

# Interleukin-4 Rapidly Inhibits Calcium Transients in Response to Carbachol in Bovine Airway Smooth Muscle Cells

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To assess interleukin (IL)-4 effects on calcium signaling, bovine airway smooth-muscle (ASM) cells were loaded with fura-2 and cytosolic calcium ( $[Ca^{2+}]_i$ ) was measured in single cells by digital microscopy. Human recombinant IL-4 (50 ng/ml) caused small increases in  $[Ca^{2+}]_i$ . For single cells, carbachol-stimulated calcium transients were compared before (S1) and after (S2) exposure to IL-4 or IL-13. When cells were treated with IL-4 (50 ng/ml) for 20 min, the S2/S1 ratio was  $0.17 \pm 0.04$  ( $n = 7$ ) even though IL-4 had been washed from the chamber for 10 min before the S2 response. In contrast, controls not treated with IL-4 had S2/S1 of  $0.70 \pm 0.04$  ( $n = 13$ ,  $P < 0.01$ ). Lower concentrations of IL-4 variably decreased transients and IL-13 had no effect. In other experiments, 5 min of IL-4 did not immediately decrease transients but did after a 25-min delay. Goat antihuman IL-4 antibody abolished the effect of IL-4. IL-4 (50 ng/ml) also inhibited responses to caffeine (S2/S1:  $0.30 \pm 0.04$  and  $0.54 \pm 0.06$  for IL-4-treated versus control). We conclude that IL-4 rapidly inhibited calcium transients. Because caffeine-stimulated transients were inhibited, IL-4 may act, at least in part, by depleting calcium stores. IL-4 inhibition of cholinergic signaling may be important for modulating ASM responses during inflammation.

Asthma is characterized by airway inflammation with CD4+ T lymphocytes secreting a T helper (TH) 2-like cytokine profile (1). One of the TH2 cytokines expressed in asthmatic airways is interleukin (IL)-4 (2). Two roles for IL-4 are stimulating T cells to differentiate into TH2 cells (3, 4) and promoting isotype switching to immunoglobulin (Ig) E in plasma cells (5). However, IL-4 also has effects on nonhematopoietic cell types that could be important for modulating inflammation and its consequences. For example, IL-4 stimulates fibroblast (6) and endothelial cell (7) growth and upregulates vascular cell adhesion molecule-1 on endothelial cells (8). IL-4 also stimulates and inhibits secretion of cytokines by epithelial cells (9) and smooth-muscle cells (10), respectively. Finally, IL-4 inhibits growth of vascular and airway smooth-muscle (ASM) cells (11, 12). Therefore, IL-4 has many cellular targets in the airways, including smooth-muscle cells.

Two important intracellular signaling pathways stimulated by IL-4 involve signal transducers and activators of transcription-6 and insulin receptor substrate (IRS)-1 or -2 (13). These pathways mediate many of the effects that IL-4

has on growth, gene expression, and cell survival (13). However, in some cells, other signaling pathways also may be activated directly or indirectly by IL-4 receptor (IL-4R) activation. For example, in B lymphocytes, IL-4 rapidly stimulated inositol trisphosphate ( $IP_3$ ) synthesis and increased cytosolic calcium ( $[Ca^{2+}]_i$ ) (14). Such findings suggest that, in at least some cell types, IL-4 can rapidly alter calcium homeostasis.

Whether IL-4 rapidly alters calcium homeostasis in ASM is not known. Such regulation could have potentially important implications for smooth-muscle growth and contractility in the presence of airway inflammation. The goal of this study was to assess whether a relatively brief exposure to IL-4 altered regulation of  $[Ca^{2+}]_i$  in ASM cells. To test this, we applied IL-4 to freshly dispersed bovine ASM cells loaded with fura-2, recorded changes in  $[Ca^{2+}]_i$  in single cells, and then characterized the effects that IL-4 treatment had on carbachol-stimulated calcium transients in these cells.

## Materials and Methods

### Cell Isolation

Smooth-muscle cells were dispersed from bovine trachealis (15). Minced trachealis tissue was placed in a Coulter counter vial containing a stirring bar and 2.5 ml of physiologic salt solution (PSS) (in mM: NaCl 118, KCl 4.7,  $KH_2PO_4$  1.2,  $MgSO_4$  1.2,  $NaHCO_3$  25.6, glucose 11.1, and  $CaCl_2$  2.5) modified to have no added  $CaCl_2$  and containing collagenase D (6 mg) and elastase (grade II) (3 mg). The minces were incubated at 37°C with constant stirring for 12 min and then transferred to a second vial containing enzyme, and the incubation was repeated until cells began to be released from the mince. The partially digested mince then was transferred to 3 ml of PSS modified to contain 0.1 mM  $CaCl_2$  and incubated for 3 min with constant stirring. The cells released during this and one subsequent identical incubation were used for studies. The dispersed cells were loaded with 0.5  $\mu$ M fura-2-AM in the presence of pluronic F-127 (0.004%) for 60 min at room temperature and then introduced into a perfusion chamber having a bottom cover glass. After adherence to glass for 10 min at room temperature, PSS perfused the chamber at 1 ml/min.

### Calcium Measurement

Fura-2 loaded into cells was excited by computer-controlled 337- and 380-nm ultraviolet light generated by a nitrogen laser and a nitrogen laser-pumped dye laser, respectively (Laser Science, Franklin, MA). Each laser alternately fired pulses (3 ns) at 30 Hz; these were guided by a bifurcated quartz fiber to a neutral-density filter at the epiport of the microscope and then focused on cells through a  $\times 40$  objective lens (Nikon, Melville, NY). The fluorescent signals emitted by the fura-2 were passed back through the objective to a 455-nm dichroic mirror, a 475-nm barrier filter (Omega Optics, Brattleboro, VT), and an image intensifier (Xybion Electronic Systems, San Diego, CA), and were captured by a Philips-based frame transfer charge-coupled device camera (CCTV, New York, NY). The analog signals from the camera were digitized and stored in an imaging board, and digital outputs from this board

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Abbreviations: airway smooth muscle, ASM; cytosolic calcium,  $[Ca^{2+}]_i$ ; interleukin, IL; IL-4 receptor, IL-4R; inositol trisphosphate,  $IP_3$ ; physiologic salt solution, PSS; standard error of the mean, SEM; sarcoplasmic reticulum, SR; T helper, TH.

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were transferred to a personal computer with software by Recognition Technology, Inc. (Framingham, MA).

As described previously (15), to measure  $[Ca^{2+}]_i$  in cells loaded with fura-2, background from a cell-free region of the cover glass was subtracted before data acquisition and an 11-by-11-pixel area was selected over each cell studied, avoiding the area of the nucleus. The fluorescence emissions stimulated by alternating pulses of 337 and 380 nm light were recorded and their ratios plotted. Ratios were converted to calcium concentrations by using the formula  $[Ca^{2+}]_i = K_D \cdot \beta \cdot (R - R_{min}) / (R_{max} - R)$ , where  $R_{max}$  and  $R_{min}$  are the fluorescence ratios measured *in situ* with permeabilized (4-bromo A-23187) fura-2-loaded cells exposed to high (2.5 mM  $CaCl_2$ ) and zero calcium, respectively;  $\beta$  is the ratio of fluorescence stimulated by 380 nm light in zero versus high calcium; and  $K_D$  is the equilibrium dissociation constant describing calcium binding to fura-2. On the basis of an *in situ* determination in bovine trachealis cells (16), a  $K_D$  value of 386 nM was used in converting fluorescence ratios to  $[Ca^{2+}]_i$ .

### Protocols

Cells loaded with fura-2 and attached to the glass coverslip of the perfusion chamber (0.3 ml volume) were perfused with PSS containing carbachol ( $10^{-5}$  M) for 2 min and then washed for at least 30 min with PSS before the start of experiments. In all experiments, cell chambers were continuously superfused with PSS containing the specified reagents, and a four-way valve allowed changing of the perfusate without disturbing the recordings made from single cells. There was cell-to-cell variability in the magnitude of calcium transients in response to carbachol. To deal with this variability, a protocol was devised such that responses to carbachol after cytokine treatment could be normalized to the responsiveness of that same cell before treatment with cytokine. Therefore, to test the effects of IL-4 or IL-13 on responses to carbachol, chambers were perfused with carbachol ( $10^{-5}$  M) for 2 min and the resulting calcium transient was recorded (S1). After washing the carbachol from the chambers for 15 min, a time interval sufficient for refilling of intracellular calcium stores in these cells (15), the chambers were perfused with PSS containing IL-4 (5–50 ng/ml) or IL-13 (50 ng/ml) for 5 to 20 min. Finally, after again washing with PSS for the time specified, the cells were stimulated with carbachol ( $10^{-5}$  M) and a second calcium transient for the same cell (S2) was recorded. An S2/S1 ratio was then calculated for each cell as an index of cell responsiveness. In some experiments, caffeine (10 mM) was substituted for carbachol for the S2 stimulus. For all experiments, controls were exposed to vehicle (PSS) instead of IL-4 or IL-13 for the same lengths of time and according to the same protocol. Up to four chambers were prepared from each cell isolation, allowing each chamber to be treated with IL-4 or IL-13 only once. A total of 55 separate tracheas were used in these studies. In experiments testing the effect of antibody to IL-4, the antibody (60  $\mu$ g/ml) and IL-4 (50 ng/ml) were preincubated together for 1 h at 37°C in a total volume of 5 ml.

### Data Analysis

In measuring S1 and S2 responses, the magnitude of the calcium transient was measured from the peak  $[Ca^{2+}]_i$  level recorded in response to carbachol or caffeine, and basal  $[Ca^{2+}]_i$  was subtracted. All data are expressed as means  $\pm$  standard error of the mean (SEM) and  $n$  indicates the number of cells studied. Analysis of variance was used for multiple comparisons between means. For comparing two groups, an unpaired Student's *t* test was used.

### Materials

Collagenase, elastase, and IL-4 were obtained from Roche Molecular Biochemicals (Indianapolis, IN). Fura-2-AM and pluronic

F-127 were obtained from Molecular Probes, Inc. (Eugene, OR). IL-13 and goat antihuman IL-4 neutralizing antibody (IgG) were obtained from R&D Systems, Inc. (Minneapolis, MN). Other reagents were obtained from Sigma (St. Louis, MO).

### Results

IL-4 caused small and variable increases in  $[Ca^{2+}]_i$ . When 35 cells with a resting  $[Ca^{2+}]_i$  level of  $181 \pm 11$  nM were perfused with IL-4 (50 ng/ml), there was a small but significant increase in  $[Ca^{2+}]_i$  to  $260 \pm 26$  nM within approximately 1 min of initial IL-4 exposure ( $P < 0.01$ ). The increase in  $[Ca^{2+}]_i$  was transient such that  $[Ca^{2+}]_i$  decreased to  $163 \pm 9$  nM after 5 min of IL-4 exposure (Figure 1). When intracellular calcium stores were first decreased by a 30-min exposure to thapsigargin (0.3  $\mu$ M), an inhibitor of sarcoplasmic reticulum (SR)  $Ca^{2+}$ -adenosine triphosphatase (ATPase) activity, responses to IL-4 were not detectable ( $15 \pm 13$  nM increase above basal,  $n = 6$ , not significant).

Initial responses to carbachol (S1) averaged  $1,428 \pm 338$  nM (range 315 to 4,628 nM,  $n = 13$ ) in magnitude and, for each individual cell, strongly predicted the magnitude of a second response to carbachol (S2) (Figure 2). Consequently, for controls, S2/S1 ratios had little variability and averaged  $0.70 \pm 0.04$  ( $n = 13$ ). In contrast, S2/S1 was significantly decreased when cells were exposed to IL-4 for 20 min. Specifically, after S1 testing, cells were perfused with IL-4 (50 ng/ml) for 20 min and then washed for 10 min with PSS before S2 testing. The calculated S2/S1 ratios were significantly decreased to  $0.17 \pm 0.04$  ( $n = 7$ ,  $P < 0.01$ ) (Figure 3A). The initial responsiveness of a cell (S1) did not predict the effect that IL-4 would have on the S2/S1 ratio in that cell (Figure 3B). Also, the magnitude of the small, transient increase in  $[Ca^{2+}]_i$  in response to IL-4 alone (Figure 1) did not correlate with the effect that IL-4 exposure had on S2/S1 in a given cell (not shown). When thapsigargin was administered during the interval between the S1 and S2 stimulus, the S2/S1 ratio was decreased markedly (Figure 2), consistent with recovery of S2 responses being strongly dependent on refilling of SR calcium stores. The inhibitory effect of IL-4 was concentration-dependent (Figure 3A).

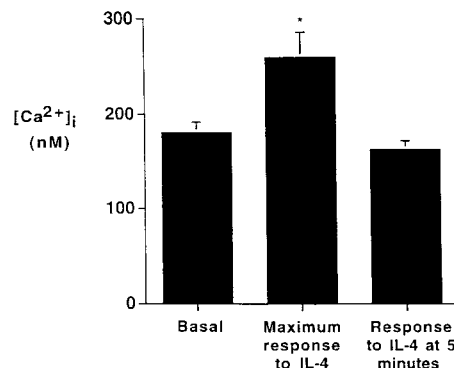
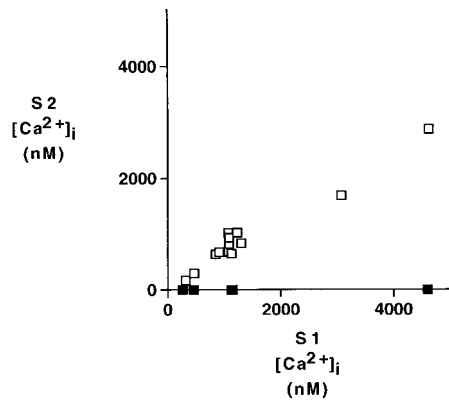


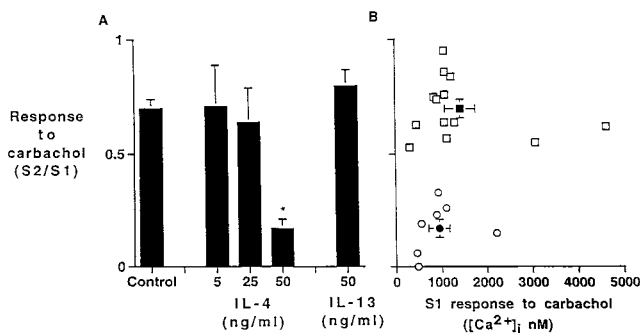
Figure 1. Effect of IL-4 on  $[Ca^{2+}]_i$  in bovine ASM cells. Cells loaded with fura-2 were exposed to human recombinant IL-4 (50 ng/ml) and changes in  $[Ca^{2+}]_i$  were recorded in single cells. The maximum response to IL-4 occurred within one minute. Data are means  $\pm$  SEM,  $n = 35$ . \*Significantly different from basal ( $P < 0.05$ ).



**Figure 2.** S2/S1 ratio. For control cells (*open squares*) not exposed to IL-4, each cell was stimulated with carbachol ( $10^{-5}$  M) and the maximum change in  $[Ca^{2+}]_i$  during the resulting transient was recorded and defined as S1. After washing with PSS for 45 min, the same cell was stimulated a second time with carbachol and the magnitude of the resulting transient was recorded and defined as S2. The figure shows that the magnitude of the S1 response did predict the magnitude of the S2 response in that same cell. For each control cell, an S2/S1 ratio was calculated as an index of cell responsiveness and averaged  $0.70 \pm 0.04$ . Other cells (*filled squares*) were treated identically except that thapsigargin was present during the recovery period between S1 and S2 stimuli.

When cells were perfused by 5 and 25 ng/ml IL-4, the mean S2/S1 ratios were not significantly changed but there was increased variability in responses among cells. After 5 and 25 ng/ml IL-4, S2/S1 ratios varied between 0.29 and 1.16, and 0.01 and 0.92, respectively. When cells were perfused by IL-13 (50 ng/ml) for 20 min according to the same protocol used for IL-4, no inhibition of S2/S1 was observed (Figure 3A).

The effects that IL-4 had on calcium responses to carbachol ( $10^{-5}$  M) were time-dependent (Figure 4). After S1

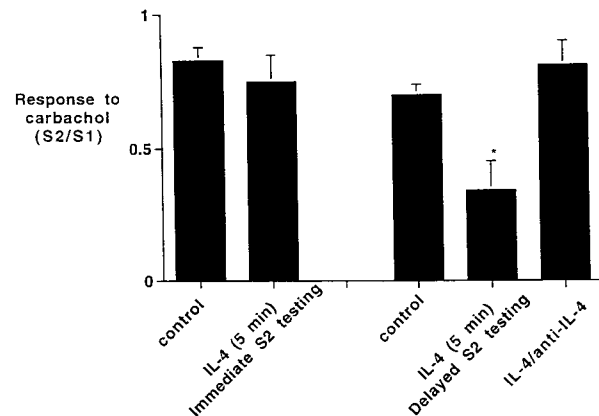


**Figure 3.** Effects of IL-4 and IL-13 on responses to carbachol. (A) After the S1 stimulus with carbachol ( $10^{-5}$  M), the cells were washed for 15 min and then exposed to IL-4 (5, 25, and 50 ng/ml) ( $n = 5-7$ ) or IL-13 (50 ng/ml) ( $n = 7$ ) for 20 min. Finally, cells were washed for 10 min before being stimulated a second time with carbachol (S2). Mean data are presented. \*Significantly different from control ( $P < 0.01$ ). (B) Data from A are presented to show the individual responses of control cells (*open squares*) versus those cells exposed to IL-4 (50 ng/ml) (*open circles*). Filled symbols show mean values for control (*square*) and IL-4-treated (*circle*) cells.

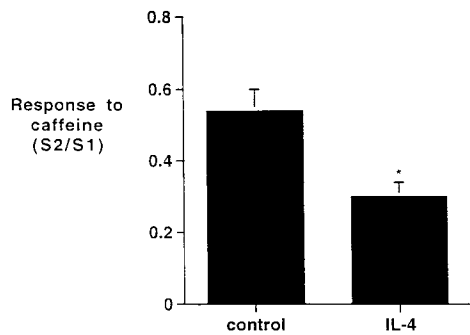
testing, cells were exposed to IL-4 (50 ng/ml) for only 5 min and then immediately exposed to carbachol ( $10^{-5}$  M) a second time to test the S2 response. Under these conditions the S2/S1 ratio was  $0.75 \pm 0.10$  ( $n = 6$ ) for IL-4-treated cells, not significantly different from controls. In contrast, when cells were exposed to IL-4 (50 ng/ml) for the same 5-min interval, then perfused with PSS and tested 25 min later, the S2/S1 ratio was decreased to  $0.34 \pm 0.11$ , significantly different from controls ( $n = 6$ ,  $P < 0.01$ ). IL-4 (50 ng/ml) did not significantly inhibit S2/S1 ratios when the IL-4 was administered along with goat antihuman IL-4 (Figure 4).

The effects that IL-4 had on responses to caffeine were also tested. After S1 testing with carbachol, cells were exposed to IL-4 (50 ng/ml) for 20 min, washed for 10 min, and then stimulated with caffeine (10 mM) to assess an S2 response. The S2/S1 ratio was decreased in IL-4-treated cells ( $0.30 \pm 0.04$ ,  $n = 5$ ), and this was significantly different from controls not treated with IL-4 ( $0.54 \pm 0.06$ ,  $n = 5$ ,  $P < 0.01$ ) (Figure 5).

To further assess mechanisms, we characterized the effect that SR calcium content had on the time course of IL-4 inhibition of S2/S1 ratios (Figure 6). One group of cells was treated with IL-4 (50 ng/ml) for 2 min immediately after the S1 response (carbachol) and then the S2 stimulus (carbachol) was delivered. S2/S1 ratios were  $0.16 \pm 0.05$  ( $n = 4$ ), significantly different from time-matched controls not exposed to IL-4 ( $0.49 \pm 0.09$ ,  $n = 7$ ,  $P < 0.05$ ). A second group of cells was treated with IL-4 for 2 min, but only after washing with PSS for 10 min to allow refilling of intracellular stores. For this group of cells, the S2/S1 ratio was



**Figure 4.** Time dependence of IL-4 effect on S2/S1 ratios. After the S1 stimulus with carbachol ( $10^{-5}$  M) and a 15-min wash, cells were exposed to IL-4 (50 ng/ml) for 5 min. In one group of cells ( $n = 6$ , *left side* of figure), the second stimulus (S2) with carbachol ( $10^{-5}$  M) followed immediately after the 5-min exposure to IL-4. In that case, IL-4 had no apparent effect on the S2/S1 ratio compared with time-matched controls. For other cells ( $n = 6$ , *right side* of figure), the S2 stimulus with carbachol ( $10^{-5}$  M) was applied after the cells had been exposed to IL-4 for 5 min but then washed for 25 min before S2 testing. In this case, the S2/S1 ratio was significantly decreased compared with time-matched controls. \*Significantly different from control ( $P < 0.01$ ). The presence of goat antihuman IL-4 neutralizing antibody inhibited the effect that IL-4 had on S2/S1 ( $n = 4$ ).



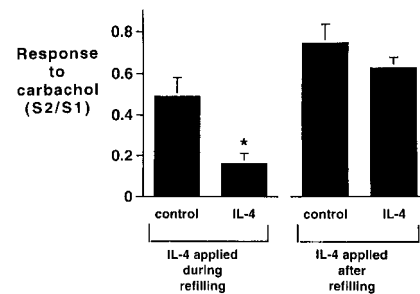
**Figure 5.** Effects of IL-4 on responses to caffeine. After the S1 stimulus with carbachol ( $10^{-5}$  M) and a 15-min wash, cells were exposed to IL-4 (50 ng/ml) for 20 min. Cells then were washed for 10 min before being stimulated with caffeine (10 mM) (S2). Time-matched controls were not exposed to IL-4. Data are means  $\pm$  SEM,  $n = 5$ . \*Significantly different from control ( $P < 0.01$ ).

$0.62 \pm 0.05$  ( $n = 4$ ), not significantly different from time-matched controls. In these experiments, under control conditions, S2 responses were inhibited ( $S2/S1 = 0.11 \pm 0.03$ ,  $n = 3$ ) when thapsigargin was present during the 10-min recovery period between the S1 and S2 stimuli.

## Discussion

Recombinant human IL-4 rapidly inhibited calcium transients in response to carbachol in bovine ASM cells *in vitro*. To our knowledge, this is the first demonstration that a relatively brief exposure to IL-4 inhibits agonist-stimulated calcium transients in ASM or in any cell type. These new findings are potentially important because of the known role that calcium plays in regulating contractions of ASM and because TH2 cytokines, including IL-4, are thought to play a regulatory role in asthmatic airway inflammation.

IL-4 alone caused a small, transient increase in  $[Ca^{2+}]_i$  that depended on SR calcium stores. However, the main finding was that 30 min after the introduction of IL-4 to the perfusate, carbachol-stimulated calcium transients were significantly inhibited. This effect of IL-4 on calcium transients could not be explained by random differences in S1 responses for the treated versus untreated cells because S1 responses were comparable for the two groups of cells and there was no correlation between the magnitude of S1 responses and the S2/S1 ratios observed. The inhibitory effect of IL-4 on responses to carbachol was dose-dependent in that it was not consistently observed at concentrations below 50 ng/ml. The observed increased variability in S2/S1 ratios when cells were exposed to lower concentrations of IL-4 may be an indication of cell heterogeneity in sensitivity to IL-4. Alternatively, the variability might be caused by effects of dispersal enzymes on cell-surface receptors. Other experiments showed that the inhibition of calcium transients by IL-4 occurred rapidly but did require a finite period of time to become manifest. From the time of first exposure to IL-4, the inhibitory effect required more than 5 but less than 30 min. A notable feature of this inhibitory effect was that after the first 5 min of IL-4 exposure IL-4 could be washed from the chambers and, still, calcium transients would be inhibited 25 min later. This suggests that a



**Figure 6.** Effect of SR filling state on IL-4 inhibition of transients. After the S1 stimulus with carbachol ( $10^{-5}$  M), cells were treated in two different ways. One group of cells was washed immediately for 2 min with buffer containing IL-4 (50 ng/ml) and then the S2 stimulus with carbachol was applied. The other group of cells was washed with buffer alone for 10 min to allow refilling of intracellular stores and then the cells were exposed to IL-4 for 2 min before the S2 stimulus with carbachol. For both groups, controls were identically treated cells not exposed to IL-4. Data are means  $\pm$  SEM,  $n = 4-7$ . \*Significantly different from control ( $P < 0.05$ ).

5-min exposure to IL-4 inhibits calcium transients by activating processes that are slowly reversible or irreversible. In aggregate, our findings were consistent with IL-4 activating, over a 30-min interval, an IL-4R-mediated process that impaired calcium signaling by muscarinic agonists.

Several findings argued against a nonspecific effect of IL-4 in these experiments. First, whereas some cells, especially hematopoietic cells, do respond to much lower concentrations of IL-4, the maximally effective concentration of IL-4 used in this study (50 ng/ml) was in a range consistent with previous reports for B lymphocytes, pre-B cells, fibroblasts, osteoclasts, endothelial cells, and ASM (10, 12, 14, 17-19). Second, IL-4 did not cause a sustained increase in resting  $[Ca^{2+}]_i$  levels, as might be expected if IL-4 were exerting a nonspecific cytotoxic effect. Third, the effect of IL-4 was specific because inhibitory effects on calcium transients were not observed with the closely related TH2 cytokine, IL-13. Fourth, potential contaminants in the IL-4 preparation seemed an unlikely explanation because goat antihuman IL-4 antibody abolished the inhibitory effect of IL-4. Fifth, the inhibitory effect of IL-4 was evident even when IL-4, and potential contaminants, had been washed from the chamber before S2 testing with carbachol. Finally, the fact that human IL-4 had effects on bovine cells was not surprising because cross-reactivity for IL-4 between these species has been reported previously (20).

The specificity of the IL-4 effect is supported by the observation that the closely related cytokine IL-13 did not inhibit calcium transients in ASM. Usually these two cytokines share biologic activities in nonhematopoietic cells, but there is some precedent for differential effects (21-23). In human ASM cells, IL-4, but not IL-13, inhibited release of monocyte chemoattractant protein-1 and -2 (23). These differential effects were clearly complex, however, because IL-4 and IL-13 both inhibited release of RANTES and IL-8 from these cells (10, 24).

Comparing the effects of IL-4 and IL-13 is of interest because IL-13 has been shown to be an important mediator of the asthma phenotype in murine models (25, 26),

and these two cytokines bind to a family of shared heteromultimeric receptor complexes (27). The receptor complexes specifically expressed by ASM are not known, but two major receptors for IL-4 have been described and those are the type I (IL-4R $\alpha$ / $\gamma$ c) and type II (IL-4R $\alpha$ /IL-13R $\alpha$ 1) receptors (13). Because the type II receptor also binds IL-13, the current results are consistent with the type I receptor mediating the inhibition of calcium transients. However, it is important to note that our experiments tested IL-13 effects at only a single concentration and time interval. Also, the common  $\gamma$  chain ( $\gamma$ c) component of the type I receptor is predominantly expressed in hematopoietic cells (28) and it is the type II receptor that has been proposed to couple to calcium mobilization (13). Therefore, it is possible that the type II receptor mediated the effects of IL-4 in our experiments, but that IL-13 has slower, more rapidly reversible, or different effects due to concomitant binding at additional types of IL-13R complexes (27–29). Also, it is possible that the apparent differential effects of IL-4 and IL-13 in the current study were due to poor species cross-reactivity for human IL-13. Against this, however, is the substantial homology between bovine and human IL-13 and the observation that human IL-13 stimulates bovine B-cell proliferation (30). On the basis of these considerations, our conclusions are cautious regarding the subtype of IL-4R that mediates inhibition of calcium transients.

Additional experiments were done to begin to characterize mechanisms linking IL-4R activation to inhibition of calcium mobilization by carbachol. We tested the effects that IL-4 had on calcium mobilization by caffeine, an agent that causes rapid release of calcium from intracellular stores by mechanisms independent of IP<sub>3</sub> synthesis. Our finding that IL-4 inhibited responses to caffeine did not exclude the possibility that IL-4 has inhibitory effects on muscarinic receptor coupling to IP<sub>3</sub> synthesis, but it did suggest that, at least in part, IL-4 acts either by inhibiting release of calcium from the SR or by depleting SR stores. Because carbachol and caffeine mobilize calcium through different SR channels, and because IL-4 alone caused increases in [Ca<sup>2+</sup>]<sub>i</sub> that depended on SR calcium stores, depletion of SR stores, rather than inhibition of calcium release, is the more likely mechanism.

Because SR content is determined by a dynamic equilibrium between calcium release and uptake, stores could be depleted by an increased rate of calcium release, decreased SR Ca<sup>2+</sup>-ATPase activity, or decreased entry of calcium into the cell along SR refilling pathways. Previous studies of IL-4 in B lymphocytes and osteoclasts have demonstrated rapid changes in [Ca<sup>2+</sup>]<sub>i</sub> that depended on calcium mobilization from intracellular stores. In osteoclasts, IL-4 caused a rapid and sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> that depended on both calcium influx and mobilization of intracellular stores (19). In B lymphocytes, IL-4 rapidly stimulated IP<sub>3</sub> synthesis and calcium transients that depended on mobilization of calcium from intracellular stores (14). A link between IL-4R activation and IP<sub>3</sub> synthesis has not been established for ASM, but one possibility is IL-4R activation of IRS, phosphatidylinositol 3-kinase, and phospholipase C $\gamma$  (13, 31). By this pathway, IL-4 could inhibit calcium transients by rapidly causing a sustained IP<sub>3</sub>-

mediated calcium release that gradually depletes the SR over approximately 30 min. Consistent with this, we showed that the rapidity with which IL-4 inhibited transients was dependent on the filling state of the SR (Figure 6). IL-4 inhibited S2/S1 ratios within 2 min when SR calcium was decreased and the SR was in the process of refilling. However, that same 2-min exposure to IL-4 had no detectable effect on S2/S1 ratios when SR stores were filled.

Although the gradual depletion of the SR as the result of an increased rate of calcium release could explain the inhibition of transients by IL-4, it is important to acknowledge that, for single cells, there was no correlation between the magnitude of the acute effect of IL-4 alone on [Ca<sup>2+</sup>]<sub>i</sub> and the magnitude of the inhibitory effect on calcium transients 30 min later. Also, gradual depletion of SR calcium would not necessarily explain the observation that IL-4 could be washed from the cells for 10 to 25 min and still calcium transients were inhibited. Therefore, it remains possible that the immediate effects of IL-4 alone on [Ca<sup>2+</sup>]<sub>i</sub> and its acute (< 2 min) effects on S2/S1 ratios are unrelated to, or only a partial explanation for, the inhibition of transients 30 min later.

The novel findings of the current study have potential implications for regulation of smooth-muscle function in asthma, a disease characterized by a TH2 cytokine profile (2). Prior studies by others have shown that IL-4 inhibits growth (12) and release of cytokines by ASM cells (10, 23, 24). Our *in vitro* data now identify yet another inhibitory effect of IL-4 on ASM function, namely the inhibition of calcium transients in response to a muscarinic agonist. Although the *in vivo* context and duration of this IL-4 effect on calcium mobilization are not known, inhibition of calcium mobilization in response to vagal stimulation could conceivably play some role in modulating smooth-muscle contraction, growth, and secretory activity in the presence of airway inflammation. In this regard, it is notable that studies of immunized, IL-4-deficient mice showed that IL-4 is necessary for the development of bronchial hyperresponsiveness (32, 33). However, considering that some studies suggested that IL-4-deficient mice have increased baseline bronchial responsiveness (33, 34) and one other study did show increased responsiveness after allergen challenge (35), it is likely that the roles of IL-4 in the development of bronchial hyperresponsiveness are complex.

In summary, recombinant, human IL-4 rapidly (< 30 min) inhibited carbachol-stimulated calcium mobilization in freshly dispersed bovine ASM cells. Because calcium transients in response to caffeine were also inhibited by IL-4, it may be that IL-4 inhibits transients, at least in part, by depleting SR calcium stores. This rapid effect of IL-4 on calcium transients in ASM cells may be an important factor modulating airway remodeling and contraction during inflammation.

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