lowing internalization of the beads. The significance of our observation, that ATP bound to agarose can promote a contractile response in the guinea-pig vas deferens that is blocked by $ANAPP_3$, is several-fold. First, it strengthens the concept that in this tissue there exists cell surface receptors for ATP and that occupation of these receptors promotes smooth muscle contraction. To this extent it implies that the ATP-mediated contraction of the guinea-pig vas deferens adheres, in part, to the pattern well-documented for other smooth muscle agonists. Second, it provides evidence that the blockade of AGATP-4-induced responses, and ATP-induced responses as well, by $ANAPP_3$ occurs at a receptor level. Since ATP will protect against the blockade [1], the effect of $ANAPP_3$ is not the result of an intracellular action of this antagonist. Third, it offers a methodological approach whereby the pharmacological and biochemical actions of ATP in the extracellular space of tissues can be explored free from the constraints that apply to soluble ATP.

In summary, ATP covalently linked to agarose beads via ribose, but not via $N^6$ or $C^8$, caused contractions of the vas deferens which were antagonized by $ANAPP_3$, a specific ATP antagonist. Responses of the vas deferens to adenine nucleotides were mediated, at least in part, by a cell surface $P_2$-purinergic receptor. The antagonism by $ANAPP_3$ occurred at this site.

†*Department of Pharmacology and Toxicology and*
‡*School of Pharmacy*
*West Virginia University Medical Center*
*Morgantown, WV 26506, and*
§*Physiology Section*
*NIOSH*
*Morgantown, WV 26505, U.S.A.*

RICHARD J. HEAD†
JOHN P. O'DONNELL‡
G. KURT HOGABOOM†
JEFFREY S. FEDAN†§||

|| Address all correspondence to: Jeffrey S. Fedan, Physiology Section, NIOSH, 944 Chestnut Ridge Road, Morgantown, WV 26505, U.S.A.

REFERENCES

1. G. K. Hogaboom, J. P. O'Donnell and J. S. Fedan, *Science* **208**, 1273 (1980).
2. D. P. Westfall, R. E. Stitzel and J. N. Rowe, *Eur. J. Pharmac.* **50**, 27 (1978).
3. S. J. Jeng and R. J. Guillory, *J. supramolec. Struct.* **3**, 448 (1975).
4. G. Burnstock, in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Eds. H. P. Baer and G. I. Drummond), p. 3. Raven Press, New York (1979).
5. G. Burnstock, *Nature New Biol.* **229**, 282 (1971).
6. I. H. Chaudry and A. E. Baue, *Biochim. biophys. Acta* **628**, 336 (1980).
7. H. Kostron, H. Winkler, L. J. Peer and P. König, *Neuroscience* **2**, 159 (1977).
8. J. Christie and D. G. Satchell, *Br. J. Pharmac.* **70**, 512 (1980).
9. S. Cockcroft and B. D. Gomperts, *Biochem. J.* **188**, 798 (1980).
10. J. P. Bennett, S. Cockcroft and B. D. Gomperts, *J. Physiol., Lond.* **317**, 335 (1981).
11. R. Dahlquist, *Acta pharmac. tox.* **35**, 368 (1974).
12. J. C. Parker, V. Castranova and J. M. Goldinger, *J. gen. Physiol.* **69**, 417 (1977).
13. J. P. O'Donnell, G. K. Hogaboom and J. S. Fedan, *Eur. J. Pharmac.* **73**, 261 (1981).
14. J. S. Fedan, D. P. Westfall and W. W. Fleming, *J. Pharmac. exp. Ther.* **207**, 356 (1978).
15. W. T. Gerthoffer, J. S. Fedan, D. P. Westfall, K. Goto and W. W. Fleming, *J. Pharmac. exp. Ther.* **207**, 356 (1978).
16. J. S. Fedan, G. K. Hogaboom, J. P. O'Donnell, J. Colby and D. P. Westfall, *Eur. J. Pharmac.* **69**, 41 (1981).
17. D. P. Westfall, J. Colby, J. S. Fedan, G. K. Hogaboom and J. P. O'Donnell, *Fedn Proc.* **40**, 709 (1981).
18. B. Levitt, R. J. Head, R. E. Stitzel, J. P. O'Donnell and D. P. Westfall, *Fedn Proc.* **41**, 1056 (1982).
19. J. S. Fedan, G. K. Hogaboom, D. P. Westfall and J. P. O'Donnell, *Eur. J. Pharmac.* **81**, 193 (1982).
20. J. F. Manery and E. E. Dryden, in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Eds. H. P. Baer and G. I. Drummond). Raven Press, New York (1979).
21. N. C. R. Merrillees, *J. Cell Biol.* **37**, 794 (1978).

*Biochemical Pharmacology*, Vol. 32, No. 3, pp. 565–567, 1983.
Printed in Great Britain.

0006–2952/83/030565–03 $03.00/0


## Inhibition of normal and leukemic lymphocyte proliferation by compound 48/80*

(*Received* 15 *March* 1982; *accepted* 28 *July* 1982)

Polycations of various types have proven to be extremely useful pharmacological and biochemical agents whose full potential is still being evaluated. Basic polyamino acids promote the leakage of small molecules from cells [1, 2], cause the degranulation of mast cells [3], serve as effective drug carriers into cells [4–6], and can both stimulate and inhibit the mitogen stimulation of lymphocytes [7, 8]. Increasing the molecular weight (and the degree of polymerization) results in increased toxicity [9] and leakage of small molecules [1, 2]. Usually, significant activity is not observed until molecular weights greater than 10,000 have been achieved; lower molecular weights are usually inactive.

Considerable research has also been done uitilizing the low molecular weight polycation, compound 48/80. This

* Supported by NIH Biomedical Research Support Grant RR05588, the Office of Naval Research, and the Poncin Scholarship Fund.

agent, originally synthesized as one of a series of hypotensive compounds [10], and its analogs [11] have been shown to exert their effects in part by histamine release from mast cells [11, 12]. In fact, compound 48/80 has become the standard compound to use in studying the degranulation of mast cells. Compound 48/80 is composed of oligomers of *p*-methoxyphenethylmethylamine, with the hexameric to octameric forms being the most active [13, 14]. In this communication, we have examined the effect of compound 48/80 on another aspect of the immune response, the proliferation of lymphocytes stimulated by alloantigens and, in addition, the proliferation of leukemic lymphocytes. The effect of compound 48/80 is compared with polycations of similar or higher molecular weight.

*Methods*

The mixed lymphocyte culture (MLC) was performed exactly as described [15, 16]. The murine leukemia cell line, L1210, was obtained from the American Type Culture Collection. These cells were maintained in Eagle's Minimum Essential Medium (MEM) containing 10% heat-inactivated horse serum. Stock cultures were maintained by dilution of the L1210 cells to 40,000 cells/ml in fresh medium and subcultured when the density reached 600,000–800,000 cells/ml. The proliferation assay of L1210 cells was performed by placing 10,000 L1210 cells in each well of 96-well microtiter plates. Incubations were continued for 48 hr. The L1210 cells were labeled and harvested as were the MLCs [15, 16]. The average [$^3$H]thymidine incorporated in control cultures was 30,000 cpm/well for the MLC and 40,000 cpm/well for the L1210 cells. The standard deviation for replicate samples was usually within 10% of the measured value.

Gel filtration of compound 48/80 was performed on a 0.9 × 45 cm column of Sephadex G-25 equilibrated and eluted with 0.03 N acetic acid titrated to pH 3.0 with HCl as described [13]. Fractions of 2.0 ml were collected, lyophilized, and redissolved in 20 mM acetic acid for assay in the MLC and with L1210 cells.

Polycations were obtained from the following sources: poly-L-lysine of average 4,000, 12,000 and 30,000 molecular weights and compound 48/80 were from the Sigma Chemical Co., St. Louis, MO,; hexadimethrine (Polybrene) of average molecular weight 6,000 was from the Aldrich Chemical Co. Milwaukee, WI; spermine-derived polycations, fraction III and fraction IV, were synthesized and purified as described [16].

*Results and discussion*

Two general classes of polycation have been tested for their abilities to inhibit the proliferation of normal murine lymphocytes stimulated by alloantigens (MLC) and leukemic lymphocytes (L1210). These were the high molecular weight polylysines and hexadimethrine and the low molecular weight compound 48/80 and spermine-derived polycations, Fxn III and Fxn IV. The results of these comparisons are shown in Table 1, which lists the $IC_{50}$ in both μg/ml and estimated μM concentration. The molecular weight estimates were either from the averages supplied by the manufacturers and literature values or from elution from standardized G-25 columns [16]. It is important to note that, on a molar basis, the low molecular weight polycations were similar in activity to the 12,000 mol. wt polylysine or the 5,000 to 7,000 hexadimethrine. This is contrary to the inhibition shown by the poly-L-lysine whose inhibitory activity increased with increasing molecular weight. The 4,000 mol. wt polylysine was active only at extremely high concentrations. All the polycations were equally active in inhibiting both the MLC and L1210 cells, with the exception of hexadimethrine which showed an approximately 8-fold difference in the $IC_{50}$ for MLC as compared to L1210 cells.

It has been shown that the most active forms of compound 48/80 are minor components of the mixture and correspond in molecular weight from hexamers to octamers [13]. We have separated compound 48/80 on Sephadex G-25 columns and found similar results. The most active fractions in inhibiting the MLC (Fig. 1) were found to migrate ahead of the major absorbance peak. Similar results were obtained using the L1210 cells. The most active fraction had an $IC_{50}$ in the MLC of 1.5 μg/ml. If we assume an average molecular weight of 1,000–2,000, this is in the range of 1–2 μM, or as active as 12,000 mol. wt poly-L-lysine. These results suggest that the polycationic nature, while necessary, is not the only criterion for growth inhibition and that a proper molecular size, as evidenced by compound 48/80, may also contribute. Higher molecular weight species of compound 48/80, which elute ahead of the activity peak, were less active, suggesting that the activity does not continue to increase with increasing polymerization in the case of compound 48/80. The spermine-derived polycations, Fxn III and Fxn IV, have been characterized as probable dimers and trimers of the polyamine spermine [16] and elute from Sephadex G-25 columns in the same areas as the most active species of compound 48/80. These low molecular weight polycations had activities similar to that of compound 48/80 in this system. These polycations may also have a proper enough size to be more potent inhibitors than their charge alone would suggest.

Relative to this argument is the recent finding that com-

Table 1. Inhibition of the MLC and of L1210 cells by various polycations*

| Compound | Average mol. wt | $IC_{50}$ (μg/ml) MLC | L1210 |
|---|---|---|---|
| Poly-L-lysine | 30,000 | 5.0 ± 2.2 (0.2) | 5.8 ± 2.4 (0.2) |
| | 12,000 | 25.3 ± 6.3 (2.0) | 18.9 ± 8.2 (1.6) |
| | 4,000 | 300 ± 90 (72) | 190 ± 100 (48) |
| Hexadimethrine | 6,000 | 79 ± 2 (13) | 11.6 ± 4.4 (1.9) |
| Compound 48/80 | 1,000 | 11.8 ± 3.4 (12) | 14.8 ± 0.8 (15) |
| | 1,500, G-25 Fxn | 1.5 ± 0.2 (1) | NT† |
| Spermine-derived | | | |
| Fxn III | 500 | 13.3 ± 1.3 (16) | 7.5 ± 1.4 (9) |
| Fxn IV | 700 | 4.2 ± 0.7 (4) | 3.8 ± 0.9 (3) |

* The 50% inhibitory concentration ($IC_{50}$) was calculated from concentration curves obtained by [$^3$H]thymidine incorporation assays. The data shown are the averages ± S.D. of three to six separate assays of each polycation. The numbers in parentheses are the micromolar (μM) concentrations of polycation calculated from the molecular weight of the salt of the polycation obtained from the data of the manufacturer or from G-25 elution data. The assay methods and cpm incorporated in control cultures are described in Methods.

† Not tested.

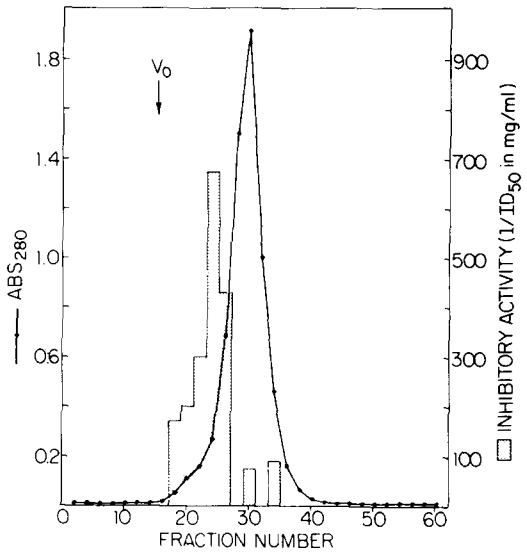


Fig. 1. Sephadex G-25 elution profile of compound 48/80. A 20-mg sample of compound 48/80 (Sigma Lot 20F-0396) was applied, and fractions of 2 ml were collected. The fractions were lyophilized and redissolved in 20 mM acetic acid for biological assay. The bars represent the relative inhibitory activities, $1/IC_{50}$ expressed in mg/ml, calculated from concentration curves obtained in the MLC assay.

pound 48/80 and somatostatin, a peptide hormone, while differing greatly in cationic charge, have similar molecular shapes and mast cell degranulating activity [17]. The concept is supported by calculations of the $IC_{50}$ based on concentration of amine groups (used here as a measure of relative cationic nature) which indicate that 12,000 molecular weight poly-L-lysine requires about 120 $\mu$M amine. The most active G-25 fraction of compound 48/80 requires an estimated 6–10 $\mu$M amine, or less than one-tenth that of 12,000 molecular weight poly-L-lysine. Thus, it would seem that the growth inhibition exhibited by the low molecular weight polycations is not simply due to binding up of cell surface negative charges. These polycations may have a shape similar enough to polypeptides such that they can fit into receptor sites, inhibit enzymes such as transglutaminase, or cause other membrane or enzyme perturbations [3]. Further experiments will be necessary to elaborate the exact mechanism and other variables of this inhibition.

In summary, compound 48/80 and other low molecular weight polycations have been found to be potent inhibitors of normal and leukemic lymphocyte proliferation. On a molar basis these polycations were as active as poly-L-lysine or hexadimethrine, polycations many times larger. These results suggest that certain low molecular weight polycations have a molecular shape/size which makes them more potent inhibitors of proliferation than their degree of cationicity would indicate. Such low molecular weight polycations may provide a route to new antimitotic or immunosuppressive drugs.

*Acknowledgement*—We thank Ms. Janelle Taylor-Norton for excellent technical assistance.

*Virginia Mason Research Center*
*Seattle, WA 98101, U.S.A.*

LEONARD M. PATT*
JOHN C. HOUCK

* Address all correspondence to: Dr. Leonard M. Patt, Virginia Mason Research Center, 1000 Seneca St., Seattle, WA 98101, U.S.A.

REFERENCES

1. E. Mayhew, J. P. Harlos and R. L. Juliano, *J. memb. Biol.* **14**, 213 (1973).
2. S. E. Kornguth and M. A. Stahmann, *Cancer Res.* **21**, 907 (1961).
3. T. C. Theoharides, *Life Sci.* **27**, 703 (1980).
4. L. J. Arnold, A. Dagan, S. D. Simon and N. O. Kaplan, *Proc. Am. Ass. Cancer Res.* **19**, 114 (1978).
5. H. J. P. Ryser and W. C. Shen, *Proc. natn. Acad. Sci. U.S.A.* **75**, 3867 (1978).
6. W. C. Shen and H. J. P. Ryser, *Proc. natn. Acad. Sci. U.S.A.* **78**, 7589 (1981).
7. A. Novogradsky, *Nature, Lond.* **250**, 788 (1974).
8. B. Larsen and I. Heron, *Experientia* **34**, 1224 (1978).
9. L. J. Arnold, A. Dagan, J. Gutheil and N. O. Kaplan, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2346 (1979).
10. R. Baltzly, J. S. Buck, E. J. DeBeer and F. J. Webb, *J. Am. chem. Soc.* **71**, 1301 (1949).
11. E. F. Kiefer and J. F. Weber, *J. med. Chem.* **16**, 1292 (1973).
12. W. D. M. Paton, *Br. J. Pharmac.* **6**, 499 (1951).
13. G. W. Read and J. F. Lenney, *J. med. Chem.* **15**, 320 (1972).
14. M. J. Ortner, R. H. Sik, C. F. Chignell and E. A. Sokoloski, *Molec. Pharmac.* **15**, 179 (1979).
15. L. M. Patt, J. M. Gleisner, D. M. Barrantes and J. C. Houck, *Pharmacology* **23**, 117 (1981).
16. L. M. Patt, D. M. Barrantes and J. C. Houck, *Biochem. Pharmac.* **31**, 2353 (1982).
17. T. C. Theoharides and W. W. Douglas, *Eur. J. Pharmac.* **73**, 131 (1981).

*Biochemical Pharmacology*, Vol. 32, No. 3, pp. 567–570, 1983.
Printed in Great Britain.

0006-2952/83/030567-04 $03.00/0


## Further evidence that vascular serotonin receptors are of the $5HT_2$ type

(*Received* 27 *March* 1982; *accepted* 5 *August* 1982)

Serotonergic receptor mechanisms in vascular tissue have been studied extensively, but their characterization in relation to brain serotonin receptors has been attempted only recently. In brain, two subtypes of serotonin receptors