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IL-4 inhibits calcium transients in bovine trachealis cells by a ryanodine receptor dependent mechanism

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Abstract

IL-4 and IL-13 have important roles in the pathogenesis of asthma. A novel finding was that brief exposure of airway smooth muscle cells to IL-4 inhibited carbachol-stimulated calcium transients. We hypothesized that IL-4 inhibits transients by decreasing calcium store content and tested this by measuring the effects of IL-4 on transients induced by a nonspecific ionophore. Bovine trachealis cells were loaded with fura 2-AM and cytosolic calcium concentrations ($[Ca^{2+}]_i$) were measured in single cells by digital microscopy. Stimulation (S1) with carbachol (10 μ M) caused rapid, transient increases in $[Ca^{2+}]_i$ to 1299 ± 355 nM (n=5). After recovery of calcium stores, stimulation (S2) of the same cells with ionomycin (10 μ M), in the absence of extracellular calcium, also increased $[Ca^{2+}]_i$ to give S2/S1 ratio of 1.03 ± 0.29 . However, after 20 min of IL-4 (50 ng/ml), but not IL-13, ionomycin transients were decreased to 0.50 ± 0.16 (S2/S1, $P=0.02$, n=6). IL-4 did not inhibit transients with ryanodine receptor calcium release channels (RyR) blocked by ryanodine (200 μ M) (S2/S1= 1.01 ± 0.11), but still did in the presence of 8-bromo cyclic ADP-ribose, an antagonist of cyclic ADP-ribose (cADPR) signaling at RyR (S2/S1= 0.48 ± 0.13). Together, findings suggest that IL-4 decreases intracellular calcium stores by mechanisms dependent on RyR, but not on cADPR signaling.

Keywords

interleukin (IL)-4; interleukin (IL)-13; calcium; ryanodine receptor; airway smooth muscle; ionomycin; caffeine; carbachol; cyclic ADP-ribose

INTRODUCTION

In airway smooth muscle, the main intracellular store for rapidly mobilizable calcium is the sarcoplasmic reticulum (SR). Store content of calcium is determined by a dynamic balance between uptake of calcium into the SR and release via inositol 1,4,5 trisphosphate receptors (IP₃R), ryanodine receptors (RyR) and possibly other channels (1-6) of the SR. Modulating this dynamic balance between uptake and release of calcium in the SR can alter the amount of calcium available for rapid mobilization by inositol 1,4,5 trisphosphate (IP₃) and cyclic ADP-ribose (cADPR) (7) and, therefore, affect the magnitude of the calcium transients elicited by agents such as carbachol and caffeine (8-10).

In vitro studies have shown that the T helper-2 cytokine, IL-4, has effects on airway smooth muscle cell functions, including cell proliferation (11) and secretion of inflammatory mediators (12-19), and that many of these effects are maximal at approximately 50-100 ng/ml. These observations by others are important because these same concentrations of IL-4 have been shown to elicit effects in other non-hematopoietic cell types and IL-4 is increased in the

bronchial lavage fluid of subjects with asthma (20). Evidence also suggests that IL-4 modulates calcium signaling in airway smooth muscle (21,22). In single, bovine trachealis cells, IL-4 (50 ng/ml) had only small effects on cytosolic calcium concentrations ($[Ca^{2+}]_i$) by itself, but yet, over 20 min time, significantly inhibited the magnitudes of both carbachol- and caffeine-stimulated calcium transients (21). Notably, the closely related T helper 2 cytokine, IL-13, did not share these inhibitory effects with IL-4. Because carbachol activates IP₃R to release SR calcium while caffeine acts by a different mechanism at the RyR calcium release channel (23), we hypothesized that IL-4 inhibits calcium transients by reducing the amount of calcium in the SR. An alternative hypothesis was that IL-4 inhibits calcium release through both IP₃R and RyR simultaneously, thereby inhibiting calcium mobilization by both carbachol and caffeine. Therefore, the first goal of this study was to distinguish these two possibilities by testing whether IL-4 and IL-13 inhibit transients in response to ionomycin, a calcium ionophore that mobilizes SR calcium independent of IP₃R or RyR function (24,25).

Because subsequent results showed that IL-4 does inhibit calcium mobilization by ionomycin, a finding consistent with store depletion, a second goal was to determine the mechanism. Importantly, a prior study showed that an effect of IL-4 on calcium transients could still be observed in the presence of cyclopiazonic acid (CPA), a selective inhibitor of the sarcoplasmic reticulum calcium ATPase (SERCA) responsible for uptake of calcium into the SR (1,22,26). This observation suggested that IL-4 does not deplete SR calcium by inhibiting SERCA activity, but, instead, increases the rate of calcium release to cause a net loss of calcium from the SR. That study also showed that the IP₃R antagonist, xestospongin C, did not inhibit the effect of IL-4, suggesting that RyR and cADPR signaling might be involved in IL-4 inhibition of calcium transients. Therefore, for the current study, we hypothesized that IL-4 depletes SR calcium by a mechanism dependent on cADPR and RyR calcium release channels and, consequently, tested whether inhibition of this signaling pathway blocked the effect of IL-4 on SR calcium stores.

METHODS

Cell isolation

Bovine trachealis tissue was minced and placed in 2.5 ml physiologic salt solution (PSS, in mM: NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.6, glucose 11.1, CaCl₂ 2.5) modified to have no added CaCl₂ and containing collagenase A (6 mg) and elastase (3 mg) (10,21). The mince was digested for 12 min at 37°C and then again with an additional aliquot of enzyme. The partially digested mince then was transferred to 3 ml of PSS modified to contain 0.1 mM CaCl₂ and incubated for 3-6 min with constant stirring. Released cells were loaded with 0.5 μ M fura 2-AM (pluronic F-127 0.004%) for 60 min at room temperature and placed in a glass perfusion chamber (volume 0.3 ml). After cells attached for 10 min, PSS perfused the chamber at 1 ml/min.

Calcium measurement

Fura 2 was excited by computer-controlled 337 and 380 nm UV light generated by a nitrogen laser and a nitrogen laser-pumped dye laser, respectively (Laser Science, Franklin, MA). Each laser alternately fired pulses (3 nsec) at 30 Hz and the pulses were directed at the cells through a 40x objective lens (Nikon, Melville, NY). The fluorescent signals emitted by fura 2 were passed back through the objective to a 455 nm dichroic mirror, a 475 nm barrier filter (Omega Optics, Brattleboro, VT), an image intensifier (Xybion Electronic Systems, San Diego, CA) and captured by a Philips-based frame transfer charge coupled device (CCD) camera (CCTV, New York, NY). The analog signals from the camera were digitized with outputs to a personal computer with software by Recognition Technology, Inc (Framingham, MA).

As described previously (10,21), background from a cell free region of the cover glass was subtracted prior to data acquisition and then an 11×11 pixels area was selected over the cell. The fluorescence stimulated by alternating pulses of 337 and 380 nm light were recorded and their ratios plotted. Ratios were converted to calcium concentrations (27): $[Ca^{2+}]_i = K_D * \beta * (R - R_{min}) / (R_{max} - R)$ where R_{max} and R_{min} are the fluorescence ratios measured in high and zero calcium, respectively; β 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. Based on an *in situ* determination in bovine trachealis cells (28), a K_D value of 386 nM was used in converting fluorescence ratios to $[Ca^{2+}]_i$.

Protocols

Cells were stimulated with PSS containing carbachol (10 μ M) for 2 min and then washed at least 30 min with PSS before the start of experiments. In all experiments, cell chambers were superfused with PSS containing the specified reagents and a 4-way valve allowed changing of the perfusate without disturbing the recordings. There was cell to cell variability in the magnitude of calcium transients. Therefore, ionomycin-stimulated calcium transients following IL-4 (50 ng/ml, recombinant human IL-4) or IL-13 (50 ng/ml, recombinant human IL-13) treatment (S2) were normalized to the magnitude of a carbachol transient in that same cell before cytokine treatment (S1). The S2/S1 ratio for each cell was used as an index of cell responsiveness to ionomycin (9,10,29). For all experiments, controls were exposed to vehicle, instead of cytokines, for the same lengths of time and according to the same protocols. In measuring S1 and S2 responses, the magnitude of the calcium transient was measured from the peak $[Ca^{2+}]_i$ level and basal $[Ca^{2+}]_i$ was subtracted. All data were expressed as a mean $\pm$ SEM and n indicates the number of cells studied. ANOVA with Newman-Keuls follow-up test was used for multiple comparisons between means.

Gel electrophoresis and western blotting

Finely minced bovine trachealis tissue was suspended in ice-cold homogenizing buffer containing 25 mM HEPES, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1.0 mM sodium vanadate, 0.2 M sucrose, 1 mM dithiothreitol, 1 mM ATP, 0.01 mg/ml tosylargininemethylester, 4 μ g/ml leupeptin, 1 mM benzamidine, 1 mM 1,10 phenanthrene, 0.2 mM phenylmethylsulfonyl fluoride, and homogenized (3×20 s at setting 40) with a Virtishear homogenizer (Virtis Co., Inc., Gardiner, NY). The homogenate was centrifuged at $5000g \times 20$ min and the supernatant collected and centrifuged at $27,200g \times 50$ min. The microsomal pellet was resuspended in a small volume of homogenizing buffer, quick frozen in liquid nitrogen, and stored at -80°C until use.

For SDS-PAGE, microsomes were denatured for 4 min at 95°C in loading buffer according to Laemmli (30). Electrophoresis was performed using 4-15% precast gels (Bio-Rad, Hercules, CA) and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). Membranes were blocked in 5% DIFCO skim milk (Becton, Dickinson and Co., Sparks, MD) and incubated overnight at 4°C in mouse anti-RyR monoclonal antibody (MA3-925, clone 34C, Affinity BioReagents, Golden, CO). Secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG (Promega, Madison, WI) and this was visualized by enhanced chemical luminescence (PerkinElmer Life Sciences, Boston, MA).

Materials

Collagenase and elastase were obtained from Roche Molecular Biochemicals (Indianapolis, IN). Ryanodine, fura 2-AM and pluronic F-127, were obtained from Molecular Probes, Inc. (Eugene, OR). IL-4 and IL-13 were obtained from R&D Systems, Inc. (Minneapolis, MN). Other reagents were obtained from Sigma (St. Louis, MO).

RESULTS

Bovine airway smooth muscle cells were exposed to carbachol (10 μ M) and a rapid, transient increase in $[Ca^{2+}]_i$ was recorded (S1 response)(Fig. 1A). For 5 cells, the magnitude of the S1 response to carbachol was 1299 ± 355 nM. After S1, the same cells were washed with PSS for 20 minutes to allow recovery and then the cells were stimulated with ionomycin (10 μ M) in nominally calcium free buffer (PSS, 0 Ca^{2+}) to elicit a second response (S2). Under these calcium-free conditions, ionomycin caused a rapid, transient increase in $[Ca^{2+}]_i$ that was comparable to that observed with carbachol ($S2/S1 = 1.03 \pm 0.29$) and had an absolute magnitude of 1219 ± 388 nM. In contrast, in cells that were pretreated with a SERCA inhibitor, CPA (30 μ M)(1,10,22,26), responses to ionomycin were decreased (Fig. 1B). For these cells pretreated with CPA, the $S2/S1$ ratio was 0.11 ± 0.09 ($P = 0.02$, $n = 5$).

In other experiments, after the S1 stimulus with carbachol, each cell was washed for 10 min to allow recovery of SR stores and then treated with either IL-4 (50 ng/ml) or vehicle control for 20 min. Then each cell was exposed to ionomycin (10 μ M in PSS, 0 Ca^{2+}) and the resulting response (S2) recorded (Fig. 2A). Treatment with IL-4 decreased $S2/S1$ ratios (0.50 ± 0.16 , $n = 6$) and this was significantly different than that observed in vehicle controls (1.02 ± 0.12 , $P = 0.02$, $n = 7$)(Fig. 2B). The S1 responses to carbachol were similar in the treated (1431 ± 363 nM) and untreated cells (1163 ± 170 nM).

Rabbit anti-RyR identified a protein of high molecular mass (~ 565 Kd) consistent with the presence of RyR calcium release channels in airway smooth muscle (Fig. 3A). In single, isolated cells, caffeine (10 mM) caused a rapid, transient increase in $[Ca^{2+}]_i$ ($S2/S1$ ratio of 0.71 ± 0.10 , $n = 3$) and this too was consistent with the presence of functional RyR calcium release channels (Fig. 3B). However, when 4 cells were pre-treated with a relatively high concentration of ryanodine (200 μ M) to block RyR calcium release channels and then exposed to caffeine, the magnitude of the resulting transient was, as expected, significantly decreased with an $S2/S1$ ratio of 0.17 ± 0.02 ($P = 0.001$)(Fig. 4A,B). In other experiments, the same high concentration of ryanodine that blocked responses to caffeine had no significant effect on $[Ca^{2+}]_i$ by itself and did not inhibit control responses to ionomycin (10 μ M) (Figs. 4A and 5).

Cells were stimulated with carbachol (10 μ M) (S1) and then washed for 10 min to allow recovery. Cells then were pre-treated with ryanodine (200 μ M) or 8-bromo cADP-ribose (8Br-cADPR)(100 μ M) (or vehicle) for 10 min before IL-4 (50 ng/ml) was added for 20 min. Finally, S2 responses were elicited with ionomycin (10 μ M) and recorded. The presence of ryanodine, but not 8Br-cADPR, significantly inhibited the effect of IL-4 on $S2/S1$ ratios (Fig. 5). Specifically, in the presence of ryanodine, treatment with IL-4 did not alter the $S2/S1$ ratio (1.01 ± 0.11 , $n = 5$), but in the presence of 8Br-cADPR, the $S2/S1$ ratio was only 0.48 ± 0.13 , $n = 5$ after treatment with IL-4. In 3 cells, a longer pre-treatment with 8Br-cADPR (15 min) before exposure to IL-4 and a higher concentration of 8Br-cADPR (200 μ M) still did not inhibit the effect of IL-4. Like ryanodine, 8Br-cADPR by itself had no significant effect on responses to ionomycin (Fig. 5).

In separate experiments, after recovery from the S1 response to carbachol, cells were pre-treated with IL-13 (50 ng/ml) (or vehicle) for 20 min and then stimulated with ionomycin. Unlike IL-4, the cytokine IL-13 did not decrease responses to ionomycin (Fig. 6).

DISCUSSION

For bovine airway smooth muscle cells, this study shows that IL-4 inhibits the magnitude of calcium transients stimulated by ionomycin and that this effect of IL-4 depends on the function of RyR calcium release channels. Because the calcium transients elicited by ionomycin

depended on the mobilization of calcium from intracellular stores, these findings suggest that, over a relatively brief interval of time, IL-4 decreases intracellular calcium stores by causing a net release of calcium from the SR through RyR calcium release channels. Notably, IL-13, a cytokine closely related to IL-4, did not deplete SR calcium stores.

In an early study of IL-4 signaling in osteoclasts, IL-4 stimulated small increases in $[Ca^{2+}]_i$ that depended on both calcium influx and mobilization of SR calcium stores (31). In airway smooth muscle cells, IL-4 did cause similar small increases in $[Ca^{2+}]_i$, but these were highly variable among individual cells and were not consistently observed (21,22). It was suggested that even though IL-4 caused only very small and inconsistent increases in $[Ca^{2+}]_i$, IL-4 still might be causing a substantial mobilization of calcium from the SR but into a localized, subplasmalemmal region of the cytoplasm that is difficult to detect by our whole cell imaging techniques. Consistent with this, calcium transients in response to maximally effective concentrations of carbachol (10 μ M) (28) and caffeine (10 mM) (9) were both significantly inhibited by only 20 min pre-treatment with IL-4 (21). Since carbachol and caffeine mobilize calcium from the SR by distinct mechanisms (1), an IL-4 effect on both agents was consistent with IL-4 decreasing SR calcium stores. However, another possibility was that IL-4 simultaneously inhibited both IP₃R and RyR-mediated calcium release from the SR. To distinguish these possibilities, in the present study, we tested whether IL-4 inhibits transients elicited by ionomycin (10 μ M), a concentration of calcium ionophore that mobilizes SR calcium by mechanisms independent of IP₃R and RyR (24,25). To eliminate any contribution that calcium influx might make to the magnitude of the transients recorded, extracellular calcium was absent during ionomycin exposure in all experiments. To be certain that the ionomycin response recorded was specifically due to release of calcium from the SR, in preliminary experiments, we showed that pre-treating cells with CPA to deplete SR calcium abolished ionomycin-stimulated transients. Then, in the main experiments, ionomycin was used to mobilize SR calcium. The data show that pre-treatment with IL-4 did inhibit responses to ionomycin and, therefore, that IL-4 does not act by inhibiting IP₃R and RyR simultaneously. Instead, the results are most consistent with IL-4 partially depleting SR stores of calcium.

The calcium content of the SR is determined by a dynamic balance between rates of uptake and leak of calcium from the SR (1). Theoretically, IL-4 could decrease calcium in the SR by inhibiting calcium uptake via SERCA and/or by stimulating the release of calcium from the SR. In a prior study of these same cells, IL-4 inhibited calcium transients in response to carbachol even in the presence of a high concentration of CPA (30 μ M), an inhibitor of SERCA (22). This finding suggested that IL-4 did not decrease SR calcium content by inhibiting calcium uptake and, therefore, must be increasing the rate of release of calcium from the SR. However, subsequent experiments in that same study, showed that xestospongin C, an inhibitor of IP₃R, did not block the effect of IL-4 on calcium transients. Therefore, in the current study, we hypothesized that IL-4 decreases SR calcium by stimulating release of calcium ions through RyR calcium release channels. Consistent with that possibility, RyR channels are present in bovine trachealis as evidenced by Western blotting and the presence of calcium transients in response to caffeine.

Although low concentrations of ryanodine cause release of SR calcium through RyR, high concentrations of ryanodine have been used to selectively block calcium release through RyR (1,2,32-34). To assess whether RyR function is necessary for IL-4 to decrease SR calcium stores, concentrations of ryanodine high enough to inhibit responses to caffeine were identified and then applied to the cells during IL-4 exposure. Ryanodine, 200 μ M, did not increase $[Ca^{2+}]_i$ or inhibit responses to ionomycin under control conditions, but did inhibit caffeine-stimulated transients, findings that agreed well with previous studies (2,32-34). When ryanodine (200 μ M) was present, IL-4 no longer inhibited transients in response to ionomycin. Therefore, the aggregate data suggest that IL-4 causes a net loss of SR calcium and that this

depends on the release of calcium ions through RyR calcium release channels. In these same experiments, ryanodine (200 μ M) alone had no effect on $[Ca^{2+}]_i$ or on calcium responses to ionomycin.

RyR are highly regulated channels modulated by cytosolic calcium, luminal calcium, ATP/ Mg^{2+} , redox status, phosphorylation, cADPR, and associated proteins such as calmodulin, calsequestrin, and FK-506 binding protein (2). Although the mechanism by which cADPR regulates RyR has not been determined, cADPR has been shown to cause calcium release in many cell types including airway smooth muscle (1,3,7,35,36). To assess whether cADPR plays a role in linking IL-4 to RyR function, we tested the effects of 8Br-cADPR (100 μ M), a cell permeable specific antagonist of cADPR at the RyR calcium release channel in airway smooth muscle (1,36-38). 8Br-cADPR (100 μ M) did not block the inhibitory effect of IL-4 on calcium transients and a higher concentration of 8Br-cADPR (200 μ M) also did not block the effect. Therefore, although the effect of IL-4 on SR calcium does depend on functional RyR calcium release channels, it does not appear to depend on cADPR signaling. Possibly, IL-4 is regulating RyR by stimulating calcium influx to trigger calcium-induced calcium release, by promoting phosphorylation of RyR, or by modifying proteins associated with RyR such as calmodulin, calsequestrin or FK-506 binding protein (2).

A prior study showed that the phosphatidylinositol 3-kinase (PI3K) antagonists, wortmannin and deguelin, both inhibited the effect of IL-4 on calcium transients (22). Because the current experiments showed that IL-4 causes a net loss of SR calcium by mechanisms dependent on RyR calcium release channels, the simplest explanation or model for our findings is that IL-4 stimulates signaling pathways involving PI3K to increase the release of calcium through RyR, thereby causing a net loss of calcium from the SR. However, two features need to be considered in this overall model. First, the fact that IL-4 inhibits calcium transients in response to ionomycin, carbachol and caffeine could indicate that IL-4 does not actually deplete SR calcium, but, instead, somehow renders the calcium unreleasable by any agent. This possibility seems especially unlikely in view of our results with ryanodine. Ryanodine inhibited the effect of IL-4 in this study, preserving responses to ionomycin at control levels. If IL-4 had rendered calcium unreleasable by ionomycin, then one would not have expected simple blockade of RyR to preserve releasability.

A second feature of the model to consider is that our results with ryanodine show that RyR function is required for IL-4 to decrease SR calcium, but that does not necessarily mean that IL-4 signaling pathways directly modulate RyR channel function in these experiments. For example, it is theoretically possible that basal leak of calcium ions through RyR is not directly modulated by IL-4 signaling, but still required for enough SR calcium depletion to occur and be detectable in our experimental protocols. However, regardless of whether or not IL-4 signaling directly modulates the specific activity of RyR, the data do support the conclusion that some amount of calcium ions must be able to transit through RyR in order for IL-4 to decrease SR calcium stores enough to affect the magnitude of transients.

The functional implications of IL-4 causing release of calcium from the SR are not addressed in this study, and this is an area of current investigation. Because IL-4 by itself does not consistently increase $[Ca^{2+}]_i$ (21,22), we suggest that the calcium ions are released from the SR into poorly imaged, near-plasma membrane regions of the cytosol and then pumped from the cell by calcium pumps. Consistent with this, a preliminary report showed that IL-4 caused a rapid increase in large-conductance, calcium-activated potassium channel (BK_{Ca}) activity in cultured airway smooth muscle cells (39). The release of calcium ions into near-membrane regions of the cytosol and partial depletion of SR calcium stores could both have implications for the regulation of a variety of cell functions.

IL-13 is a T helper-2 cytokine that shares many properties with IL-4, including binding to a shared family of plasma membrane receptors (40). However, in the current study, IL-4, but not IL-13, decreased intracellular calcium stores. The basis of this differential effect for IL-4 and IL-13 is not established but is consistent with prior studies in airway smooth muscle cells. For example, differential effects for these two cytokines have been described for the kinetics of STAT6 activation, modulation of beta-adrenergic receptor responsiveness and secretion of inflammatory mediators (14-16).

In summary, a relatively brief exposure of cells to IL-4 inhibited the magnitude of calcium transients elicited by ionomycin. Moreover, the inhibitory effect of IL-4 was antagonized by the presence of high concentrations of ryanodine that blocked RyR calcium release channels. These findings suggest that, for bovine airway smooth muscle cells, IL-4, but not IL-13, stimulates the release of enough SR calcium through RyR calcium release channels that stores are decreased and subsequent calcium transients are attenuated.

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Figure 1.

Ionomycin stimulates the release of SR calcium stores. A) Bovine trachealis cells were loaded with fura 2-AM and single cells were imaged ratiometrically. In the presence of extracellular calcium, cells were stimulated with carbachol (10 μ M, cch) for 2 min and the resulting transient was designated S1. Cch was then washed from the cells and cells were perfused by vehicle control (PSS buffer) for 20 min. Then, in the absence of extracellular calcium, cells were stimulated with ionomycin (10 μ M) for 2 min and the resulting transient recorded. S2/S1 ratios for cells treated with vehicle control were 1.03 ± 0.29 , $n=5$. B) Cells were similarly treated except that cyclopiazonic acid (30 μ M, CPA) was applied for 20 min instead of vehicle control. S2/S1 ratios for cells treated with CPA were 0.11 ± 0.09 , $n=5$. Representative traces are shown and, for schematic purposes, time is not to scale.

Figure 2.

IL-4 inhibits calcium transients in response to ionomycin. Cells were treated as described in figure 1 except that IL-4 (50 ng/ml) was applied after recovery from the S1 response. The maximum increase in $[Ca^{2+}]_i$ during the calcium transient was less after IL-4 treatment. A) Representative traces are shown and, for schematic purposes, time is not to scale. B) S2/S1 ratios for individual cells are compared for vehicle (open square) versus IL-4 treated (closed squares) cells. Mean $\pm$ SEM for control (open triangle) and IL-4 treated (closed triangle) cells are shown.

Figure 3. Identification of RyR calcium release channels in bovine trachealis cells. A) Western blot analysis from a microsomal preparation of bovine trachealis subjected to SDS-PAGE on a 4-15% polyacrylamide gradient gel and probed with antibody directed against RyR calcium release channels. Rabbit anti-RyR identified a protein of high molecular mass (~565 Kd) consistent with the presence of RyR calcium release channels. Data are representative of three independent experiments. B) Functional RyR calcium release channels are demonstrated by a calcium transient in response to 10 mM caffeine.

Figure 4. Blockade of RyR calcium release channels with a high concentration of ryanodine. A) Ryanodine (200 μ M) inhibited calcium transients in response to caffeine but did not stimulate an increase in $[Ca^{2+}]_i$ by itself. Representative traces are shown and, for schematic purposes, time is not to scale. B) Caffeine response (S_2/S_1) for control (n=3) versus ryanodine-treated (n=4) cells. Data are mean $\pm$ SEM. * indicates $P=0.001$ compared to vehicle controls.

Response to Ionomycin (S2/S1)

Vehicle	+	-	-	-	-	-
IL-4	-	+	-	+	-	+
Ryanodine	-	-	+	+	-	-
8-bromo-cADPR	-	-	-	-	+	+

Figure 5.

Ryanodine inhibits the effect of IL-4 on transients. After the S1 response to carbachol, cells were washed 10 min and then exposed to vehicle or IL-4 for 20 min in the absence and presence of ryanodine (200 μ M) or 8-bromo-cADPR (100 μ M). The S2 response to ionomycin then was recorded and S2/S1 ratio calculated as an index of cell responsiveness. Ryanodine, but not 8-bromo-cADPR, inhibited the effects that IL-4 has on calcium transients (* indicates $P < 0.05$, $n = 5-7$).

Figure 6.

IL-13 does not inhibit responses to ionomycin. After the S1 stimulation with carbachol, cells were washed and exposed to vehicle or IL-13 for 20 min before stimulation with ionomycin (S2). S2/S1 ratios were calculated as an index of cell responsiveness. IL-13 did not inhibit responses to ionomycin. Data are mean $\pm$ SEM (n=4).