THE EFFECT OF PAPAVERINE ON THE FATE OF THYMIDINE PHOSPHATES IN ISOLATED MOUSE THYMUS CELLS

Herbert Sheppard, Wen-Hui Tsien and Suzanne Sass

Department of Cell Biology, Roche Research Center
Hoffmann-La Roche Inc., Nutley, New Jersey 07110

Received January 17,1977

SUMMARY

The burst of incorporation of ^{3}H into DNA of mouse thymocytes during an incubation at 37° for 5 min. following a preincubation at 4° for 30 min. is markedly inhibited by papaverine (0.1 mM). This event is accompanied by an efflux of ^{3}H into the medium, largely in the form of thymidine. No enhanced efflux of ^{3}H is detected when DNA synthesis is blocked by hydroxyurea (1 mM). While it is uncertain that papaverine has a separate effect on DNA synthesis, the reduced incorporation into DNA could be explained by its ability to increase the breakdown of intracellular thymidine phosphates.

INTRODUCTION

It has been demonstrated that papaverine along with several other cyclic AMP phosphodiesterase inhibitors, inhibited nucleoside transport into a variety of cell types by what appeared to be a cyclic AMP-independent mechanism (1). When these studies were carried out with ^{3}H-thymidine (^{3}H-Tdr), the inhibition of transport was reflected in a decreased incorporation into DNA but it was not clear that these agents were without effect on DNA synthesis. By using the technique of prelabeling the dTTP pool at 4° with no incorporation into DNA (2), it was found that papaverine quite readily inhibited the incorporation of thymidine phosphates into DNA and at the same time promoted the efflux of ^{3}H from isolated murine thymocytes.

MATERIALS AND METHODS

Preparation of the cells: Thymus glands were removed from decapitated 5-8 week old male C57 BL/6 mice obtained from Jackson Laboratories, Bar Harbor, Maine and placed in cold glucose-salt buffer (5.5 mM Glucose; 5.5 mM KCl; 1 mM $MgSO_4$; 5 mM Na_2HPO_4; 120 mM NaCl; 5 mM Tris; adjusted to

ISSN 0006-291X

pH 7.2 with HCl). This buffer was used for all manipulations. The tissues were rinsed several times, trimmed, minced with scissors and pressed through a 200 mesh stainless steel sieve (W.S. Tyler, Inc., Mentor, Ohio) using a polyethylene spatula. The cell suspension was centrifuged unrefrigerated at 600 g in 5 min. The cell pellet was washed twice, filtered through a nylon cloth and diluted to approximately 2 x 10^7 cells per ml. Cell counts were performed with a hemocytomer (American Optical Co.) according to the procedure outlined by the manufacturer.

Labeling of cells: Anywhere from 10-40 ml of the cell suspension, were transferred to a 50 ml round bottom plastic tube and incubated in an ice bath (4°) in the presence of 4 μc/ml of (6-^{3}H) thymidine (Amersham, S.A.: 21-23 C/mole). After 30 min., the cells were centrifuged in an IEC PR-6 refrigerated centrifuge at 150 g for 5 min., washed twice with vortexing and resuspended to its original volume.

Incubations: A 0.25 ml aliquot of the cold-prelabeled cell suspension was placed in a glass culture tube (12 x 75 mm) containing 0.25 ml of cold buffer with test agents. After 5 or 15 min. of incubation at 37° (run in quadruplicate) the cells were centrifuged at 150 g for 5 min. at 5°. The medium was poured into tubes containing 0.5 ml of 0.8 N $HClO_4$ and the cells were rinsed once without resuspending, centrifuged and the supernatent combined with the previous one. This acid fixed solution was centrifuged at 900 g for 10 min. and 0.25 ml of the supernatent was added to 5 ml Aquasol (NEN Corp.) and counted in a liquid scintillation spectrometer.

The cell pellet was vortexed with one ml of 0.4 N $HClO_4$ and the suspension was allowed to stand in the refrigerator for an hour or longer. Carrier BSA (0.1 ml of 1% solution) was added and each tube was centrifuged at 900 g for 10 min. The supernatent and a subsequent wash (1 ml of 0.2 N-$HClO_4$) made up the cold acid soluble (CAS) fraction of the cells. The residue was then extracted with 2 ml of 0.6 N $HClO_4$ at 85° for 20 min., centrifuged at 900 g for 10 min. and a 0.25 ml aliquot of the extract counted as above. This represented the hot acid soluble (HAS) or DNA containing fraction.

Chromatography: The CAS extracts of medium and cells were neutralized with 2.5 M K_2CO_3, let stand for an hour in an ice bath and centrifuged to remove the $KClO_4$. These neutralized extracts were chromatographed overnight with carrier dTTP, dTDP, dTMP, Tdr and thymine (T) (Schwarz-Mann, Orangeburg, N.Y.) on 3MM Whatman filter paper using a solvent system of 1 M NH_4Ac (pH 5): absolute ethanol (3:7) in an ascending manner. The U.V. absorbing areas were cut out and counted in toluene-0.4% dibutyl-PBD.

RESULTS

After prelabeling the cells with ^{3}H-Tdr for 30 min. at 4° and washing as described, each aliquot of the cell suspension was found to contain 25,900 cpm in the cold acid soluble (CAS) fraction and only 240 cpm in the hot acid soluble (HAS) fraction containing the DNA. Of this CAS-^{3}H, 60% was found by paper chromatography to be dTTP, 39% was dTDP and 1% was dTMP. No Tdr or thymine (T) was detected. Five minutes after exposing the cells to 37° (Table 1) there was a burst of incorporation of ^{3}H into the HAS of the control cells such that 42% was associated with DNA. This value in-

TABLE I

Effect of 0.1 mM papaverine (P) and 1 mM hydroxyurea (HU) on the fate at 37° of mouse thymus cell-^{3}H accumulated from ^{3}H-thymidine during a prelabeling period at 4° for 30'

	10^3 CPM/incubation					
	5'			15'		
FRACTION	C	P	HU	C	P	HU
MEDIUM	4.52	11.85***	5.46	5.23	18.18***	5.89
CAS[1]	9.08	11.08*	14.58***	6.88	2.55***	12.28***
HAS[2]	9.76	3.40***	3.61***	10.92	5.28***	5.86***

[1]CAS refers to the cold acid soluble fraction of the cells

[2]HAS refers to the hot acid soluble fraction of the cells

Statistically significant differences from control values are designated by p values of < 0.001 (***) and < 0.05 (*).

creased only slightly over the next ten minutes. There was a surprisingly large fraction of the total ^{3}H found in the medium. This did not change significantly with time and subsequent work has demonstrated that most if not all was liberated during the vortexing associated with the washing of the prelabeled cells and was already extracellular at the start of the 37° incubation. This phenomenon resembles the reported loss of intracellular ^{3}H when mouse L929 cells were scraped from a plate (3).

The samples incubated with papaverine for 5 min., showed a 65% inhibition of incorporation into the DNA fraction, a 2.6 fold increase in the medium-^{3}H and a slightly but statistically significant elevation of CAS-^{3}H. After 15 min. of incubation, however, the CAS-^{3}H was reduced well below the control values, with more ^{3}H appearing in the medium and slightly more in DNA.

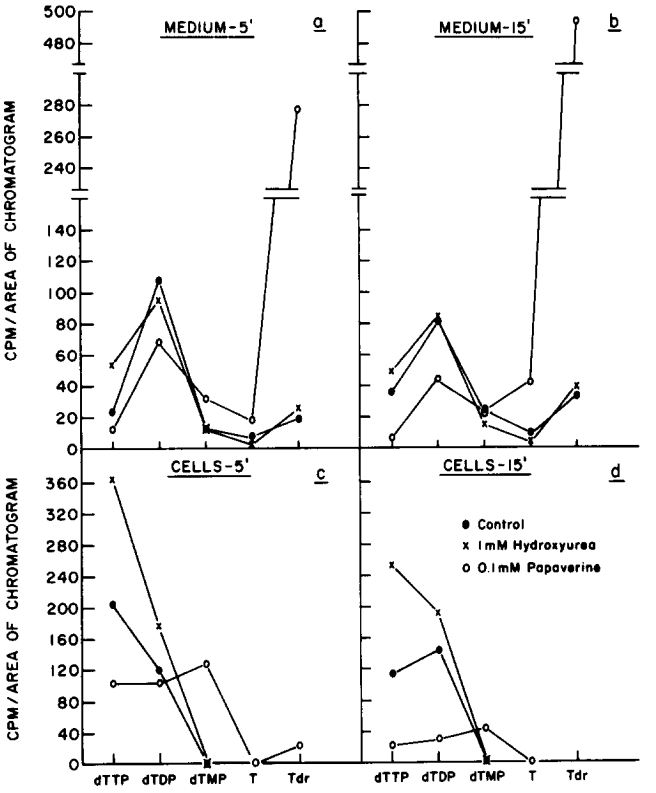

Figure 1. Distribution of ^{3}H among thymine (T), thymidine (Tdr) and thymidine mono-, di- and triphosphates (dTMP, dTDP and dTTP) contained in cold acid extracts of medium (a & b) and cells (c & d). These substances were separated by ascending paper chromatography with carriers using 1M NH_4Ac (pH 5): absolute ethanol (3:7) as the solvent.

Hydroxyurea (HU) similarly inhibited the incorporation of ^{3}H into DNA but did not produce an increase in the ^{3}H content of the medium. Rather, all of the ^{3}H not incorporated into the DNA could be found in the CAS fraction of the cells. As with papaverine, some further incorporation into DNA occurred over the next 10 min. at 37°.

It was important to determine the nature of the ^{3}H extruded from the papaverine treated cells and accumulated in the CAS of the HU treated cells. The control medium after 5 min. of incubation (Fig. 1a) contained mostly dTDP

and dTTP with the amounts of ^{3}H in dTDP > dTTP > dTMP ≥ Tdr > T. The pattern of the HU-medium was essentially the same as the controls and neither changed appreciably over the next ten min. (Fig. 1b). During that longer period of incubation there appeared to be a slight decrease in dTDP with concommitant increases in dTMP and dTdr. A markedly different pattern was noted with the papaverine cells in that the increased ^{3}H was accounted for mainly in the Tdr area and this increased with time of incubation. Simultaneously the dTTP and dTDP-^{3}H levels fell while dTMP-^{3}H rose. The value for thymine was uncertain since that area was probably contaminated with Tdr.

The picture with the CAS-^{3}H was somewhat different (Fig. 1c,d). The 5 min. controls contained only dTTP (63%) and dTDP (37%). The 5 min. HU pattern was essentially the same although the values were appreciably higher as would be expected from the higher CAS-^{3}H in Table 1. The 5 min. papaverine samples were quite different with the bulk of the ^{3}H distributed almost equally among the three Tdr-phosphates and a small amount of Tdr. Over the next ten minutes there occurred a marked fall in dTTP and a slight increase in TDP for the control and HU samples. In the papaverine CAS, however, the 15 min. values for Tdr and its phosphates were very much reduced.

DISCUSSION

As with chick embryo cells, intracellular Tdr-phosphate pools of mouse thymocytes can be prelabeled with ^{3}H-Tdr at 4° and used for DNA synthesis during a subsequent incubation at 37° (2). The pattern of labeling differs in that the chick embryo cells accumulate dTMP and dTTP in a ratio of 2/1 (4) while the mouse thymus cells accumulate dTTP and dTDP in a ratio of 2/1. Though this ratio in thymus cells varies from experiment to experiment, significant levels of dTMP are rarely found.

The papaverine-induced increase in medium ^{3}H-Tdr accompanied by a cellular increase in ^{3}H-dTMP and decrease in ^{3}H-dTTP suggests that the intracellular hydrolytic cleavage of the Tdr-phosphates is being stimu-

lated. The Tdr produced probably leaves the cells rapidly and accumulates in the medium because 0.1 mM papaverine is a good blocker of deoxynucleoside transport and/or phosphorylation (1,5).

It is possible that the breakdown of Tdr-phosphates increased with papaverine because of a reduced synthesis of DNA. The data obtained with HU, however, shows that inhibition of DNA synthesis comparable to that obtained with papaverine results in an intracellular accumulation rather than an enhanced breakdown of Tdr-phosphates. This finding is in agreement with studies using mouse embryo cells (6) but not mouse L929 cells (3).

The 5 min. fall in DNA synthesis was initially thought to be independent of increased breakdown of intracellular Tdr-phosphates because the CAS-^{3}H of the papaverine-treated cells was greater than that of the controls. However, chromatographic analysis demonstrated that the amount of dTTP and dTDP was less and dTMP more than in the controls and that this could have accounted for the papaverine-induced depression in DNA synthesis.

The papaverine-enhanced reduction of nucleoside tri-phosphate pools has been reported for ATP (7) and UTP (8) and the former change appeared to result from a decreased O_2 uptake. In our hands, however, the breakdown of Tdr-phosphates proceeds unaltered under N_2 (data not shown). These changes could account for the reported cytotoxicity of papaverine (9,10). The well known vasodilatory activity (11) of papaverine is thought to result from its inhibition of cyclic AMP phosphodiesterase (12) but it is possible that nucleotide breakdown could play a role.

It would seem, therefore, that enhancement of the breakdown of nucleoside phosphates may be a general response to papaverine. Whether this agent has an effect on DNA synthesis separate from its effects on deoxyribonucleotide pools remains to be demonstrated. In this regard it should be noted that efforts to demonstrate such an action on DNA polymerases and isolated nuclei have proved unsuccessful (13).

REFERENCES

1. Sheppard, J.R. and Plagemann, P.G.W. (1975) J. Cell. Physiol. 85, 163-172.
2. Scholtissek, C. (1968) Biochim. Biophys. Acta 158, 435-447.
3. Adams, R.L.P., Berryman, S. and Thomson, A. (1971) Biochim. Biophys. Acta 240, 455-462.
4. Scholtissek, C. (1967) Biochim. Biophys. Acta 145, 228-237.
5. Sheppard, H., Burghardt, C.R. and Tsien, W.H. (1976) 25th Annual Conference of the Rutgers Bureau of Biological Research. (In Press).
6. Skoog, L. and Nordenskjold, B. (1971) Eur. J. Biochem. 19, 81-89.
7. Browning, E.T., Groppi, V.E. Jr. and Kon, C. (1974) Mol. Pharmacol. 10, 175-181.
8. Glazer, R.I. and Schneider, F.H. (1975) J. Biol. Chem. 250, 2745-2749.
9. Prasad, K.N. (1975) Biol. Rev. 50, 129-265.
10. Plagemann, P.G.W. and Richey, D.P. (1974) Biochim. Biophys. Acta 344, 263-305.
11. The pharmacological Basis of Therapeutics, 4th Ed. Ed. L.S. Goodman and A. Gilman (1970), pp. 753-754.
12. Pöch, G., Juan, H. and Kukovetz, W.R. (1969) Naunyn-Schmiedebergs Arch. Pharmakol. 264, 293-294.
13. Weissbach, A. Personal Communication.