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Laboratory Report

Halothane Action on Lymphocytes Does Not Involve Cyclic AMP

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Both theophylline and halothane inhibited transformation of human lymphocytes by phytohemagglutinin (PHA). Theophylline did not augment the inhibitory action of halothane and depressed PHA transformation of halothane-treated cells to the same extent as that of air-treated cells. Halothane- and air-treated lymphocytes, prior to PHA addition, had the same content of cyclic AMP. The addition of PHA to these cultures raised cyclic-AMP concentrations to the same extent in halothane- and air-treated lymphocytes. Halothane action on PHA-stimulated lymphocytes appears not to involve changes in cyclic nucleotide metabolism. (Key words: Anesthetics, volatile, halothane; Blood, lymphocytes; immune response, phytohemagglutinin; Metabolism, cyclic adenosine monophosphate.)

HALOTHANE decreases the responsiveness of human lymphocytes to the mitogen, phytohemagglutinin (PHA).[1,2] As judged by changes in RNA and protein synthesis, this anesthetic effect was significant 16 hours after PHA was added to the cultures,[3] and nuclear volume increases were significantly inhibited 24 hours post-PHA in the presence of 2 per cent halothane.[4] In searching for a mechanism by which halothane caused these effects, we have questioned whether changes in intracellular 3′,5′-adenosine monophosphate (cyclic AMP; cAMP) might be caused by the PHA and/or the halothane, so that one agent interfered with the action of the other. Such an interaction of anesthetics and cyclic nucleotides would have practical implications,[5] so we report that these studies in lymphocytes failed to reveal a relationship of halothane action to cAMP.

Materials and Methods

Methods for preparation of lymphocyte cultures, stimulation with PHA, exposure to halothane, and harvest of cultures have been described.[3,4] Culture volumes were 3 ml, containing 10^6 lymphocytes, for the theophylline experiments, or 20 to 24 ml, containing 20 to 24 × 10^6 lymphocytes, for the cAMP assays. Replicate cultures of 10^6 lymphocytes were equilibrated for two hours in either air–5 per cent CO_2 or air–halothane–5 per cent CO_2 at 37 C. CO_2 and halothane concentrations in the incubators were verified gas-chromatographically. After this two-hour period, PHA-P (Difco) was added; theophylline was added either at that time or later, depending upon the experiment. Theophylline (1,3 dimethylxanthine, Sigma) was dissolved in MEM and this solution was sterilized by Millipore filtration.

At 71 hours post-PHA, tritiated thymidine (^{3}H-Tdr, Amersham-Searle, sp. act. 150 mC/mM, 0.5 μCi) was added to each culture and the cells harvested and acid-washed one hour later. Radioactivity in the acid-insoluble precipitate from 10^6 cells was counted in a Packard Model 3385 Tri-Carb liquid scintillation spectrometer and counts converted to dpm. For each experiment, five to ten replicate cultures, prepared from the same blood sample, were exposed to the control condition and to the variable conditions being studied. Data were normalized to 100 per cent dpm for the controls, and percentage changes from controls were calculated and compared statistically by the t test for non-paired data.

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TABLE 1. Percentage Decreases in ^{3}H-Tdr Uptake*

	Halothane Per Cent in Air	Theophylline, mM in Culture				
		0	0.06	0.11	0.22	0.33
Donor 1	None	0	30	29	44	58
Donor 2	None	0	22	28	38	37
Donor 3	None	0	7	9	35	39
Mean ± SE		0	19.7 ± 6.8	22.0 ± 6.5	39.0 ± 2.6	44.7 ± 6.7
Donor 4	0.6	0	23	35	51	51
Donor 5	1.0	0	34	55	56	38
Donor 6	1.5	0	17	17	31	40
Mean ± SE		0	24.7 ± 5.0	35.7 ± 11.0	46.0 ± 7.6	43.0 ± 4.0
P, halothane *vs.* air		—	N.S.	N.S.	N.S.	N.S.

* Results from six experiments in which lymphocytes prepared from a single donation were divided into five cultures of 10^6 lymphocytes each for the five concentrations of theophylline. The end point measured was incorporation of ^{3}H-Tdr into acid-insoluble material 72 hours post-PHA addition. In each experiment, the counts obtained in the absence of theophylline were used as the reference point for evaluating depression of the reaction by theophylline. These baseline counts were 10 to 32 per cent lower for the halothane-exposed cultures than for those in air alone. Cultures were equilibrated in their appropriate gas conditions for two hours, then PHA added, then theophylline added one hour after the PHA.

Measurements of cAMP were done by the radioimmunoassay method available in kit form from Schwarz/Mann. The principle of the procedure is that a limiting amount of antibody to cAMP is mixed with a known amount of ^{125}I-cAMP and a sample containing an unknown amount of unlabeled cAMP. These two sources of cAMP compete for binding sites on the antibody so that an increase in cAMP present in the unknown sample will cause a decrease in the formation of labeled antigen–antibody complex. The radioactivity of this complex is counted by gamma scintillation spectrometry and counts from unknown samples compared with those of a standard curve prepared for each experiment by measuring cAMP standard solutions serially diluted. Due to wide disparities in data from different donors, the numbers were normalized from picomoles cAMP/10^6 lymphocytes to percentage changes from counts obtained from 10^6 cells prior to PHA addition.

Results

The first experiments undertaken were to determine a dose–response relationship of theophylline to ^{3}H-Tdr uptake following PHA stimulation. Theophylline was added one hour after PHA. In all cases, the transformation of these cells, as judged by the end point of nucleoprotein synthesis, was depressed. The results are shown in table 1. All doses of theophylline, from none to 0.33 mM in culture, were added to five replicate cultures from a donor's cells obtained on that day. Thus, comparisons were made with the no-theophylline condition, both for air-treated and for halothane-treated cells. Such comparisons in halothane-exposed cultures are on the basis of 100 per cent response in the no-theophylline condition for those cells, even though the anesthetic by itself lowered the counts by 10 to 33 per cent from those of cells in air only.

A dose of 0.11 mM theophylline, midway in the dose–response curve, was selected for further studies of the timing of additions of PHA and theophylline. Table 2 summarizes studies done in cultures exposed only to air, and shows a significant inhibitory action of theophylline when added 1, 1½, or 2 hours after PHA, but not when added before or after these times. Further experiments were then done to investigate interaction of halothane with theophylline, and these are summarized in table 3. No significant effect of

theophylline, over that of halothane alone, was demonstrated.

Finally, cAMP was measured in cultured lymphocytes exposed to air or 1 per cent halothane, then dosed with PHA and, ½, 1 or 2 hours later, harvested for this assay. There was no difference, before PHA addition, between cAMP levels in air- and halothane-exposed cells. Table 4 summarizes experiments in which, after PHA, cAMP levels increased, but not differently from air- to anesthetic-treated cultures. As may be seen from the high error terms, there was considerable variability in values obtained from cells of different donors.

Discussion

These results are essentially negative, but this is, in itself, significant. There has recently been a great deal of interest in the "second messenger" role of cAMP in mediating intracellular reactions following signals from hormones, the "first" messengers. Since actions of catecholamines appear to be exerted through cAMP,[6] it has occurred to pharmacologists that other drugs may act by this mechanism. Potentiations of catecholamine effects by halothane[7,8] and cyclopropane[9] have been studied recently, from this point of view. In uterus and vascular smooth muscle used in those studies, it did appear that these anesthetics altered cAMP metabolism. We had hoped that the PHA-stimulated lymphocyte might offer a simple cellular system in which the action of halothane on cyclic nucleotide metabolism might be studied in detail.

In simple terms, the cAMP reaction occurs in the following sequence: the messenger hormone contacts the cell membrane, activating membrane-bound adenylate cyclase; this enzyme has an intracellularly oriented "catalytic subunit" that converts ATP to cAMP; cAMP causes intracellular "kinases" to assume an active form and cause effects that depend on the nature of the kinase (*e.g.*, glycogenolysis, lipolysis, contractile or permeability changes); cAMP is inactivated by intracellular cyclic nucleotide phosphodiesterases, which are inhibited by methylxanthines such as caffeine and theophylline.[10] Thus, cAMP can be increased intracellularly either by stimulating adenylate cyclase to form more cAMP or by inhibiting phosphodiesterase to prevent its breakdown.

We used theophylline as a phosphodiesterase inhibitor, as did Hirschhorn *et al.*, who reported that 0.1 mM theophylline caused 18.1 per cent inhibition of subsequent ^{14}C-Tdr incorporation.[11] They concluded that cAMP was inhibitory to PHA-induced lymphocyte transformation. They also found that cAMP, in the absence of PHA, caused a biphasic response, stimulating ^{14}C-Tdr uptake at low, and inhibiting it at high, concentrations. Smith and co-workers[12] obtained similar results. On the other hand, Novogrodsky and Katchalski found that PHA did not increase cAMP in rat lymph node lymphocytes and concluded that cAMP did not mediate action

TABLE 2. Effect of Timing of Theophylline Addition to Air-treated Cultures*

Hours, Post-PHA, to Theophylline Addition	Per Cent Decrease in ^{3}H-Tdr Uptake Mean ± SE	Significance of Decrease
0	0	N.S.
0	7 ± 3	N.S.
1	20 ± 4	$P < .01$
1½	15 ± 4	$P < .01$
2	30 ± 3	$P < .01$
4	7 ± 4	N.S.
18	0	N.S.

* Effect on ^{3}H-Tdr uptake of timing of theophylline addition to bring culture concentration to 0.11 mM. Ten cultures with, and ten without, theophylline were prepared from the same blood donation for each of the times indicated.

TABLE 3. Effects of Halothane and Theophylline on ^{3}H-Tdr Uptake of PHA-treated Cultures*

Hours, Post-PHA, to Theophylline Addition	Per Cent Decrease in ^{3}H-Tdr Uptake, Compared with Air-treated Controls	
	1 Per Cent Halothane Mean ± SE	1 Per Cent Halothane + Theophylline Mean ± SE
1	31 ± 3	33 ± 4
1½	20 ± 3	20 ± 2
2	22 ± 2	28 ± 4
4	29 ± 3	31 ± 3

* Cultures equilibrated two hours with 1 per cent halothane–air–5 per cent CO_2, or air–5 per cent CO_2. For each experiment, ten replicate cultures from the same donor were compared after PHA addition with cells in air, halothane, or 0.11 mM theophylline plus halothane.

TABLE 4. Mean Percentage Increases of cAMP in PHA-treated Lymphocytes*

	Time, Post-PHA, Hours			
	0	½	1	2
Air	0	74 ± 58	54 ± 33	11 ± 6
1 per cent halothane	0	79 ± 24	50 ± 15	45 ± 31
P, air vs. halothane		N.S.	N.S.	N.S.

* Measured increases in values of cAMP in 10^6 lymphocytes at indicated times after addition of PHA to cultures. Cultures were equilibrated in air–5 per cent CO_2 or 1 per cent halothane–air–5 per cent CO_2 for two hours before addition of PHA. Values of PHA were measured in triplicate samples of cells prepared from seven donors. Baseline values, before PHA was added, did not differ significantly between air- and halothane-equilibrated cultures.

of PHA,[13] a conclusion also reached recently by Glasgow *et al.*[14] in studies of human lymphocytes.

Our findings suggest that PHA does elevate cAMP in human lymphocytes but that addition of the phosphodiesterase inhibitor, theophylline, antagonizes the transformation of cells by PHA rather than augmenting it. The extent of elevation of cAMP by PHA may have been in a stimulatory range, which was raised to inhibitory concentrations by the theophylline, or theophylline may have acted in some way unrelated to cAMP levels in depressing transformation. We decided not to study this further, since it seemed that the suppression of lymphocyte transformation by halothane was unrelated to factors involved in cAMP metabolism. We conclude that the action of this anesthetic on these cells is not via an alteration in cAMP.

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