Biochemical Pharmacology, Vol. 27, pp. 1385–1387.

0006-2952/78/0501-1385$02.00/0

Influence of flufenamic acid and calcium ion concentration on the histamine release from rat mast cells induced by compound 48/80 and the calcium ionophore A23187

(*Received* 1 *March* 1977; *accepted* 31 *May* 1977)

Flufenamic acid, a non-steroidal anti-inflammatory drug, inhibits compound 48/80 and antigen-induced histamine release from rat mast cells *in vitro* [1, 2]. Available cellular ATP is necessary for histamine release from mast cells by these agents [3, 4] and non-steroidal anti-inflammatory drugs (NSAID) (including flufenamic acid) are thought to inhibit histamine release by depleting mast cell ATP content [1, 5].

Some histamine-releasing agents such as antigen require the presence of extracellular calcium in the histamine release process *in vitro* [6, 7]. Calcium itself will induce degranulation and histamine release when injected directly into a rat mast cell by micropipette [8], and the calcium ionophore A23187 is a potent histamine releaser in the presence of calcium ions [9, 10]. Compound 48/80 differs from antigen and A23187 in that it lacks an absolute requirement for extracellular calcium ions when releasing histamine from mast cells [7, 11]. However, the compound 48/80 release reaction is potentiated by extracellular calcium [11] and is thought to utilize a tightly bound intracellular pool of calcium when extracellular calcium is unavailable [12], although the precise role of calcium in the histamine release reaction remains unclear [13]. Changes in calcium permeability induced by histamine liberators [14, 15] are thought to be involved intimately in the release mechanism and have been suggested to be energy dependent [16–19].

The present study describes the effect of flufenamic acid on compound 48/80- and A23187-induced histamine release from rat mast cells in the presence of variable calcium concentrations. This study was designed to investigate if part of the activity of flufenamic acid on mast cells was related to drug-induced interference with the functions of calcium in the histamine release process.

Mast cell preparation. Rat peritoneal cells were collected from male Fullensdorf albino rats essentially following the method of Johnson and Moran [20] but using a calcium-free buffered salt solution for performing peritoneal lavage (NaCl 150 mM, KCl 2.7 mM, Na_2HPO_4 3 mM, KH_2PO_4 3.5 mM, dextrose 5.6 mM, and bovine serum albumin 0.07%; pH 7.0). Further separation of the mast cells was not performed. The histamine released from suspensions of rat peritoneal cells comes from the mast cell subpopulation [21].

Mast cell incubation. The cells collected from four rats were resuspended in 3–4 ml of a calcium-free salt solution (NaCl 150 mM, KCl 2.7 mM, HEPES* buffer 20 mM, dextrose 5.6 mM, and bovine serum albumin 0.07%; pH 7.0). Aliquots (30 μl of the concentrated peritoneal cells were added to 1.5 ml of the HEPES buffer salt solution (HBSS) giving a final mast cell count of 100,000–200,000 mast cells/ml. Variations in the concentrations of calcium and dextrose were made in different experiments. The HEPES buffer allowed the calcium concentrations to be increased to 10.8 mM without precipitation of calcium. Small volumes (20–40 μl) of flufenamic acid (7.5 mM) dissolved in calcium and dextrose-free HBSS were added prior to cell incubation. After incubation at 37° (15 min unless otherwise stated), 22.5 μl of an aqueous solution of compound 48/80 (final concentration, 150 ng/ml) or 16.5 μl of the diluted stock solution of A23187 (final concentration, 10^{-6} M) was added for a further 5-min incubation. A23187 was dissolved according to Prince *et al.* [22] and a stock solution of 0.91×10^{-3} M dissolved in 5% acetone and 95% ethanol was kept at −20°. A one in ten dilution of the stock solution in ethanol was made prior to use. The concentrations of ethanol and acetone did not influence the release of histamine or its measurement. The cells were centrifuged at 250 *g* for 5 min at 4°.

Histamine measurement. The histamine released into the supernatant was measured directly using an automated fluorometric procedure† based on the method of Shore *et al.* [23]. The alterations in calcium and dextrose concentrations of the media studied and the presence of flufenamic acid in the media at the concentrations used did not interfere with the measurement of histamine. Maximum releasable histamine was determined by disrupting mast cells by boiling and then measuring the histamine in the supernatant. Control incubations with and without flufenamic acid and in the absence of releasing agent were performed in all experiments. After subtraction of the relevant control measurements (mean ± S. E. of three estimations), histamine release was expressed as the percentage of maximum releasable histamine.

ATP measurement. Small aliquots (50 μl) of the cell incubation mixture were removed after 10 min for measurement of ATP content as previously described [5]. The ATP content was expressed as ng/ml of the incubation mixture. Variation of calcium content in the media or the presence of 0.2 mM flufenamic acid did not interfere with the bioluminescence assay for ATP.

Chemicals. Flufenamic acid was kindly donated by Parke Davis & Co., Australia, and A23187 by Eli Lilly Co., Australia. Compound 48/80 and HEPES buffer were purchased from Burroughs Welcome, Australia, and bovine serum albumin, ATP and buffered firefly lantern extract from the Sigma Chemical Co., (St. Louis, MO, U.S.A.). All other reagents were of the highest analytical grade commercially available.

Submaximal histamine release from rat mast cells by compound 48/80 (150 ng/ml) declined rapidly as the calcium concentration approached 9 mM (Fig. 1). In the absence of dextrose, flufenamic acid inhibited compound 48/80-induced histamine release over the range of calcium concentrations studied (Fig. 1, panels (a) and (b)). The presence of dextrose in the medium opposed the inhibitory activity of flufenamic acid at low and near physiological (0.9 mM) calcium concentrations as expected (Fig. 1, panels (c) and (d)). However, at higher calcium concentrations, the drug enhanced the compound 48/80 histamine release compared to controls (Fig. 1, panels (c) and (d)). This effect was not due to enhanced spontaneous histamine release by flufenamic acid.

* HEPES.

† R. O. Day, P. D. Paull, J. E. Ray and D. N. Wade, manuscript submitted for publication.

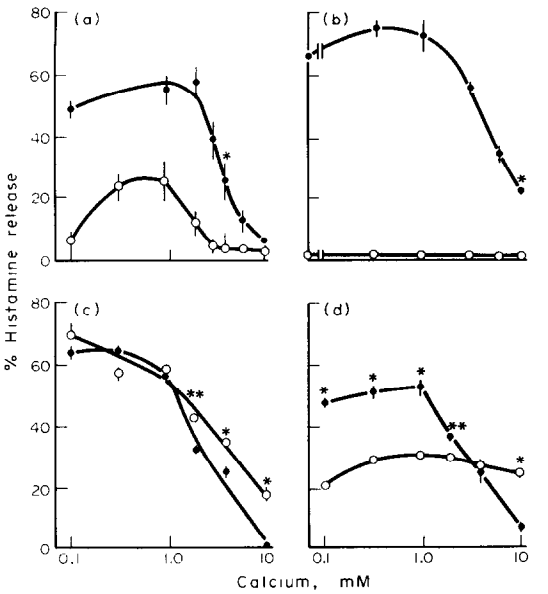

Fig. 1. Effect of flufenamic acid (O——O) at concentrations of 0.08 mM (a and c), 0.125 mM (b) and 0.2 mM (d) on compound 48/80-induced (final concentration, 150 ng/ml) histamine release (control histamine release, (●——●) in the presence (c and d) and absence (a and b) of dextrose, 5.6 mM. Each point is the mean ± S. E. of four estimations from which the appropriate spontaneous histamine release (mean ± S. E. of three estimations) has been subtracted. Spontaneous histamine release at "high" or "low" calcium concentrations was not significantly different from controls ($P > 0.05$) for any of the concentrations of flufenamic acid studied, although greater than controls in some experiments. Spontaneous histamine release was 3–13 per cent for these experiments. Maximum histamine release ranged from 1 to 3.5 μg/ml (10^5 mast cells/ml) for all experiments. Significant differences between drug and control means are labeled (* = $P < 0.01$; ** = $P < 0.05$; unpaired two-tail *t*-test). Drug and control means were significantly different ($P < 0.01$) for all calcium concentrations less than 3.6 mM calcium in Fig. 1a and less than 9 mM calcium in Fig. 1b.

The facilitation of compound 48/80-induced histamine release by preincubation of the cells with flufenamic acid (0.15 mM) in the presence of 9 mM calcium was apparent after 30 sec, progressed for 10 min and was sustained for at least 30 min preincubation. The presence of dextrose was necessary to demonstrate this facilitation of histamine release, the optimal concentration being greater than 1 mM. The amount of facilitation observed varied somewhat with different cell preparations but was consistently present.

Flufenamic acid inhibited histamine release induced by the calcium ionophore A23187 at physiological calcium concentrations (Fig. 2). However, at low calcium ion concentrations and with dextrose in the medium, facilitation of release occurred (Fig. 2a).

ATP levels of the peritoneal cells incubated in 0, 0.9 and 9 mM $CaCl_2$, respectively, were not significantly different (Table 1). Flufenamic acid (0.15 mM) significantly reduced the cellular ATP content independent of the calcium content of the medium (Table 1). A greater drug-induced reduction in ATP content was observed if dextrose was absent from the incubation medium (Table 1).

These data indicate that low concentrations of flufenamic acid oppose the inhibitory effects of high concentrations of calcium on 48/80-induced histamine release and low concentrations of calcium on A23187-induced histamine release but only if dextrose is present in the media. These effects of flufenamic acid occur despite concomitant drug-induced reductions in peritoneal cell ATP levels. Normally a fall in peritoneal mast cell ATP levels would tend to reduce histamine release as there is a linear relationship between the amount of histamine released from rat mast cells and their ATP content prior to challenge with a histamine liberator [5, 24, 25]. In the presence of dextrose, the effect of the drug-induced reduction of cellular ATP apparently is overshadowed by the drug-induced removal of the inhibitory effects of the "high" and "low" calcium concentrations on 48/80 and A23187 histamine release respectively. Histamine release is thus enhanced. However, when dextrose is absent, flufenamic acid causes a greater fall in ATP levels and consequently any drug-induced interference with the inhibitory activity of high and low calcium concentrations is masked, as the cellular ATP level finally determines the amount of histamine that can be released. It should be noted that, although the mast cells are only a subpopulation of the peritoneal white cells used to measure ATP levels in this study, it is unlikely that there would be differential alterations in cellular ATP levels in such a cell population [5].

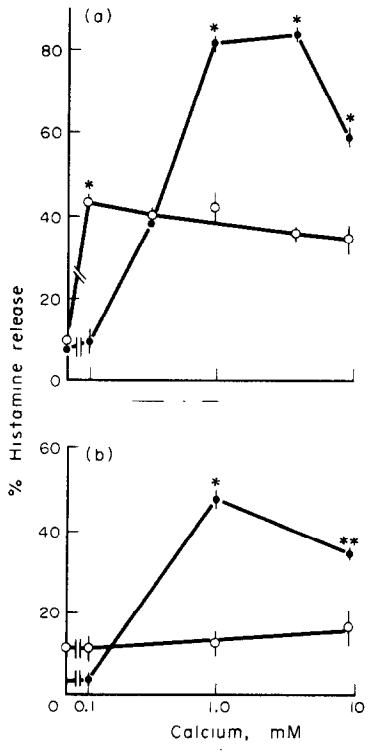

Fig. 2. Influence of flufenamic acid (O——O), 0.15 mM, on A23187-induced (final concentration, 10^{-6} M) histamine release (control release, ●——●) in the presence (a) and absence (b) of dextrose, 5.6 mM. Each point is the mean ± S. E. of three estimations minus the relevant spontaneous histamine release (mean ± S. E. of three estimations). Significant differences between drug and control are labeled (* = $P < 0.01$; ** = $P < 0.05$; unpaired two-tail *t*-test). Absolute mast cell histamine content was as described in Fig. 1. Spontaneous histamine release was less than 7 per cent for these experiments.

Table 1. Effect of flufenamic acid on ATP levels of the rat peritoneal cell suspension at 0, 0.9 and 9 mM calcium with and without dextrose in the medium*

Calcium (mM)	ATP (ng/ml supernatant) No Dextrose Control	No Dextrose Flufenamic acid (0.15 mM)	Dextrose (5.6 mM) Control	Dextrose (5.6 mM) Flufenamic acid (0.15 mM)
0	30.7 ± 0.5	6.0 ± 0.2	34.6 ± 0.3	20.0 ± 0
0.9	29.0 ± 0.9	6.4 ± 0.2	35.4 ± 0.8	20.6 ± 0.5
9	31.0 ± 1.5	5.6 ± 0.4	34.0 ± 2.0	19.3 ± 0.5

* Each value is the mean ± S. E. of four estimations. Significant differences between controls and flufenamic acid-treated cells were noted ($P < 0.01$, two-tail unpaired t-test). There were no significant differences ($P > 0.05$, one-way analysis of variance) between control values at 0, 0.9 and 9.0 mM calcium for dextrose and non-dextrose treated cells respectively.

The explanation for the divergent actions of flufenamic acid on 48/80- and A23187-induced histamine release, respectively, is unknown but may reflect the differences in the release mechanisms of these two histamine liberators (apparent from this work and that of Diamant and Patkar [16]) and/or different roles for calcium in the release processes. However, these observations do suggest that this drug may interfere with the movements and/or functions of calcium in the histamine release reactions induced by compound 48/80 and A23187. There is evidence that flufenamic acid and other NSAID can affect cation transport across membranes [26, 27]. Northover [28] demonstrated that flufenamic acid and other NSAID can inhibit the ATP-dependent uptake of calcium by the membranes of endothelial cells and suggested that this activity of NSAID may be related to their anti-inflammatory action. Whether the demonstrated effects of the interaction of flufenamic acid and calcium on histamine release from mast cells are common to other NSAID or of importance in the mode of action of NSAID requires further study.

Acknowledgements—R. O. Day is supported by the National Health and Medical Research Council of Australia. The authors would like to thank Miss L. Dennis for secretarial assistance.

University of New South Wales, Department of Clinical Pharmacology, St. Vincent's Hospital, Sydney, N.S.W. 2010, Australia

RICHARD O. DAY
DENIS N. WADE

REFERENCES

1. H. Yamasaki and K. Saeki, *Archs. int. Pharmacodyn. Thér.* **168**, 166 (1967).
2. S. Norn, *Acta pharmac. tox.* **30** (suppl. 1), (1971).
3. B. Diamant and B. Uvnäs, *Acta physiol. scand.* **53**, 316 (1961).
4. B. Uvnäs, *Ann. N.Y. Acad. Sci.* **103**, 278 (1963).
5. G. D. Champion, R. O. Day, J. E. Ray and D. N. Wade, *Br. J. Pharmac.* **59**, 29 (1977).
6. J. C. Foreman and J. L. Mongar, *J. Physiol., Lond.* **224**, 753 (1972).
7. K. Saeki, *Jap. J. Pharmac.* **14**, 375 (1964).
8. T. Kanno, D. E. Cochrane and W. W. Douglas, *Can. J. Physiol. Pharmac.* **51**, 1001 (1973).
9. J. C. Foreman, J. L. Mongar and B. D. Gomperts, *Nature, Lond.* **245**, 249 (1973).
10. D. E. Cochrane and W. W. Douglas, *Proc. natn. Acad. Sci. U.S.A.* **71**, 408 (1974).
11. B. Uvnäs and I. L. Thon, *Expl Cell. Res.* **23**, 45 (1961).
12. W. W. Douglas and Y. Ueda, *J. Physiol., Lond.* **234**, 97P (1973).
13. R. P. Rubin, *Pharmac. Rev.* **22**, 389 (1970).
14. J. C. Foreman, M. B. Hallet and J. L. Mongar, *Br. J. Pharmac.* **55**, 283P (1975).
15. R. Dalquist, *Acta pharmac. tox.* **35**, 1 (1974).
16. B. Diamant and S. A. Patkar, *Int. Archs Allergy appl. Immun.* **49**, 183 (1975).
17. W. Hasselbach and M. Makinose, in *Role of Membranes in Secretory Processes* (Eds. L. Bolis, R. O. Keynes and W. Wilbrandt), pp. 158–69. North-Holland, Amsterdam (1972).
18. M. Klingenberg, in *Essays in Biochemistry* (Eds. P. N. Campbell and F. Dickens), Vol. 6, pp. 119–59. Academic Press, London (1970).
19. S. G. Schultz and P. F. Curran, *Physiol. Rev.* **50**, 637 (1970).
20. A. Johnson and N. Moran, *Proc. Soc. exp. Biol. Med.* **123**, 886 (1966).
21. S. Norn, *Acta pharmac. tox.* **26**, 373 (1968).
22. W. T. Prince, H. Rasmussen and M. J. Berridge, *Biochim. biophys. Acta* **329**, 98 (1973).
23. P. A. Shore, A. Burkhalter and V. H. Cohn, *J. Pharmac. exp. Ther.* **127**, 182 (1959).
24. T. Johansen and N. Chakravarty, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **275**, 457 (1972).
25. T. Johansen and N. Chakravarty, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **288**, 243 (1975).
26. J. L. Barker and H. Levitan, *Biochim. biophys. Acta* **274**, 638 (1972).
27. H. Levitan and J. L. Barker, *Science, N.Y.* **178**, 63 (1972).
28. B. J. Northover, *Br. J. Pharmac.* **48**, 496 (1973).