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ACCELERATED COMMUNICATION

(*S*)-Albuterol Increases Intracellular Free Calcium by Muscarinic Receptor Activation and a Phospholipase C-Dependent Mechanism in Airway Smooth Muscle

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ABSTRACT

Racemic albuterol has been one of the most widely used β_2-adrenoceptor agonists for the relief of the symptoms of asthma, yet the use of β_2 agonists has been known to induce bronchial hyperresponsiveness. To probe a possible role of the *S*-enantiomer for hyperresponsiveness, we determined the effects of (*S*)-albuterol on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in dissociated bovine tracheal smooth muscle cells. Both (*S*)-and (*R*,*S*)-albuterol increased $[Ca^{2+}]_i$ at concentrations of >10 pM and 1 nM, respectively, with a maximal response by 150 and 100 nM, respectively. (*S*)-Albuterol (1 and 10 μM) induced Ca^{2+} oscillations, reaching 1–2 μM $[Ca^{2+}]_i$. This response is in a stark contrast to that of (*R*)-albuterol, which decreased $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was blocked by 100 nM atropine or 500 nM 4-diphenylacetoxy-*N*-methylpiperidine but was insensitive to the β_2 antagonist ICI 118,551 (10 μM). (*S*)-Albuterol (10 μM) increased inositol-1,4,5-trisphosphate levels by 213 ± 34.4% ($p < 0.05$, four experiments) in cells exposed for 30 sec. The sustained phase of the Ca^{2+} increase was absent in Ca^{2+}-free solution, suggesting that Ca^{2+} influx was responsible for the sustained Ca^{2+} response. The results also suggest that (*S*)-albuterol may cross-react with muscarinic receptors. As a Ca^{2+} agonist in airway smooth muscle, (*S*)-albuterol may have profound clinical implications because 50% of prescribed racemic albuterol is composed of (*S*)-albuterol.

Racemic albuterol has been one of the most widely used β_2-adrenoceptor agonists for the relief of the symptoms of asthma. However, the use of β_2 agonists has been linked to bronchial hyperresponsiveness (Kerrebijn *et al*., 1987; Sears *et al*., 1990; Taylor *et al*., 1993; Wahedna *et al*., 1993) and a paradoxical increase in the mortality rate known as the "asthma paradox" (Sears *et al*., 1990; Grainger *et al*., 1991; Spitzer *et al*., 1992; Suissa *et al*., 1994; Barrett and Strom, 1995). The mechanism underlying this β_2-adrenergic agonist-induced hyperresponsiveness is not known. The β_2 agonists generally possess two stereoisomers due to an asymmetrical carbon adjacent to the aromatic ring. The *R*-enantiomer binds to the β_2-adrenoceptor, promoting formation of cAMP and bronchodilation, whereas the *S*-enantiomer has been considered to be a less effective agonist (Johnson *et al*., 1993). Therefore, tachyphylaxis to β_2 agonist has been suggested to be the cause for the loss of the protective effects of the β_2 agonist after prolonged or excessive use of a β_2 agonist (Gibson *et al*., 1978; Cockcroft *et al*., 1993). Desensitization of β_2-adrenoceptors through receptor internalization (Straser *et al*., 1985), together with subsequent reduction in receptor mRNA expression (Nishikawa *et al*., 1994), offers one plausible explanation for the loss of protective effects. However, a recent *in vivo* study in guinea pigs suggests that acute airway hyperresponsiveness may be characteristic of the *S*-enantiomers of β_2 agonists (Mazzoni *et al*., 1994). Because *the in vitro* effects of the *S*-enantiomer of β_2 agonists on $[Ca^{2+}]_i$ are unknown and the effector of the spasmogens is airway smooth muscle, we studied the effects

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ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; PSS, physiological salt solution; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; QNB, quinuclidinyl benzilate; PLC, phospholipase C; PKC, protein kinase C.

of (*S*)-albuterol in detail at the cellular level to understand its action in dissociated smooth muscle cells; the purpose of the current study was to gain further insight into the possible role of (*S*)-albuterol in airway hyperresponsiveness.

Materials and Methods

Chemicals and reagents. We obtained (*S*)-, (*R*,*S*)-, and (*R*)-albuterol from Sepracor (Marlborough, MA). (*S*)-Isoproterenol was purchased from Sigma Chemical. ICI 118,551 was purchased from Tocris Cookson (St. Louis, MO). Nimodipine, U73,122, atropine, and 4-DAMP were purchased from Research Biochemicals (Natick, MA). [^{3}H]QNB was purchased from DuPont-New England Nuclear (Boston, MA). The Ins(1,4,5)P_3 assay kit was from Amersham Life Science (Clearbrook, IL).

Cell isolation. Tracheal smooth muscle cells were dispersed from small pieces (1 × 1 × 5 mm) of bovine trachealis cut while under observation with the use of a dissecting microscope. Approximately 0.5 g of tissue was placed into 2.5 ml of nominally Ca^{2+} free solution, which consisted of PSS (containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.6 mM $NaHCO_3$,11.1 mM glucose, 2.5 mM $CaCl_2$) without 2.5 mM $CaCl_2$, plus 2.5 mg of collagenase (Boehringer-Mannhein Biochemicals, Indianapolis, IN), 2 mg of elastase (Boehringer-Mannheim), and 0.5 mg of DNase (Boehringer-Mannheim). The mixture was stirred gently with a microstirrer for 15 min at 37°. The tissue slices then were transferred to fresh enzyme solution and gently stirred for an additional 15 min. The digested pieces were recovered by a nylon mesh (hole size, 0.5 mm) and resuspended in Ca^{2+}-free PSS after repeated washing. Gentle spinning of the tissue in Ca^{2+}-free PSS for 3 min released ≈1–3 × 10^5 cells/ml, which were equilibrated in low Ca^{2+} (0.1 mM) PSS.

Ca^{2+} measurements. The measurement of $[Ca^{2+}]_i$ was carried out with fluorescent imaging microscopy as described previously (Kajita and Yamaguchi, 1993; Yamaguchi *et al.*, 1995). The dispersed smooth muscle cells were loaded with 0.5 μM Fura-2 acetoxymethyl ester for 60 min at room temperature. Fura-2-loaded cells were transferred to a recording chamber (volume, 150 μl) and allowed to settle to the bottom coverglass before superfusion of normal PSS at 37°. These cells were 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, Newton, MA). Each laser alternately fired short pulses (3 nsec) at 30 Hz. These alternating pulses of light 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 40× lens (NA, 1.3; Nikon). The emitted fluorescent signals were passed back through the objective to a 455-nm dichroic mirror and a 475-nm barrier filter (Omega Optics, Brattleboro, VT) and imaged by a frame transfer, charge-coupled device camera (FTM 800; Philips Component, Slatersville, RI). The signals were digitized and stored in an imaging board (Recognition Technology, Westborough, MA), and the digital outputs from the board were transferred to a personal computer. For each set of experiments, the responses from one to seven cells were taken from one animal, and the final data usually represented the measurements from at least two animals.

The gray levels of fluorescence emissions were recorded continuously from the selected area (usually 8 × 6 pixels) within each cell, and their ratios were plotted for ≈9 min after subtraction of background fluorescence. These ratios were converted to Ca^{2+} concentrations using the equation (Grynkiecz *et al.*, 1985): $[Ca^{2+}] = K_d \times \beta \times [(R - R_{min})/(R_{max} - R)]$, where R_{max} and R_{min} are the fluorescence ratios measured in high and zero Ca^{2+}, respectively, and β is the ratio of emitted fluorescence with 380-nm excitation in high and zero Ca^{2+}. K_d is the equilibrium dissociation constant for free and bound dye concentrations defined as $K_d = C_{free} \cdot [Ca^{2+}]/C_{bound}$ for 1:1 complexion, and the value 386 nM was derived from *in situ* calibration in bovine tracheal cells (Kajita and Yamaguchi, 1993). During a 9-min recording of the response, 16 manually selected or automatically timed images were stored for later analysis.

Binding protocol. Membranes were prepared from trachealis trimmed of mucosa and connective tissue, minced with scissors in ice-cold phosphate-buffered saline, and repeatedly washed with phosphate-buffered saline. The minced tissue then was homogenized in two volumes of homogenization buffer (containing 5 mM Tris·HCl, pH 7.4, 2 mM EDTA, 0.5 mM dithiothreitol) and 0.2 mM phenylmethylsulfonyl fluoride (Sigma Chemical, St. Louis, MO) using an Ultra-turrax Tissuemizer (Tekmar, Cincinnati, OH). The crude homogenate was filtered through cheesecloth and centrifuged twice at 900 × *g* for 10 min to remove undisrupted cells and nuclei. The supernatant was centrifuged twice at 40,000 × *g* for 40 min. The pellet was resuspended in a buffer containing 30 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, and 25% sucrose (w/v) at an approximate protein concentration of 1 mg/ml; frozen rapidly; and stored at −80° until use. Protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Receptor binding assays with ^{125}I-cyanopindolol were performed in triplicate at a final volume of 300 μl. Diluted membranes (10 μg/tube) were incubated in binding buffer [50 mM Tris·HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 0.1% bovine serum albumin, and 100 μM guanosine-5′-*O*-(3-thio)triphosphate] with the ligands and ^{125}I-cyanopindolol (50,000–60,000 cpm/tube) at room temperature for 150 min. Reaction was terminated by the addition of 3 ml of ice-cold buffer followed by filtration through Whatman (Clifton, NJ) GF/C filters. The filters then were washed with 15 ml of ice-cold buffer, and radioactivity on the filters was considered to be bound ligand.

Binding assays with [^{3}H]QNB were carried out with the same protocol except the binding medium was replaced by 1 ml of the solution containing 10 mM Tris·HCl, pH 7.5, and 1 mM $MgCl_2$. The membranes were incubated with varying amounts of (*S*)-albuterol and [^{3}H]QNB (≈5000 cpm) for 1 hr with constant shaking. The mixtures were filtered over GF/C filters followed by three washes with 5 ml of ice-cold buffer. The bound count on the filter were performed with a scintillation counter. The nonspecific count was assumed to be cpm bound in the presence of 10 or 20 μM atropine and subtracted from each point to determine the specific binding. The binding data were analyzed with use of InPlot or Prism (GraphPAD Software, San Diego, CA), and the plot selected exhibited the K_d value closest to the mean K_d value of three similar experiments. The affinity of the radioligand for the receptor (K_d) was calculated with PRISM using the equation for enzymatic reactions (Cheng and Prusoff, 1973).

Ins(1,4,5)P_3 measurements. Dispersed cells were placed into six-well culture plates (≈10^5 cells/well). These cells were incubated with serum-free RPMI media in an incubator for 4 hr and equilibrated with 95% air/5% CO_2 at 37°. The reactions were performed on a warm plastic plate. The agonist was added to a final concentration of 10 μM. The generation of Ins(1,4,5)P_3 was followed at different time periods ranging from 10 sec to 10 min. At each time point, the reaction was stopped by the addition of an equal volume of 10% ice-cold trichloroacetic acid (Sigma Chemical), which subsequently was removed before assay by partitioning three times with five volumes of diethyl ether. After adjustment of the samples to pH 7.5 with 1 M $NaHCO_3$, 25- and 100-μl volumes of each sample were assayed by using a radiometric Ins(1,4,5)P_3 assay kit. The assays were done in triplicate, and data are presented as percentage increase over the basal value.

Data analysis. All values are expressed as mean ± standard error. Statistical significance was assessed by paired or unpaired Student's *t* test, and $p < 0.05$ was considered significant.

Results

The effects of (*S*)-, (*R*,*S*)-, and (*R*)-albuterol on $[Ca^{2+}]_i$ in tracheal smooth muscle cells were determined by using an

imaging microscope. The response to superfusion of either (*S*)- or (*R*,*S*)-albuterol was characterized as an increase in $[Ca^{2+}]_i$ as shown in Fig. 1, A and B. The increased in $[Ca^{2+}]_i$ occurred at (*S*)-albuterol concentrations of >10 pM and increased maximally by 149.6 ± 16.1 nM (12 cells) at 1 μM with an EC_{50} value of 3.3 nM (Figs. 1A and 2C). Likewise, (*R*,*S*)-albuterol increased $[Ca^{2+}]_i$ at concentrations of >1 nM and had the maximum increase of 99.6 ± 16.6 nM at 100 μM with an EC_{50} value of 12.9 nM (Fig. 1B). At 1 and 10 μM (*S*)-albuterol, a fraction of the cells (≈10% and ≈25%, respectively) induced Ca^{2+} oscillations that reached 1–2 μM (Fig. 1C). In contrast, (*R*)-albuterol decreased $[Ca^{2+}]_i$ in these cells (Fig. 1D). At a concentration of 10 μM (*S*)-albuterol, the increases in $[Ca^{2+}]_i$ were accompanied by cell shortening. The effect was observed in all cells, averaging 10.1 ± 0.96% (range, 5–21%; 20 cells) of the preexposure length as determined from stored images before and after a 5-min exposure to the *S*-enantiomer. The dose response for (*R*)-albuterol is not shown because the affinity sites we detected for the decrease in $[Ca^{2+}]_i$ should themselves be the subject of detailed study. However, the cells responded with a decrease in $[Ca^{2+}]_i$ at all concentrations of >5 nM, with a maximum decrease in the range of 105–106 nM.

A number of mechanisms may be involved in the elevation of $[Ca^{2+}]_i$ by (*S*)-albuterol: (1) $Ins(1,4,5)P_3$-mediated Ca^{2+} release (Berridge, 1993), (2) depletion-induced influx of Ca^{2+} (Putney, 1986; Randriamampita and Tsien, 1993), and (3) activation of voltage-gated Ca^{2+} channels through modulation of the membrane potential. To investigate these potential mechanisms, we examined the response to (*S*)-albuterol under various conditions, including Ca^{2+}-free PSS and in the presence of a PLC or PKC inhibitor. When (*S*)-albuterol (10 μM) was superfused in nominally Ca^{2+}-free PSS, there was only a transient increase in $[Ca^{2+}]_i$ (133 ± 12.6 nM, six cells) and the steady state levels were decreased by 72.3 ± 12.5 nM (six cells) below the levels before the test (Fig. 2A). To test the possibility of phosphatidyl-inositol turnover and $Ins(1,4,5)P_3$-mediated Ca^{2+} release, we examined the effects of the PLC inhibitor U73,122 (Bleasdale *et al*., 1990). Fig. 2B illustrates the simultaneous application of 10 μM U73,122 and (*S*)-albuterol, showing the decrease in steady state $[Ca^{2+}]_i$. This approach was successful because the mean onset time for the (*S*)-albuterol (10 μM)-induced response from randomly chosen cells was 29.6 ± 2.6 sec (17 cells), which was ≈15 sec slower than that for carbachol. Fig. 2C shows another Ca^{2+} response to (*S*)-albuterol that was reversed by subsequent U73,122. We further tested the effects of calphostin C, the blocker of PKC on (*S*)-albuterol-induced Ca^{2+} response. When applied simultaneously with (*S*)-albuterol (10 μM), calphostin C (100 nM) induced only an transient rise in Ca^{2+} and eliminated the anticipated Ca^{2+} increase. Instead, the steady state $[Ca^{2+}]_i$ was decreased (Fig. 2D). The histograms summarize the effects of (*S*)-albuterol on the transient (peak) and steady state $[Ca^{2+}]_i$ in the presence of U73,122 and calphostin C and with Ca^{2+}-free solution (Fig. 2E).

To obtain evidence for the phosphatidyl-inositol turnover, we measured $Ins(1,4,5)P_3$ levels in smooth muscle cells treated with (*S*)-albuterol at various times. Exposure to 10 μM (*S*)-albuterol for 30 sec increased $Ins(1,4,5)P_3$ by 213 ± 34.4% ($p < 0.05$, four experiments) (Fig. 2F).

To assess the role of extracellular Ca^{2+}, we measured the response to (*S*)-albuterol in the presence of nimodipine, the antagonist of L-type Ca^{2+} channels. Nimodipine (100 nM) alone decreased basal $[Ca^{2+}]_i$ from 134.3 ± 23.4 to 100.6 ± 20.7 nM ($p > 0.05$) within 3 min, but when tested at 5 min after the introduction of nimodipine, the (*S*)-albuterol (10 μM)-induced net increase in $[Ca^{2+}]_i$ was decreased from a paired control value of 99.7 ± 10.2 to 9.6 ± 12.5 nM ($p < 0.001$, six cells).

The affinity of (*S*)-albuterol for β-adrenoceptor was determined by competition binding experiments using ^{125}I-cyanopindolol, a nonspecific β-adrenoreceptor ligand, with a bovine tracheal membrane preparation. The IC_{50} value of (*S*)-albuterol for displacement of ligand (average, 293 μM; range, 263–323 μM; three cells) was >100-fold higher than that of (*R*)-albuterol (average, 1.66 μM; range, 0.5–5.5 μM; three cells), indicating that (*S*)-albuterol has very low affinity for β-adrenoreceptors in these cells (Fig. 3A).

To identify possible receptors for (*S*)-albuterol, we considered muscarinic receptors. The competition binding against [^{3}H]QNB with atropine or (*S*)-albuterol is shown in Fig. 3B. The IC_{50} value for (*S*)-albuterol was 21 μM. We previously found that the IC_{50} values for atropine and 4-DAMP in tracheal smooth muscle membranes and single cells were 10 and 45 nM, respectively (Lucchesi *et al*., 1990); therefore, we used 100 nM atropine or 500 nM 4-DAMP to determine whether these antagonists had an effect on (*S*)-albuterol-induced changes in $[Ca^{2+}]_i$. Fig. 3C shows the effect of the simultaneous superfusion of 100 nM atropine and 10 μM (*S*)-albuterol on $[Ca^{2+}]_i$; the increase in $[Ca^{2+}]_i$ was absent, and steady state $[Ca^{2+}]_i$ decreased. We observed a similar response with 500 nM 4-DAMP (Fig. 3D). We then tested whether the (*S*)-albuterol-induced increase in $[Ca^{2+}]_i$ was sensitive to the specific β_2-adrenoceptor antagonist ICI 118,551(Bilski *et al*., 1983). In the presence of 10 μM ICI 118,551, which alone had no effect on steady state $[Ca^{2+}]_i$, (*S*)-albuterol (10 μM) still increased $[Ca^{2+}]_i$ by 170.5 ± 43.5 nM (10 cells), a rise virtually identical to that of controls (153.5 ± 41.1 nM, 10 cells). The additional test was conducted to determine whether the decrease in $[Ca^{2+}]_i$ observed with atropine was sensitive to ICI 118,551. In seven cells, a decrease in $[Ca^{2+}]_i$ was prevented when the β_2-adrenoceptor antagonist ICI 118,551 was applied to cells with atropine and (*S*)-albuterol (Fig. 3E). These results of the response to 10 μM (*S*)-albuterol with atropine, 4-DAMP, ICI 118,551, and ICI 118,551 plus atropine are summarized in Fig. 3F.

Discussion

Our data indicate that the albuterol stereoisomers [(*S*)- and (*R*)-] exhibited opposite effects on $[Ca^{2+}]_i$ in airway smooth muscle cells. The unexpected finding was that (*R*,*S*)- and (*S*)-albuterol increased $[Ca^{2+}]_i$, and, in particular with (*S*)-albuterol, the increase in $[Ca^{2+}]_i$ invariably was accompanied by shortening of all cells. In contrast, (*R*)-albuterol decreased $[Ca^{2+}]_i$ and exhibited no shortening effect. These differences between the albuterol stereoisomers can affect the action of contractile agonists; for example, with muscarinic agonist, carbachol administered simultaneously with (*S*)-albuterol exacerbated Ca^{2+} mobilization in bovine tracheal cells, but carbachol administered simultaneously with (*R*)-albuterol diminished Ca^{2+} mobilization (Yamaguchi and McCullough, 1996). These results suggest that (*S*)-albuterol

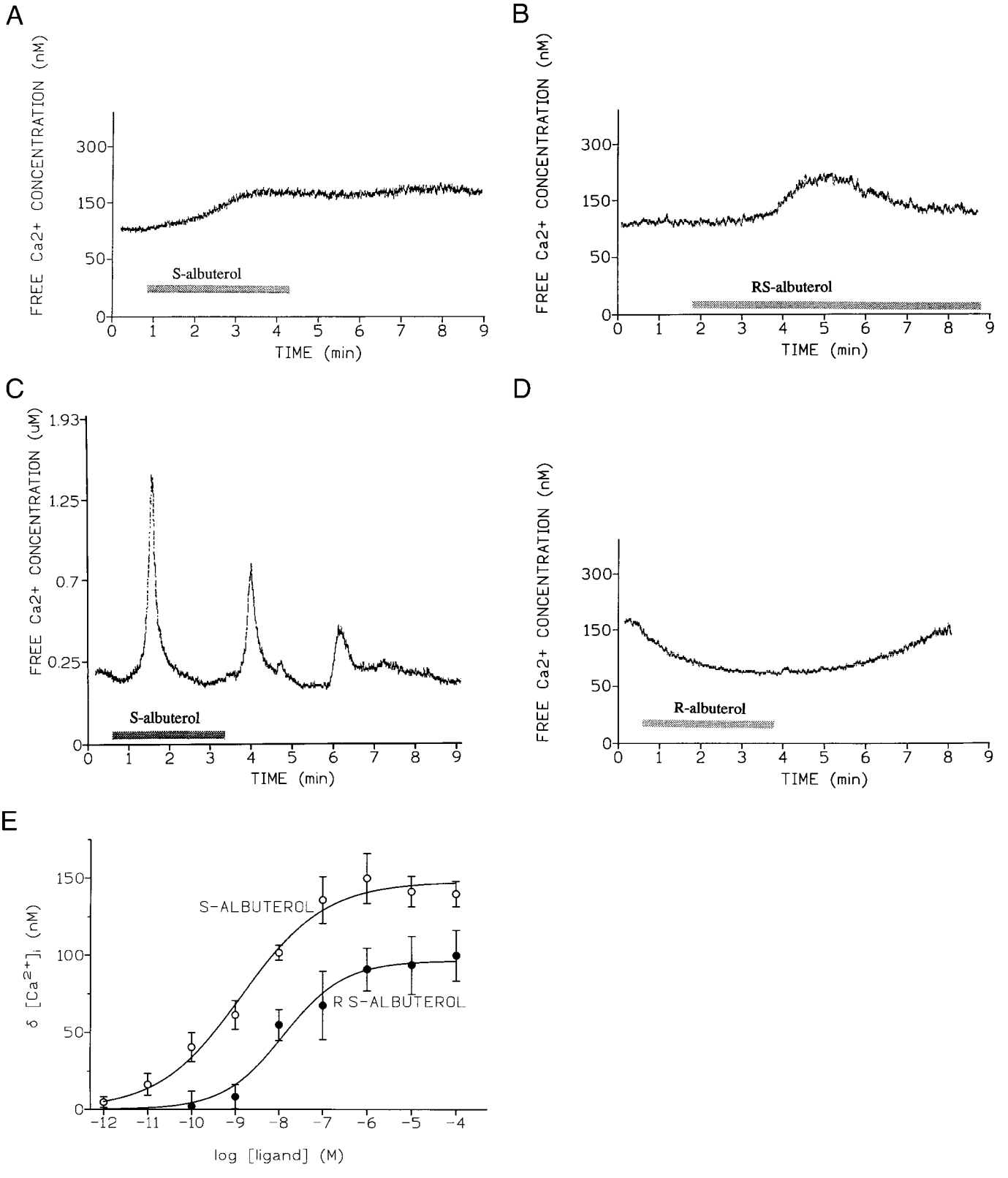

Fig. 1. A, (*S*)-Albuterol-induced increase in $[Ca^{2+}]_i$. B, (*R*,*S*)-Albuterol-induced increase in $[Ca^{2+}]_i$. C, $[Ca^{2+}]_i$ oscillations induced by (*S*)-albuterol. D, (*R*)-Albuterol (10 μM)-induced decrease in $[Ca^{2+}]_i$. In all cases, a 10-μM concentration of the *S*- or *R*-enantiomer was superfused over the cells attached to the coverglass. The delay of application due to the dead space was corrected. The expanded vertical scale of $[Ca^{2+}]_i$ below 150 nM reflects the nonlinear nature of calibration curves in these concentrations. E, Dose-response curve of (*R*,*S*)- and (*S*)-albuterol. Data represent net increases in steady state $[Ca^{2+}]_i$ above basal levels (△, $[Ca^{2+}]_i$) and exclude Ca^{2+} oscillations observed with 1 and 10 μM (*S*)-albuterol (one and seven cells, respectively). EC_{50} values for (*R*,*S*)- and (*S*)-albuterol are −7.89 and −8.48, respectively. *Solid line*, drawn from the fit of sigmoid curve by the InPlot (GraphPAD Software).

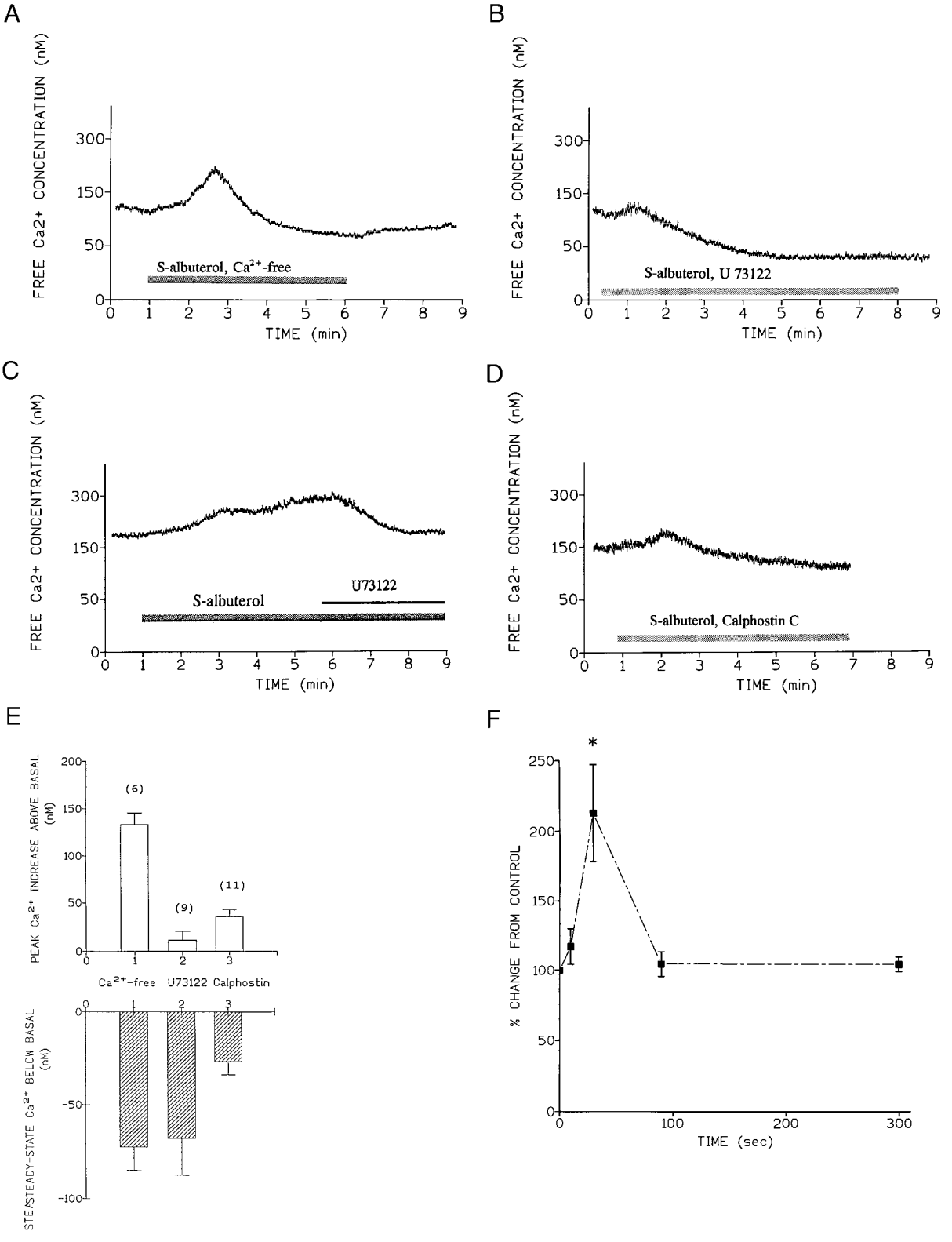

Fig. 2. A, Effect of (*S*)-albuterol in Ca^{2+}-free PSS. (*S*)-Albuterol (10 μM) induced only a transient rise in $[Ca^{2+}]_i$ in nominally Ca^{2+}-free PSS, which was followed by a decreased $[Ca^{2+}]_i$, representing steady state levels in Ca^{2+}-free PSS. B, Changes in $[Ca^{2+}]_i$ in response to simultaneously applied (*S*)-albuterol (10 μM) and U73,122 (10 μM). C, Effect of the PLC inhibitor U73,122 on (*S*)-albuterol-induced increase in $[Ca^{2+}]_i$, showing the reversal of (*S*)-albuterol (10 μM)-induced increase in $[Ca^{2+}]_i$ to the prestimulation level on the addition of 10 μM U73,122. D, Changes in $[Ca^{2+}]_i$ in response to simultaneously applied calphostin C (100 nM) and (*S*)-albuterol (10 μM). E, Changes in peak transient $[Ca^{2+}]_i$ (*top*) and steady state $[Ca^{2+}]_i$ (*bottom*) induced by 10 μM (*S*)-albuterol in Ca^{2+}-free PSS, U73,122, and calphostin C. *Bars*, number of cells used for experiments. The statistical significance was assessed only for the peak Ca^{2+} increase in Ca^{2+}-free [$p > 0.05$ (NS)], U73122 ($p < 0.001$), and calphostin C ($p < 0.001$), in comparison to control response by 10 μM (*S*)-albuterol in PSS (141 $\pm$ 9.9 nM, 15 cells). F, $Ins(1,4,5)P_3$ generation by 10 μM (*S*)-albuterol. $Ins(1,4,5)P_3$ levels were measured with radioimmunoassay in cells exposed to (*S*)-albuterol at various time points (0, 10, 30, 90, and 300 sec). An $Ins(1,4,5)P_3$ increase was significant in cells exposed for 30 sec ($p < 0.05$, four cells).

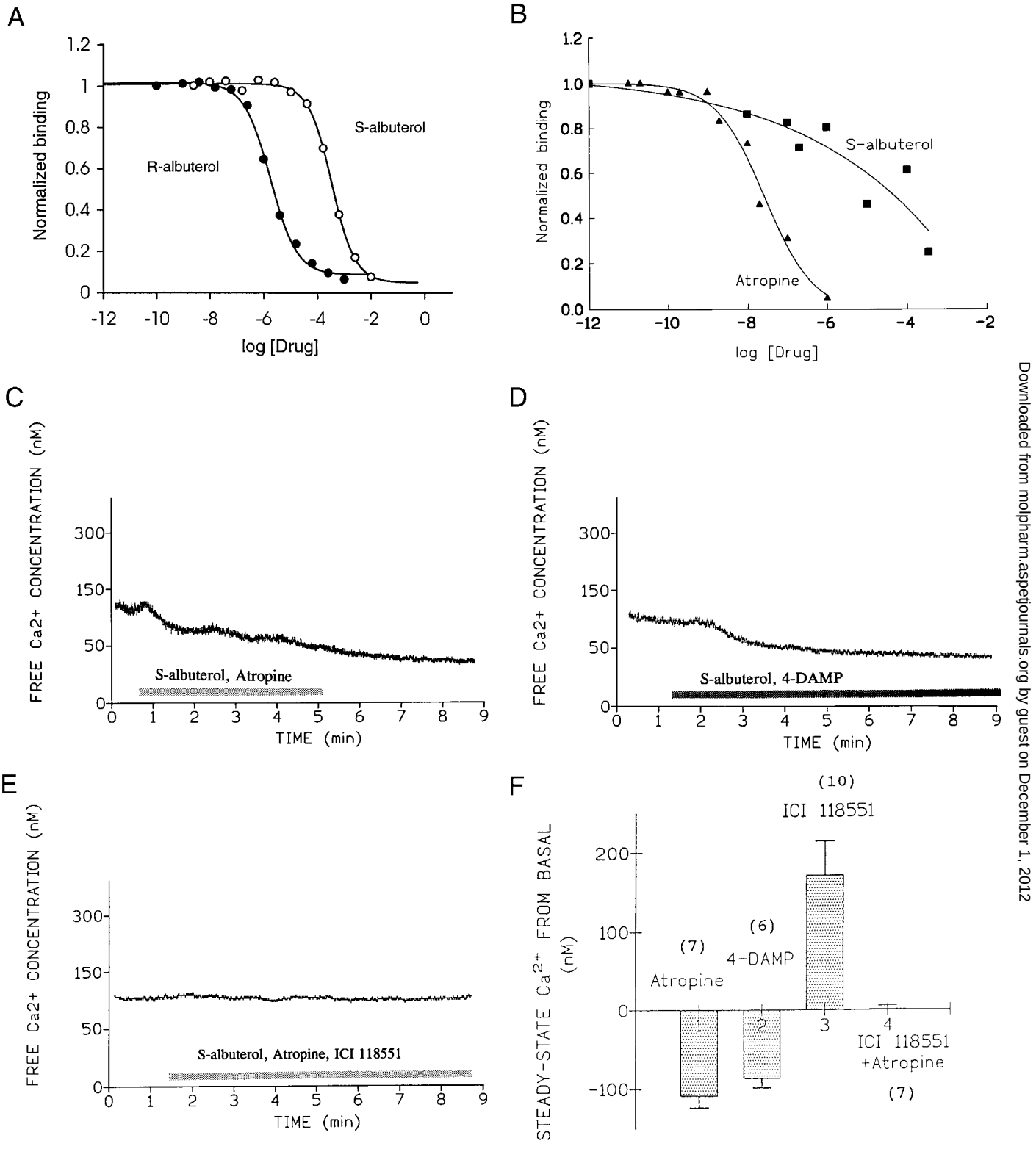

Fig. 3. A, Displacement of ^{125}I-cyanopindolol with (*S*)- or (*R*)-albuterol. Values represent the average of data measured in triplicate. The IC_{50} values for (*R*)- and (*S*)-albuterol are −5.78 and −3.53, respectively. B, Displacement of [^{3}H]QNB by atropine and (*S*)-albuterol. The IC_{50} values are 43 nM and 21 μM for atropine and (*S*)-albuterol, respectively. C, Decrease in $[Ca^{2+}]_i$ in response to simultaneously applied (*S*)-albuterol (10 μM) and atropine (100 nM). D, Decrease in $[Ca^{2+}]_i$ in response to simultaneously applied (*S*)-albuterol (10 μM) and 4-DAMP (500 nM). E, Blockade of changes in $[Ca^{2+}]_i$ in response to simultaneously applied (*S*)-albuterol (10 μM), atropine (100 nM), and ICI 118,551 (10 μM). F, *Bars*, numbers of cells used for experiments. Steady state $[Ca^{2+}]_i$ induced by (*S*)-albuterol (10 μM) simultaneously applied with atropine ($p < 0.001$), 4-DAMP ($p < 0.001$), ICI 118,551 (10 μM, $p > 0.05$), and atropine plus ICI 118,551 ($p < 0.001$). They illustrate that a decrease in $[Ca^{2+}]_i$ is sensitive to β_2-adrenoceptor antagonist, but an increase in $[Ca^{2+}]_i$ was insensitive to ICI 118,551.

directly opposes the beneficial effects of (*R*)-albuterol at a critical step for generation of force.

(*S*)-Albuterol has been considered to be a less effective β_2 agonist because of its effects on cAMP formation (Johnson *et al*., 1993). The results of our study are inconsistent with the *S*-enantiomer as a β_2 agonist because not only were the effects of (*S*)-albuterol on $[Ca^{2+}]_i$ insensitive to high doses of ICI 118,551 but also Ins(1,4,5)P_3 generation and shortening of the cell did not fit the known effects of β_2-adrenoceptor activation in smooth muscle. The β_2 agonists and their second messenger decrease $[Ca^{2+}]_i$ and relax muscle tone in tracheal and vascular smooth muscle (McDaniel *et al*., 1991; Yamaguchi *et al*., 1995), whereas we observed the opposite effects with (*S*)-albuterol. On the other hand, a decrease in $[Ca^{2+}]_i$ by (*S*)-albuterol in the presence of U73,122 (Fig. 2B), atropine, or 4-DAMP (Fig. 3 AB) was believed to result from β_2-adrenoceptor activation because with atropine, the response was blocked by ICI 118,551 (Fig. 3E). This effect can be attributed to contaminated (*R*)-albuterol and may explain why cAMP was increased by the *S*-enantiomer in the previous study.

Functional tests revealing blockade of the increased $[Ca^{2+}]_i$ with atropine and 4-DAMP implicate muscarinic receptors in (*S*)-albuterol-induced response. The finding prompted us to examine whether muscarinic receptor antagonists had direct effects on Ca^{2+} stores. We found that atropine (100 nM) exerted no effects on caffeine (10 mM)-induced Ca^{2+} release (ratio of atropine plus caffeine/caffeine, 1.07 ± 0.2; five cells). Although this was not the direct test on Ins(1,4,5)P_3-mediated stores, the total lack of response in the presence of atropine led us to believe that inhibitory effects of the muscarinic antagonists must have resulted from interactions with the receptors. The competition binding with [^{3}H]QNB may provide additional evidence for interaction of the *S*-enantiomer with muscarinic receptors (Fig. 3B). The mean K_d value from three such experiments was 8.17 ± 1.1 μM. Considering the high affinity of the EC_{50} value in the functional responses (Fig. 1D), (*S*)-albuterol is a highly efficient agonist for a muscarinic receptor. Muscarinic effects in airway smooth muscle, including Ca^{2+} mobilization (Yang *et al*., 1993) and contraction (Eglen *et al*., 1990), are known to be mediated by M_3 receptors. The blockade of 4-DAMP on (*S*)-albuterol-mediated increase in $[Ca^{2+}]_i$ in this study is consistent with the idea of the M_3 muscarinic receptors being involved with the response to (*S*)-albuterol. Our data suggest that (*S*)-albuterol cross-reacts with muscarinic receptors; however, they do not rule out a possibility that the *S*-enantiomer has its own receptors in these cells. To discover the universal nature of the effects of the *S*-enantiomer among β_2 agonists, we also tested the effect of (*S*)-isoproterenol on $[Ca^{2+}]_i$ in bovine tracheal smooth muscle cells. (*S*)-Isoproterenol (10 μM) increased $[Ca^{2+}]_i$ by 98.7 ± 24.5 nM (nine cells). Thus, the increase in $[Ca^{2+}]_i$ essentially was similar to that observed with (*S*)-albuterol. The two drugs share a chemical structure of $HOCHCH_2NH$ adjacent to the phenol ring.

The data for U73,122 and calphostin C suggest that Ca^{2+} mobilization by (*S*)-albuterol involves (1) activation of PLC and resulting Ins(1,4,5)P_3 production that induces an initial transient release of Ca^{2+} from stores and (2) PKC to induce a sustained influx of Ca^{2+} from the extracellular medium, probably through L-type Ca^{2+} channels because it is sensitive to nimodipine. A complementary mechanism for slow Ca^{2+} elevation by membrane-permeant 1,2-dioctanoyl-*sn*-glycerol was observed previously in bovine tracheal cells, providing a basis for PKC to play a role in the sustained elevation of $[Ca^{2+}]_i$ (Kajita and Yamaguchi, 1993).

Our finding that the *S*-enantiomer of albuterol primarily functions as a Ca^{2+} agonist follows a well known example of dihydropyridine enantiomers. The *S*-enantiomer of the dihydropyridine, 202–791, or Bay K-8644 enhances L-type Ca^{2+} current and acts as Ca^{2+} agonists primarily by stabilizing the open state of the channel, whereas the *R*-enantiomer favors the inactivated state and reduces whole-cell L-type Ca^{2+} current (Kokubun *et al*., 1986; Hamilton *et al*., 1987). Taken together with the current results, the future studies of racemic drugs may require identification of the pharmacological and physiological effects of each enantiomer.

In conclusion, our study revealed that (*S*)-albuterol had characteristics of a typical contractile agonist. This is mostly due to a PLC activation, which results in phosphatidyl-inositol turnover with increased Ins(1,4,5)P_3 levels. The phosphatidyl-inositol cascade further induces PKC translocation and results in nimodipine-sensitive Ca^{2+} influx, possibly through L-type Ca^{2+} channels. Many of these properties are shared by other contractile agonists, such as histamine and carbachol (Chilvers *et al*., 1990; Berridge, 1993; Yang *et al*., 1993; Kajita and Yamaguchi, 1993). This enhanced Ca^{2+} mobilization may be one important factor in the hyperresponsiveness associated with the clinical use of racemic β_2-agonists. By exhibiting deleterious effects on airway smooth muscle, the use of *S*-enantiomer albuterol may have profound clinical implications because 50% of racemic albuterol consists of (*S*)-albuterol.

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