Ionophore and Arachidonic Acid Stimulation of Airway Responses in Rhesus Monkeys

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ABSTRACT Aerosolized doses of the ionophore, A23187, and arachidonic acid individually resulted in no airway response in rhesus monkeys. When these two agents were given simultaneously, by aerosol, an airway response occurred. The pulmonary function abnormalities that occurred qualitatively simulated those of an antigen-induced airway response. This is the first demonstration in our laboratory of two agents which singly will not produce a response but which are reactive when delivered in combination. Other fatty acids did not produce a similar response. The response to A23187 and arachidonic acid occurred only in rhesus monkeys from our colony which had been demonstrated to have airway responses to aerosolized antigen challenge, a response shown previously to be associated with hyperreactive airways to pharmacologic stimuli. The A23187 and arachidonic acid response was inhibited by aerosolized 5,8,11,14-eicosatetraynoic acid, an inhibitor of the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism. Further, indomethacin, a prostaglandin synthetase inhibitor of the cyclooxygenase pathway, inhibited the response, although previous studies showed that this drug will potentiate an antigen-induced response in this animal model of asthma. The slow-reacting substance of anaphylaxis antagonist, FPL 55712, did not inhibit the A23187-arachidonic acid response under the conditions of these experiments. The mechanism of the A23187-arachidonic acid airway response in rhesus monkeys may or may not be the same as the antigen-induced response.

INTRODUCTION

In previous reports we have described the characteristics of the immediate-type airway response to aerosolized ascaris antigen in a group of rhesus monkeys (1, 2). The limited population of animals with persistent and consistent airway reactivity to inhaled antigen has been shown to have hyperreactive airways as demonstrated by increased sensitivity to aerosol challenge with analogues of acetylcholine, (2, 3) histamine, and prostaglandin $F_{2\alpha}$ (3). The immunologic, physiologic, and pharmacologic characteristics described have led to the suggestion that the rhesus monkey antigen-induced airway response constitutes a primate model of human asthma (2, 3).

The qualitative changes in pulmonary function abnormalities occurring after antigen aerosol challenge are simulated by certain immunologic stimuli (anti-IgE), histamine, carbocholine, or prostaglandin $F_{2\alpha}$ (4).

In previous studies, we found that certain agonists would mutually potentiate the action of others on an airway response. Examples are carbocholine and antigen (5) and antigen and arachidonic acid (6), although the latter alone would not stimulate an airway response under the conditions of the experiments (6). We have not found in previous studies, two aerosolized agents which produce no airway response when delivered singly but which will do so as a combination. In this study, we report that the ionophore, A23187, aerosolized simultaneously with arachidonic acid will produce an immediate-type airway response which simulates an antigen-induced response. Neither of these agents alone produced an airway response under the conditions of the experiments. Such a model of the immediate-type airway response due to nonimmunologic stimuli permits the evaluation of agents capable of blocking the airway reaction due to ionophore and arachidonic acid.

If this response to nonimmunologic stimuli is a model of immediate-type bronchial hypersensitivity, it could prove useful in evaluating blocking agents and studying pathogenetic mechanisms.

METHODS

Rhesus monkeys. Young adult female and male *Macaca mulatta* were used. These animals were in the Northwestern

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University monkey colony under continual observation for at least 2 yr. Two types of animals were studied. These included animals characterized as having antigen-induced respiratory responses. These animals have cutaneous reactivity to ascaris antigen (4) at dilutions of 1:1,000 or greater and consistent and persistent responses to repeated ascaris antigen aerosol challenge (4). These animals with consistent airway responses are termed antigen-reactive animals. Other animals have cutaneous reactivity to ascaris antigen at dilutions of 1:100 or less and have no airway responses to repeated aerosol challenge with ascaris antigen. These animals are termed nonreactive monkeys.

Pharmacologic agents. Arachidonic acid, sodium salt, linoleic acid, and linolenic acid, were obtained from the Sigmal Chemical Co., St. Louis, Mo. The calcium ionophore, A23187, dissolved in 20% ethanol in phosphate-buffered 0.15 M NaCl, pH 7.35, was a gift from Dr. Robert L. Hamill, Eli Lilly & Co., Indianapolis, Ind. FPL 55712 was kindly supplied by Dr. Philip Sheard, Fisons Limited, Pharmaceutical Div., Leicestershire, England. FPL 55712 was prepared in solution immediately before use. The indomethacin was donated by Merck Sharp & Dohme, Div. of Merck & Co., Inc., West Point, Pa. The 5,8,11,14-eicosatetraynoic acid (ETYA)[1] was provided by Dr. W. E. Scott, Roche Laboratories, Div. of Hoffman-La Roche Inc., Nutley, N. J. Indomethacin was dissolved in 50% ethanol in phosphate-buffered 0.15 M NaCl, pH 7.35. ETYA was dissolved in 40% ethanol in phosphate-buffered, 0.15 M NaCl, pH 7.35. Neither 50, 40, nor 20% ethanol in phosphate-buffered 0.15 M NaCl produces a respiratory response or inhibits an antigen-induced response in airway-reactive rhesus monkeys when aerosolized under the conditions described.

Aerosol challenge and determination of pulmonary function parameters. Animals received aerosol challenge no more frequently than every 2 wk. For an aerosol experiment, monkeys were anesthetized with pentobarbital and an endotracheal tube and an esophageal catheter were inserted. After a period of observation, the animals received a control aerosol challenge with phosphate-buffered 0.15 M NaCl, pH 7.35 (PBS). Base-line pulmonary function studies were obtained. Next the test aerosol challenge was delivered in a standard manner (7). All challenges were with an in-line nebulizer in a Bird Mark 7 (Bird Corp., Palm Springs, Calif.) respirator with all settings controlling respirations constant for each experiment in each animal. They received 15 inhalations of challenging agent and changes in pulmonary functions subsequent to this challenge were recorded. The animals are free-breathing except during the aerosol challenge. The methodology for recording changes in pulmonary functions has been described in detail (8). The following parameters of respiration were recorded: respiratory frequency (f), peak expiratory flow rate (PEFR), tidal volume (V_T), airway resistance (Raw), and dynamic compliance (Cdyn). The PEFR is the maximal expiratory flow during quiet (nonforced) expirations. The endotracheal tube inserted into each animal was the largest tube possible. The resistance of the tubes ranged from 4 to 6 cm H_2O/liters per s at 0.1 liters/s. The flow tracing was obtained by connecting the endotracheal tube to a Fleisch pneumotachograph (No. 00) (Dynasciences Corp., Instrument Systems Div., Chatsworth, Calif.) and a Statham (PM5TC) (Statham Instruments, Inc., Oxnard, Calif.) transducer. The f, and PEFR were calculated from the flow tracing. The V_T was obtained by electronic integration of the flow signal with time.

The open plastic catheter was inserted in the esophagus to the level of the nipple line and adjusted to minimize cardiac artifact. It was connected to one side of a Statham (PM5TC) transducer to determine changes in intrapleural pressure (P). The other side of the transducer was left open to the atmosphere. The Raw was determined by the method of Amdur and Mead (9) from simultaneous flow, P, and V_T signals. The Cdyn is the ratio between V_T and the change in P from the start to the end of inspiration (flow = 0).

The computation of Raw and Cdyn for each respiratory cycle was performed by an on-line analog computer (Respiratory Mechanics Computer; Buxco Electronics, Inc., Sharon, Conn.) which generates a signal proportional to the Raw and Cdyn. The flow, P, V_T, Raw, and Cdyn signals were recorded simultaneously on a Brush Mark 260 recorder (Gould Inc., Instrument Systems Div., Rolling Meadows, Ill.). All parameters were determined from this tracing by averaging five respiratory cycles.

In a series (2, 6, 8, 10), it has been established that although the absolute base-line pulmonary function levels varied between animals (8), these levels did not change significantly (>10%) in an animal that had no aerosol challenge or in an animal challenged by aerosol with PBS. Atropine did not result in more than that percentage of variation from the base-line. Because of this, the most useful system for analysis of the respiratory response was found to be the comparison of postchallenge results with each agonist and those obtained during the control period after the PBS challenge, with expression of the results as percent change from the base-line control period. The minimal criteria for positive responses in individual parameters are: f, +20%; Raw, +25%; V_T, −15%; Cdyn, −20%; PEFR, −25%. These criteria represent mean±2 SD based on 25 challenges with PBS. Four out of the five parameters must meet the above criteria to be considered a positive response in an individual experiment.

RESULTS

Effect of A23187 and arachidonic acid as single aerosol agents. When increasing concentrations of A23187 (0.2, 2, 20, and 200 μg/ml) were given by aerosol to reactive or nonreactive monkeys, no airway response occurred. Similarly, when increasing concentrations of arachidonic acid (0.1, 1.0, and 10 mg/ml) were aerosolized, no airway response in monkeys occurred. 200 μg/ml of A23187 and 10 mg/ml of arachidonic acid were the maximal concentrations of these agents used in subsequent aerosol experiments.

Effect of A23187 and arachidonic acid aerosolized simultaneously. When these agents were aerosolized simultaneously using 200 μg of A23187/ml and 10 mg of arachidonic acid/ml, an airway response occurred (Fig. 1A). This airway response is qualitatively similar to an antigen-induced response with an increase in f, Raw, and a decrease in Cdyn, PEFR, and V_T. Similar qualitative responses occurred in 11 successive experiments. The magnitude of the changes in the individual pulmonary function parameters varied in

[1] *Abbreviations used in this paper*: Cdyn, dynamic compliance; ETYA, 5,8,11,14-eicosatetraynoic acid; f, respiratory frequency; P, intrapleural pressure; PBS, phosphate-buffered, 0.15 M NaCl, pH 7.35; PEFR, peak expiratory flow rate; PG, prostaglandin; Raw, airway resistance; SRS-A, slow-reacting substance of anaphylaxis; V_T, tidal volume.

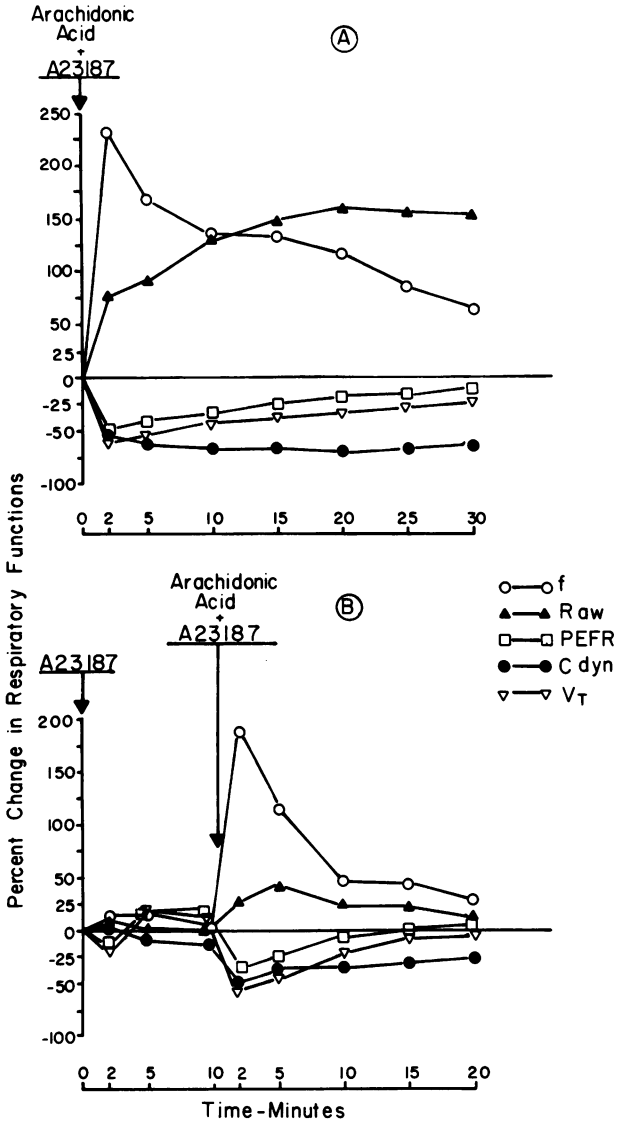


FIGURE 1 Results of aerosol challenges of rhesus monkeys. (A) Airway response in a rhesus monkey challenged with arachidonic acid (10 mg/ml) and A23187 (200 μg/ml). (B) Challenge of a rhesus monkey with A23187 (200 μg/ml) alone, with no response, followed by a second challenge in which a combination of arachidonic acid (10 mg/ml) and A23187 (200 μg/ml) results in a response.

different animals and in different challenges of the same animal. Similar variations in the magnitude of responses occur in aerosol antigen-induced responses (1). A further characteristic of the A23187 and arachidonic acid response was immediate onset (pulmonary function changes usually occurred at 2 min after completion of challenge). When either A23187 or arachidonic acid was given singly before the A23187-arachidonic acid challenge, no response occurred but this was followed by a response when the two agents were given simultaneously (Figs. 1B and 2A). The 11 successive A23187 and arachidonic acid experiments were conducted in six animals which were ascaris airway responders. When A23187 and arachidonic acid were given simultaneously to animals which were not antigen airway responders, the airway responses illustrated in Fig. 1 did not occur in four successive experiments. Thus the responses to A23187 and arachidonic acid appear to occur only in those animals which have the capability of responding to antigen and the hyperactive airway that is associated with such a response.

Other fatty acids, including linoleic acid and linolenic acid were tested in an identical manner

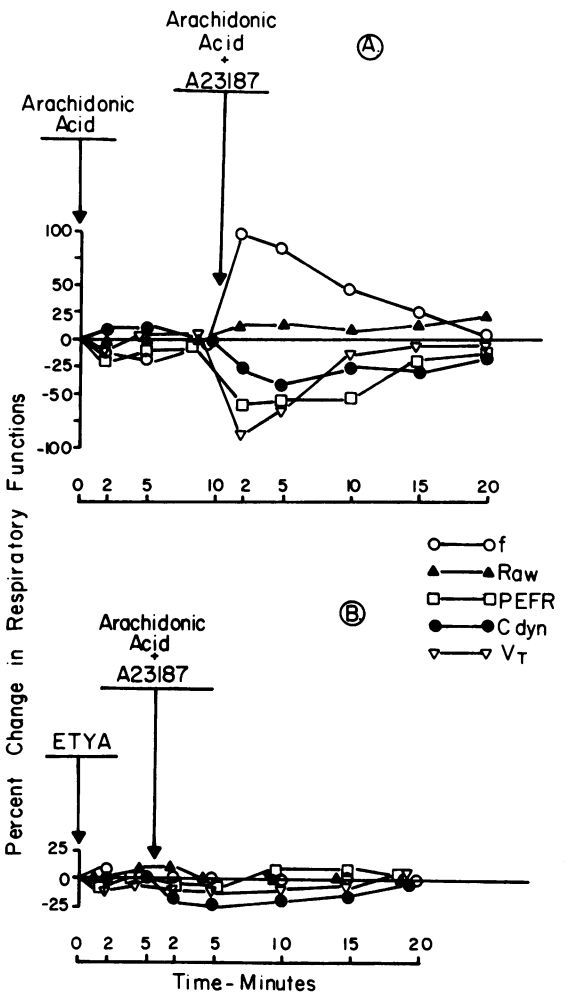


FIGURE 2 Results of aerosol challenge of rhesus monkeys. (A) Challenge of a rhesus monkey with arachidonic acid (10 mg/ml) alone, in which no response is seen, followed by a second challenge in which a combination of arachidonic acid (10 mg/ml) and A23187 (200 μg/ml) results in a response. (B) Effect of 10 mg/ml ETYA on the monkey airway response to arachidonic acid (10 mg/ml) and A23187 (200 μg/ml).

using the same molar concentrations of these two fatty acids as arachidonic acid in combination with A23187 in four experiments and no airway response to A23187 and these other fatty acids occurred.

Separate experiments demonstrated that if A23187 and arachidonic acid were given separately but in sequence at 10-min intervals with either agent given first, no reaction occurred.

Inhibition by ETYA. Animals which had demonstrated airway responses to the combination of A23187 and arachidonic acid were pretreated by aerosol of ETYA in 40% ethanol using a concentration of 10 mg/ml. After the ETYA, the A23187 and arachidonic acid were delivered by aerosol. The results of such an experiment are shown in Fig. 2B and demonstrate inhibition of the expected response to A23187 and arachidonic acid in the airway reactive monkeys. This inhibition occurred in four successive experiments.

Inhibition by FPL 55712. The slow-reacting substance (SRS) antagonist (10 mg/ml) was given simultaneously with A23187 and arachidonic acid. The results of such an experiment are shown in Fig. 3A. In a series of five experiments, no definitive inhibition of the A23187-arachidonic acid airway response could be demonstrated. The magnitude of the response of Fig. 3A as compared with Fig. 1A illustrates the variation that exists in different experiments.

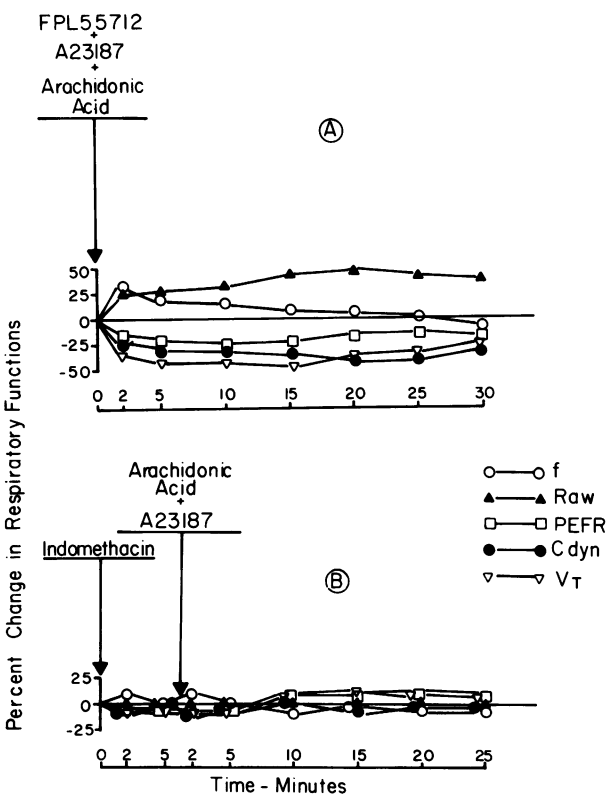


FIGURE 3 Results of aerosol challenges of rhesus monkeys. (A) Effect of the SRS-A antagonist FPL 55712 (10 mg/ml) on the monkey airway response to arachidonic acid (10 mg/ml) and A23187 (200 μg/ml). (B) Effect of indomethacin (10 mg/ml) on the monkey airway response to arachidonic acid (10 mg/ml) and A23187 (200 μg/ml).

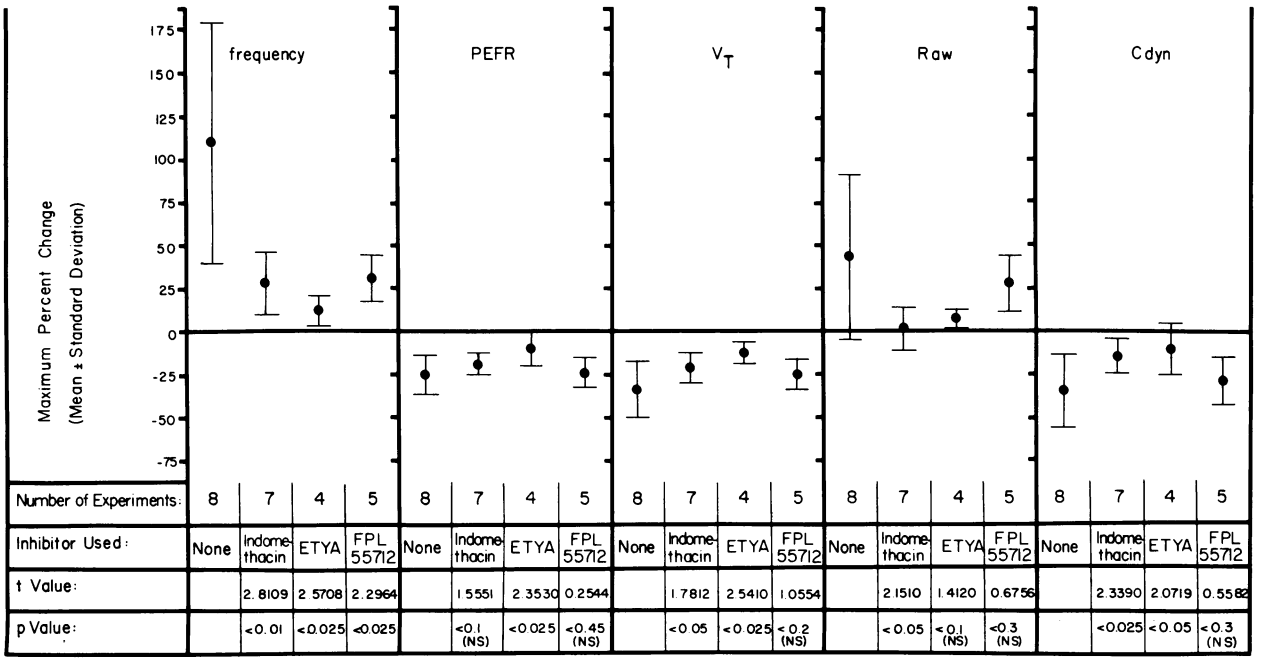
In separate experiments FPL 55712 (10 mg/ml) was given after the A23187-arachidonic airway response. The FPL 55712 did not acutely reverse the airway response.

Inhibition by indomethacin. Indomethacin (10 mg/ml) in 50% ethanol had no effect on the pulmonary response parameters of the rhesus monkey. When indomethacin was aerosolized before the delivery of A23178 and arachidonic acid by aerosol the airway response to the latter two agents was inhibited. The results of an indomethacin inhibition experiment are shown in Fig. 3B. Similar inhibition of the A23187-arachidonic acid response was seen in six additional experiments. In dose-response studies, indomethacin completely inhibited the A23187-arachidonic response at concentrations of 10, 5, and 1 mg/ml. Using a concentration of 0.1 mg/ml there was no inhibition of the response.

Analysis of inhibition experiments. The statistical analysis of all of the inhibition experiments is shown in Fig. 4. The results were obtained using the Student's *t* test for independent means (11). Using indomethacin there was statistically significant inhibition of changes in four of five pulmonary function parameters of the A23187-arachidonic acid response. Similarly ETYA inhibited four of five of the changes in pulmonary function parameters due to A23187-arachidonic acid challenge. FPL 55712 clearly inhibited only the change in frequency usually resulting from the A23187-arachidonic acid response (Fig. 4). An inhibiting effect on V_T, Raw, and Cdyn might be suggested but is clearly not statistically significant by our method of analysis (Fig. 4).

DISCUSSION

Under the conditions of these experiments, when the ionophore, A23187, and arachidonic acid were aerosolized simultaneously, an immediate-type airway response in rhesus monkeys occurred although neither agent alone induced such a response. Linoleic and linolenic acids did not produce the response seen with arachidonic acid. The airway response qualitatively simulated an ascaris antigen-induced response (1) and was demonstrated only in that group of monkeys which have airway responses to antigen, analogous to human antigen-induced asthma. These animals have hyperactive airways to challenge with analogous of acetylcholine (2, 3), histamine, and prostaglandin (PG) $F_{2\alpha}$ (3). Perhaps one of the best uses of this monkey model of asthma is to attempt to extend in vitro observations



Number of Experiments:	8	7	4	5	8	7	4	5	8	7	4	5	8	7	4	5	8	7	4	5
Inhibitor Used:	None	Indomethacin	ETYA	FPL 55712	None	Indomethacin	ETYA	FPL 55712	None	Indomethacin	ETYA	FPL 55712	None	Indomethacin	ETYA	FPL 55712	None	Indomethacin	ETYA	FPL 55712
t Value:		2.8109	2.5708	2.2964		1.5551	2.3530	0.2544		1.7812	2.5410	1.0554		2.1510	1.4120	0.6756		2.3390	2.0719	0.5582
p Value:		<0.01	<0.025	<0.025		<0.1 (NS)	<0.025	<0.45 (NS)		<0.05	<0.025	<0.2 (NS)		<0.05	<0.1 (NS)	<0.3 (NS)		<0.025	<0.05	<0.3 (NS)

FIGURE 4 Statistical analysis of the effect of potential inhibiting agents on the A23187-arachidonic acid airway response in rhesus monkeys.

of release of biologically active materials to an in vivo system in experiments that cannot be conducted in man.

The results of these experiments demonstrating that A23187 and arachidonic acid in combination, but not alone, will produce an airway response simulating an antigen-induced, immediate-type airway response are not readily explained. It appears reasonable to assume that mast cells in the lung or bronchial lumen may participate in such a reaction. We have shown that A23187 will release histamine from bronchial lumen mast cells (12). The bronchial lumen cells from monkeys with IgE-mediated airway responses will transfer airway reactivity to other monkeys (13). It is considered likely that the important cell initiating this response is the mast cells, since IgE appears to fix only to receptors of mast cells in primates. Although the mechanism of the A23187-arachidonic airway response is uncertain, it is possible that mast cells participate in the response and studies of basophils and mast cells from other species may be relevant. Some of this information may be briefly reviewed as follows. Slow-reacting substance of anaphylaxis (SRS-A) is released from rat basophilic leukemia cells and it was suggested that arachidonic acid might be a precursor of this SRS-A (14). Studies of the action of A23187 and arachidonic acid on rat peritoneal and pleural mast cells showed that this combination of agents led to the synthesis of PGD_2, PGE_2, $PGF_{2\alpha}$, thromboxanes, 6-keto-$PGF_{1\alpha}$, and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (15). SRS is released from rat peritoneal mast cells stimulated by A23187 and this was augmented by the presence of arachidonic acid (16). SRS-A is produced when mixed peritoneal cells are incubated with A23187 and cysteine (17). The formation of PGD_2 from rat basophilic leukemia cells was blocked by indomethacin at a concentration which did not block SRS-A formation (18). ETYA which blocks both the lipooxygenase and cyclooxygenase pathways of arachidonic metabolism, blocks production of SRS-A from the basophilic leukemic cells, while indomethacin (an inhibitor of the cyclooxygenase metabolic pathway of arachidonic acid) does not (14). This has been suggested as evidence that SRS-A is formed from arachidonic acid through the lipooxygenase pathway.

The results of our studies might, in part, be related to the in vitro studies described above. Because A23187 and arachidonic acid in combination produce an airway response but not separately this suggests that the response is due to metabolites of arachidonic acid produced by cells stimulated with A23187. The inhibition of the response by ETYA would be consistent with this explanation because ETYA inhibits the known cyclooxygenase pathway and lipooxygenase pathway of arachidonic acid. An alternative explanation for the action of ETYA in our experiments is that ETYA has been shown to inhibit mediator release from mast cells (19).

Indomethacin aerosolized before A23187 and arachidonic acid inhibited the response to these agents.

Such inhibition occurred with indomethacin concentrations of 10, 5, and 1 mg/ml but not with 0.1 mg/ml. A variety of possible explanations for this inhibitory action may exist. Prostaglandins, particularly $PGF_{2\alpha}$ (3) but also PGD_2 and PGI_2[2] produce immediate-type airway responses in rhesus monkeys. Indomethacin inhibits prostaglandin synthesis in a large variety of tissues (20). The action of indomethacin is presumed to be an effect on the cyclooxygenase that converts arachidonic acid to prostaglandins (21, 22). However, indomethacin may act in a variety of systems such as inhibition of phospholipase A_2 (23) which results in release of arachidonic acid (24). Because the airway response described here with A23187 required the presence of arachidonic acid the action of indomethacin as an inhibitor of phospholipase A_2, generation of arachidonic acid is considered unlikely. The action of indomethacin may best be ascribed to inhibition of cyclooxygenase or one of the other enzyme systems that generate prostaglandins which are inhibited by indomethacin. (20). In previous studies we have shown that indomethacin will potentiate an antigen-induced airway response (6). In more recent unpublished dose-response studies of the potentiation of the Raw change occurred at concentrations of indomethacin of 0.1 and 0.01 mg/ml but not with a concentration of indomethacin of 0.001 mg/ml. Our dose-response studies of the inhibitory effect of indomethacin showed inhibition over a wide range of doses except at the lowest dose. These divergent results between the A23187-arachidonic acid response and the antigen-induced response may indicate a difference between these two models of immediate airway response.

The SRS-A antagonist, FPL 55712 showed no definitive inhibitory effect on the A23187-arachidonic acid response except for the inhibition of an effect on f (Fig. 4). Because FPL 55712 has a very short half-life (25), it is possible that SRS-A activity could not be blocked by the FPL 55712 under the conditions of these experiments. However, FPL55712 did not reverse the A23187-arachidonic airway response either.

The airway response to A23187 and arachidonic acid in this study occurred only in monkeys with defined airway responsiveness to antigen. This could be due to different bioactive materials or amounts of materials produced. Alternatively, the bioactive materials produced could stimulate the hyperactive airway of these antigen-reactive monkeys but not the airways of normal monkeys. Because of the known hyperresponsiveness of this group of animals to such agents as histamine and $PGF_{2\alpha}$, the latter explanation seems more likely.

[2] Patterson, R., K. E. Harris, and P. A. Greenberger. Effect of prostaglandins D_2 and I_2 on the airways of rhesus monkeys. Manuscript in preparation.

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