; Yagi *et al.* 1988; Becker, Singer, Walsh & Fay, 1989; Gerthoffer *et al.* 1989), the sustained influx described here may have implications for tonic force generation in airway smooth muscle. Manipulation of this receptor-activated Ca^{2+} influx pathway may provide an additional means of pharmacological modulation of smooth muscle tone, and may have significance for diseases such as asthma and chronic airways obstruction.

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