Biochemical Pharmacology, Vol. 29, pp. 1843–1847.
© Pergamon Press Ltd. 1980. Printed in Great Britain.
0006-2952/80/0615-1843 $02.00/0

INCREASE IN ANTIGEN-INDUCED RELEASE OF SLOW REACTING SUBSTANCE OF ANAPHYLAXIS FROM GUINEA PIG LUNG BY SODIUM FLUORIDE

JEROME H. FLEISCH and KLAUS D. HAISCH
Lilly Research Laboratories, Indianapolis, IN 46285, U.S.A.

(*Received* 19 *October* 1979; *accepted* 25 *January* 1980)

Abstract—Some of the mechanisms involved in the release of mediators of anaphylaxis from passively sensitized guinea pig lung were examined. NaF (10 mM) markedly enhanced antigen-induced release of slow reacting substance of anaphylaxis (SRS-A), whereas release of histamine was not changed. Similar results were obtained with equimolar amounts of KF but not with NaCl. When SRS-A, released from lung in the presence of NaF, was applied to guinea pig ileum, the tissue contracted. This response was selectively antagonized by FPL 55712, an SRS-A antagonist. A23187, a divalent cation ionophore, released histamine and SRS from guinea pig lung. NaF and KF, but not NaCl, reduced equally the amounts of mediators released by A23187. These results indicate that antigen-induced release of histamine and SRS-A can be dissociated by fluoride ions. Furthermore, they suggest that NaF may prove useful in dissecting and understanding some of the complex biochemical steps involved in the release of SRS-A.

Slow reacting substance of anaphylaxis (SRS-A) is a smooth muscle stimulant released from human and animal lung upon antigen challenge [1]. A substance designated SRS, which is biologically identical to SRS-A, can be released from lung by A23187 [2], a carboxylic acid antibiotic that transports divalent cations across biological membranes [3]. Antigen and ionophore have different starting points in the SRS-A/SRS release sequences. To date, no agent has been found that can separate the events taking place within the lung cell after activation by these two substances.

Patkar *et al.* [4, 5] demonstrated that sodium fluoride (NaF) increased the permeability of rat mast cells to extracellular Ca^{2+}. Cells treated with NaF secreted histamine when exposed to Ca^{2+}. In the present study, we have investigated the effect of NaF on release of histamine and SRS-A (or SRS) in response to antigen or to A23187. NaF reduced release of histamine and SRS by A23187 and did not alter release of histamine caused by antigen. However, NaF dramatically increased antigen-induced release of SRS-A. The mechanism by which NaF produces these effects has not yet been established. Nevertheless, the present experiments, and those of Patkar *et al.* [4, 5], support the notion that simple molecules like NaF may help elucidate the events responsible for elaboration of mediators of anaphylaxis.

METHODS

Male Hartley guinea pigs (William Cavies, Fern Creek, KY), 4–6 weeks old, were decapitated. Lungs were excised and perfused through the pulmonary artery with Krebs bicarbonate solution of the following composition in mmoles/l: KCl, 4.6; $CaCl_2 \cdot 2H_2O$, 1.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; NaCl, 118.2; $NaHCO_3$, 24.8; and dextrose, 10.0. Poorly perfused and bloody areas were discarded. Normal lung was cut into 1-mm cubes with a McIlwain tissue chopper, washed with Krebs solution, divided into 400-mg aliquots, and incubated for 1 hr at 37° in vials containing 2.5 ml of antiserum diluted 1/33 with Krebs buffer. Hyperimmune serum was prepared by actively sensitizing guinea pigs with 2 mg ovalbumin in 50% Complete Freund's Adjuvant, i.p., on day 1 and day 5. On day 21, the animals were bled and serum was collected.

After passive sensitization, the tissues were washed with Krebs solution containing 1×10^{-6} M indomethacin to optimize mediator release [6]. Tissue samples were then reincubated at 37° for 15 min in 2.25 ml of indomethacin–Krebs solution containing an additional 10 mM NaF, KF or NaCl. Submaximal concentrations of antigen (250 μl, 5×10^{-5} g/ml) or A23187 (250 μl, 5×10^{-5} M) were added to make a final concentration of 5×10^{-6} g/ml and 5×10^{-6} M respectively. These suboptimal amounts of the secretagogues were specifically chosen to make it easier to quantitate a reduction or enhancement of mediator release by NaF. The incubation was continued an additional 15 min for antigen and 30 min for ionophore. The incubation medium was then decanted and centrifuged at 3000 *g* at 4° for 5 min. The supernatant solutions were collected and assayed for histamine by a modification of the automated fluorometric method of Siraganian [7] and for SRS and SRS-A by a recently developed computerized bioassay using the guinea pig ileum [8]. SRS and SRS-A are quantitated by comparison with an in-house standard, and the results are expressed in terms of arbitrary units.

Total histamine was determined by adding 2.5 ml of indomethacin–Krebs solution to the previously challenged tissues and immersing the vials in boiling water for 10 min. The amount of histamine released into the incubation medium after boiling, plus that released by antigen or ionophore challenge, represented the total amount of histamine in the tissue.

In experiments using guinea pig ileum, male or

female guinea pigs, 4–6 weeks old, were decapitated. A section of terminal ileum was removed, the lumen was cleaned, and the tissue was divided into 2.5-cm segments. The ileums were mounted in 10-ml tissue baths containing Krebs bicarbonate solution maintained at 37° and aerated with 95% O_2 and 5% CO_2. The composition of Krebs bicarbonate solution was as above except for $CaCl_2 \cdot 2H_2O$ which was 1.2 mmoles/l. All studies with the guinea pig ileum were performed in the presence of 1×10^{-6} M atropine and 1×10^{-6} M pyrilamine. Isometric measurements were made with a Grass FT03C force displacement transducer and recorded on a Grass polygraph as changes in grams of force. A passive force of 0.5 g was applied to the tissues.

The following drugs were used: ovalbumin grade 5, indomethacin, atropine sulfate, serotonin creatinine sulfate, bradykinin triacetate and EGTA* (Sigma Chemical Co., St. Louis, MO); pyrilamine maleate (ICN Pharmaceuticals, Inc.-K & K Labs, Plainview, NY); sodium fluoride (J. T. Baker Chemical Co., Phillipsburgh, NJ); A23187 (Eli Lilly & Co., Indianapolis, IN); and FPL 55712 (gift of Fisons Ltd., Leicestershire, U.K.).

RESULTS

Effect of NaF *on antigen- and ionophore-induced mediator release: preliminary observations.* Preliminary experiments on sensitized guinea pig lung suggested that NaF increased release of SRS-A but not of histamine in response to antigen. The largest potentiation of SRS-A release occurred when the tissue was challenged by submaximal concentrations of antigen; the antigen challenge was 5×10^{-6} g/ml ovalbumin for 15 min, which was previously shown to release 63 and 76 per cent of the total releasable histamine and SRS-A, respectively [8]. Potentiation of antigen-induced SRS-A release always occurred with 10 mM NaF, whereas 1 mM and 0.1 mM NaF produced erratic results, and 0.01 NaF generally was inactive. In addition, unlike 10 mM NaF, 30 mM NaF released histamine and an SRS-like substance when given alone.

We were unable to find a concentration of A23187 that would release amounts of histamine and SRS equal to those released by ovalbumin (Table 1). A concentration of A23187 that released a quantity of histamine similar to that released by ovalbumin caused the release of twice as much SRS. This same relationship was reported previously for human leucocytes by Conroy *et al.* [9].

Influence of NaF, KF, *and* NaCl *on ionophore- and antigen-induced histamine release.* NaF reduced the release of histamine after A23187 by nearly 70 per cent (Fig. 1). To determine if this was due to fluoride ions or to an osmotic effect, we examined the effects of equal concentrations of KF and NaCl on histamine release by A23187. KF inhibited histamine release to the same extent as NaF did, whereas NaCl was inactive, suggesting that the fluoride ion was the active moiety. NaF and KF had no effect on the release of histamine by ovalbumin (Fig. 2). There was, however, a small (20 per cent) inhibition of histamine release in the tissues pretreated with 10 mM NaCl.

Influence of NaF, KF *and* NaCl *on ionophore- and antigen-induced SRS/SRS-A release.* NaF and KF decreased release of SRS by A23187 to almost the same extent as with histamine (Fig. 3). In these experiments, a 20 per cent decrease in SRS release was noted in tissues pretreated with NaCl. This is similar to the result obtained above with histamine release by antigen. More significantly, NaF and KF increased antigen-induced SRS-A release 3-fold, whereas NaCl did not change the release compared to control (Fig. 4).

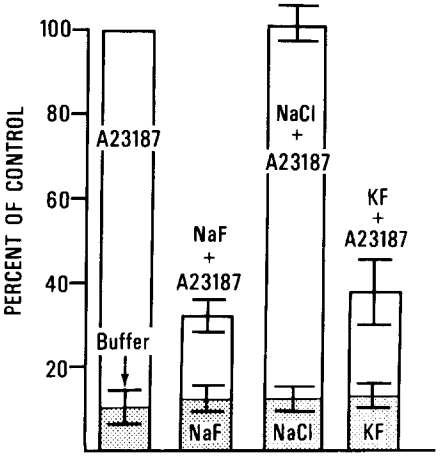

Fig. 1. A23187-induced histamine release from guinea pig lung in the presence or absence of 10 mM NaF, NaCl or KF. The shaded portions of the bars indicate that release of histamine by NaF, NaCl or KF alone did not differ from basal release. Values represent the mean ± S.E. of twelve determinations for NaF and six each for NaCl and KF.

* EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*-tetra-acetic acid.

Table 1. Histamine and SRS/SRS-A release from guinea pig lung by A23187 or antigen*

	Histamine (% total lung histamine)	SRS/SRS-A (in-house units)
A23187 (5×10^{-6} M)	19.8 ± 1.9	70.4 ± 5.4
Antigen (ovalbumin, 5×10^{-6} g/ml)	21.2 ± 1.9	34.5 ± 4.6

* Values represent the mean ± S.E. of six experiments containing twelve determinations.

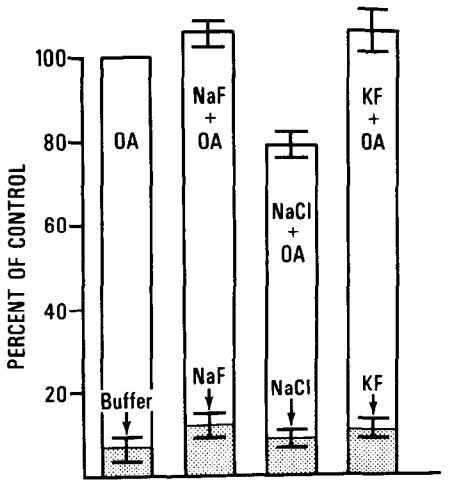

Fig. 2. Ovalbumin (OA)-induced histamine release from guinea pig lung in the presence or absence of 10 mM NaF, NaCl or KF, The shaded portions of the bars indicate that release of histamine by NaF, NaCl or KF alone did not differ from basal release. Values represent the mean ± S.E. of twelve determinations for NaF and six each for NaCl and KF.

One possible complication in these experiments, albeit a small one, was the presence of indomethacin. The results could reflect an indomethacin–fluoride interaction. To rule this out, a single experiment was run without indomethacin on triplicate samples of guinea pig lung. NaF (10 mM) enhanced SRS-A release 3-fold, wheras SRS release was reduced to 30 per cent of control; NaCl had minimal effects on release of SRS-A or SRS.

Antagonism of responses to SRS-A by FPL 55712. The increase in SRS-A release by NaF could have resulted from release of a non-SRS-A smooth muscle stimulant that contracted the ileum (bioassay). If such a substance were assayed along with SRS-A,

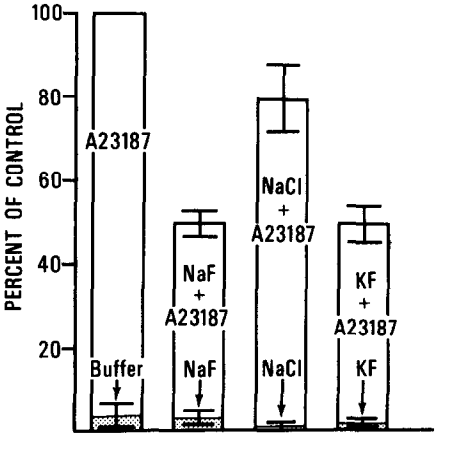

Fig. 3. A23187-induced SRS release from guinea pig lung in the presence or absence of 10 mM NaF, NaCl or KF. The shaded portions of the bars indicate that release of SRS by NaF, NaCl or KF alone did not differ from basal release. Values represent the mean ± S.E. of twelve determinations for NaF and six each for NaCl and KF.

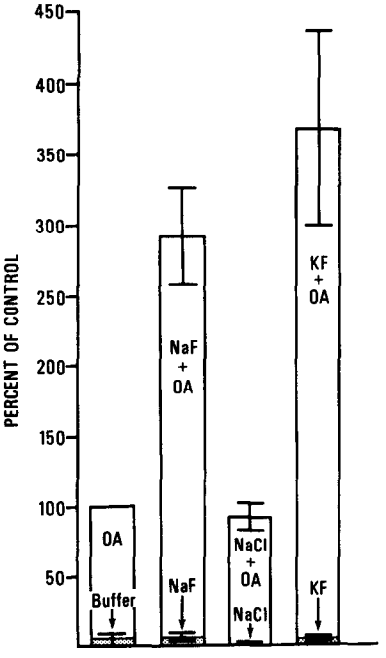

Fig. 4. Ovalbumin (OA)-induced SRS-A release from guinea pig lung in the presence or absence of 10 mM NaF, NaCl or KF. The shaded portions of the bars indicate that release of SRS-A by NaF, NaCl or KF alone did not differ from basal release. Values represent the mean ± S.E. of twelve determinations for NaF and six each for NaCl and KF.

it would appear that a greater release of SRS-A had taken place. This possibility was ruled out in the experiment shown in Fig. 5. Two ileums were contracted with bradykinin, serotonin, SRS-A released by antigen alone, and SRS-A released by antigen + NaF. FPL 55712, a specific SRS-A antagonist [10], was added to the upper tissue bath 1 min before each agonist. FPL 55712 selectively blocked responses to SRS-A generated by both procedures, indicating that a smooth muscle contracting substance other than SRS-A was not liberated by the combination of NaF + antigen (Fig. 5).

We also considered whether NaF differentially interfered with the bioassay of SRS-A and SRS. In three segments of ileum, responses to SRS-A and SRS were identical before and after pretreatment for 1 min with 1×10^{-4} M NaF, the concentration usually in the tissue baths during the bioassay. In addition, as shown above for SRS-A, we demonstrated that responses of the guinea pig ileum to SRS can be blocked selectively by FPL 55712. Therefore, at least for our purposes, SRS-A and SRS can be considered pharmacologically similar.

Effect of EGTA on antigen-induced mediator release. NaF reacts with free Ca^{2+} and Mg^{2+} to form relatively insoluble fluoride salts. Loss of these ions could dramatically influence mediator release. We therefore ran an experiment in which mediators were released by antigen in the presence of 3 or 10 mM EGTA, a chelating agent that forms a tight complex with divalent ions. For comparison, mediators were also released in the presence of 3 or 10 mM NaF.

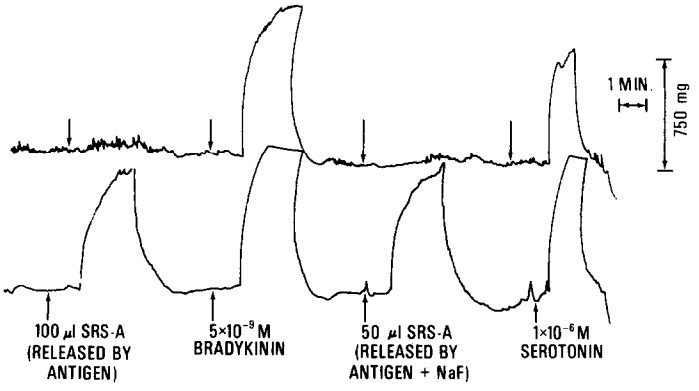

Fig. 5. Polygraph tracings of ileal contractions (two guinea pigs) produced by 100 μl SRS-A released by antigen, 5×10^{-9} M bradykinin, 50 μl SRS-A released by antigen + NaF, and 1×10^{-6} M serotonin. For the top tracing, 1×10^{-6} M FPL 55712 was added to the tissue bath 1 min prior to each agonist (at arrow).

Release of histamine and SRS-A was totally abolished from tissues treated with EGTA, whereas 3 mM NaF increased SRS-A release 2.5-fold and 10 mM NaF increased SRS-A release 3.6-fold. As previously documented (Fig. 2), NaF had no effect on histamine release.

DISCUSSION

Release of SRS-A from sensitized guinea pig lung upon antigen challenge is a complex biologic event involving *de novo* synthesis of SRS-A and numerous biochemical steps necessary for the liberation of this bronchoconstrictor agent [1]. Our study demonstrated that NaF enhanced release of SRS-A. Since SRS-A is not preformed in large quantities, its synthesis was most likely also increased. This was accomplished without altering the binding of antigen to antibody as evidenced by the lack of effect of NaF on histamine release.

Quite the opposite effects were observed when a divalent cation ionophore, A23187, was used to release histamine and SRS (to technically distinguish it from SRS-A which is released by antigen challenge). NaF decreased ionophore-induced release of SRS by 50 per cent and histamine release by nearly 70 per cent. That NaF similarly reduced ionophoric release of both mediators is suggestive of a common point of attack.

The present results were due to fluoride ions and not to either Na ions or an osmotic effect of the NaF molecule since the effects of NaF could be mimicked by KF but not by NaCl. Fluoride is known to influence cellular processes by at least three mechanisms: (1) complex formation with Ca^{2+} and Mg^{2+} in the surrounding milieu to form CaF_2 and MgF_2, which are insoluble; (2) interaction with the cyclic nucleotide system; and (3) inhibition of ATPase.

Antigen-induced release of histamine and SRS-A from lung tissue is a Ca^{2+}-dependent reaction [11, 12]. When a slight excess (3 mM) or a large excess (10 mM) of EGTA, an agent that chelates divalent cations, was added to the medium, mediator release was abolished. Obviously, NaF did not precipitate all the available Ca^{2+}, and enough remained free for release of histamine and SRS-A by antigen. We conclude, therefore, that the enhanced release of SRS-A was, in fact, due to the presence of the fluoride ion. The experiments with NaF suggest that ionophore-induced mediator release may be more Ca^{2+}-dependent than the release by antigen.

The cyclic nucleotide system plays a major role in antigen-induced mediator release from lung [13]. Although controversy exists in the literature, some studies have shown that fluoride can increase levels of cyclic AMP [14, 15] and cyclic GMP [16] in intact cells. Since increased intracellular levels of cyclic AMP are associated with a reduction and not an increase in release of SRS-A, this mechanism can probably be ruled out. Enhanced mediator release from human lung, resulting from cholinergic stimulation, was demonstrated to be accompanied by increased levels of cyclic GMP [17]. This might have provided an explanation for the action of NaF. The increased levels of cyclic GMP, however, coincided with a larger release of both histamine and SRS-A [17]. In our study, NaF increased antigen-induced release of SRS-A but not of histamine. This issue can be resolved only by further experiments on the relationship between NaF and cyclic nucleotide levels in guinea pig lung.

Fluoride ions inhibit Na^+–K^+ ATPase [18]. Thus, this action might be involved in the effect of NaF on SRS-A release. The work of Okazaki *et al.* [19] showed that ouabain, the prototypical Na^+–K^+ ATPase inhibitor, decreased histamine release from antigen-stimulated guinea pig lung. In addition, Fewtrell and Gomperts [20] demonstrated that quercetin, another ATPase inhibitor, reduced histamine release from rat peritoneal mast cells. In the present experiments, NaF did not alter antigen-induced histamine release, suggesting that inhibition of ATPase did not contribute to the mechanism of action of NaF.

In conclusion, we have demonstrated a marked enhancement of antigen-induced SRS-A release from guinea pig lung by NaF. This increased release of SRS-A is specific since NaF did not influence the simultaneous release of histamine. Our results lend support to the previous observations of Orange [21] that the immunological release of histamine and of

SRS-A from lung can be dissociated. Our results permit some interesting speculation. With regard to the ionophore, NaF may inhibit A23187-induced mediator release by lowering the extracellular concentration of Ca^{2+}. For antigen-induced mediator release, our data are consistent with either separate cellular locations or pools for histamine and SRS-A that are differentially affected by NaF, or a chain of events subsequent to antigen activation that differs for release of histamine and SRS-A with the action of NaF favoring an increased synthesis and release of SRS-A.

REFERENCES

1. K. F. Austen and R. P. Orange, *Am. Rev. resp. Dis.* **112**, 423 (1975).
2. P. J. Piper and J. P. Seale, *Br. J. Pharmac.* **63**, 364P (1978).
3. D. T. Wong, J. R. Wilkinson, R. L. Hamill and J. S. Horng, *Archs Biochem. Biophys.* **156**, 578 (1973).
4. S. A. Patkar, W. Kazimierczak and B. Diamant, *Int. Archs Allergy appl. Immun.* **55**, 193 (1977).
5. S. A. Patkar, W. Kazimierczak and B. Diamant, *Int. Archs Allergy appl. Immun.* **57**, 146 (1978).
6. J. L. Walker, *Adv. Biosci.* **9**, 235 (1973).
7. R. P. Siraganian, *J. immun. Meth.* **7**, 283 (1975).
8. J. H. Fleisch, B. R. Zaborowsky, B. J. Cerimele and S. M. Spaethe, *J. Pharmac. exp. Ther.* **209**, 238 (1979).
9. M. C. Conroy, R. P. Orange and L. M. Lichtenstein, *J. Immun.* **116**, 1677 (1976).
10. J. Augstein, J. B. Farmer, T. B. Lee, P. Sheard and M. L. Tattersall, *Nature New Biol.* **245**, 215 (1973).
11. R. P. Orange, *Monogr. Allergy* **12**, 231 (1977).
12. J. L. Mongar and H. O. Schild, *J. Physiol Lond.* **140**, 272 (1958).
13. M. Kaliner and K. F. Austen, *A. Rev. Pharmac.* **15**, 177 (1975).
14. T. P. Stossel, F. Murad, R. J. Mason and M. Vaughan, *J. biol. Chem.* **245**, 6228 (1970).
15. A. R. Shahed, A. Miller, D. Chalker and D. W. Allmann, *J. Cyclic Nucleo. Res.* **5**, 43 (1979).
16. K. Yamashita and J. B. Field, *J. biol. Chem.* **247**, 7062 (1972).
17. M. Kaliner, R. P. Orange and K. F. Austen, *J. exp. Med.* **136**, 556 (1972).
18. L. J. Opit, H. Potter and J. S. Charnock, *Biochim. biophys. Acta* **120**, 159 (1966).
19. T. Okazaki, V. S. Ilea, A. Okazaki, K. Wicher, R. E. Reisman and C. E. Arbesman, *J. Allergy clin. Immun.* **57**, 454 (1976).
20. C. M. S. Fewtrell and B. D. Gomperts, *Biochim. biophys. Acta* **469**, 52 (1977).
21. R. P. Orange, *J. Immun.* **114**, 182 (1975).