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THE EFFECT OF DIAMIDE ON CYCLIC AMP LEVELS AND CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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Summary

The effect of diamide (diazene dicarboxylic acid bis[*N*,*N*'-dimethylamide) on cyclic AMP levels and cyclic nucleotide phosphodiesterase in human peripheral blood lymphocytes was examined. In the absence of mitogenic lectins, $5 \cdot 10^{-3}$—$1 \cdot 10^{-4}$ M diamide markedly increased intracellular cyclic AMP with variable effects at higher levels. In the presence of phytohemagglutinin or concanavalin A, $5 \cdot 10^{-4}$ M or higher diamide concentrations consistently decreased cyclic AMP levels, usually to control levels or below, while $1 \cdot 10^{-4}$—$1 \cdot 10^{-5}$ M diamide augmented the lectin-induced rise in cyclic AMP. When intact lymphocytes were incubated with diamide, phosphodiesterase activity against both cyclic AMP and cyclic GMP, assayed in homogenates of these cells, was inhibited at concentrations as low as $1 \cdot 10^{-6}$ M. In contrast, when diamide was incubated with phosphodiesterase extracted from lymphocytes there was a dual effect. At low substrate concentrations and high diamide concentrations diamide was a non-competitive inhibitor of phosphodiesterase with a K_i of 1.3—2.5 mM for cyclic AMP and 3.3—10 mM for cyclic GMP. In contrast, at high substrate concentrations diamide was an 'uncompetitive' activator of phosphodiesterase activity for both cyclic AMP and cyclic GMP. The effects of diamide could be largely or completely blocked by glutathione or dithiothreitol, indicating that sulfhydryl reactivity was involved in diamide's action on lymphocyte phosphodiesterase activity and intracellular cyclic AMP levels.

These data demonstrate that diamide is a phosphodiesterase inhibitor both on phosphodiesterase extracted from lymphocytes and when incubated with intact lymphocytes and that diamide may increase or decrease intracellular cyclic AMP levels depending on the concentration of diamide used.

Introduction

Diamide (diazene dicarboxylic acid bis[*N*,*N*'-dimethylamide]) was originally described as a reagent for the specific oxidation of reduced glutathione [1]. However, recent evidence has demonstrated that diamide has other biochemical actions consistent with the oxidation of protein-bound free sulfhydryl groups or the formation of protein-glutathione mixed disulfides [2]. For example, in the kidney, diamide has been shown to be a specific antagonist of cyclic AMP-dependent protein kinase activity [3], and has been shown to inhibit the polymerization of tubulin monomers from sea urchin embryos into intact microtubules both in vivo [4] and in vitro [5]. In a recent study we demonstrated that diamide, in millimolar concentrations, was able to completely inhibit lectin-induced lymphocyte transformation when added coincidentally with the lectin or at any time thereafter; in contrast, $2 \cdot 10^{-4}$ M diamide was effective only when added during the first 30 min of the culture [6]. In these experiments measurement of intracellular non-protein sulfhydryl groups (largely glutathione) indicated that there was no significant change in the level of non-protein sulfhydryl groups in lymphocytes incubated with $2 \cdot 10^{-4}$ M diamide either in the presence or absence of lectin. In addition, there was no direct effect of diamide on lectin binding to lymphocytes. These results indicated that diamide had an intracellular action unrelated to the oxidation of glutathione, and suggested an action on protein sulfhydryl groups.

The ability of diamide to inhibit lymphocyte mitogenesis only during the first 30 min culture [6] suggested that the inhibition was related to the chemical alterations occurring in or near the external plasma membrane. Of the myriad of biochemical events which occur at the plasma membrane (see Ref. 7) we have suggested that activation of adenylate cyclase and subsequent elevation of intracellular cyclic AMP may play a central role. Other agents which elevate intracellular cyclic AMP by inhibition of phosphodiesterase or activation of adenylate cyclase are inhibitory [7]. Since sulfhydryl-reactive reagents have been shown to inhibit both adenylate cyclase [8] and phosphodiesterase [9] the possibility existed that diamide might act by inhibition of one or both of these enzymes. In this communication we have examined the effect of diamide on cyclic AMP levels in intact human peripheral blood lymphocytes incubated in the presence or absence of mitogenic lectins and on the effect of this reagent on cyclic nucleotide phosphodiesterase both in intact lymphocytes and on phosphodiesterase prepared from those cells. The effect of diamide on adenylate cyclase is currently under examination.

Materials and Methods

Materials. Materials and their suppliers were dextran (M_r 250.00), Ficol, cyclic AMP, cyclic GMP, 5'-nucleotidase from *Crotalus atrox* (100 U/mg) and diamide, Sigma Chemical Company (St. Louis, MO); Hypaque®, Winthrop Labs (New York, NY); erythroagglutinating phytohemagglutinin, Burroughs-Wellcome (Beckenham, U.K.); concanavalin A, Miles Yeda (Rehovot, Israel); cyclic [^{3}H]GMP (21 Ci/mmol) and cyclic [^{3}H]AMP (26 Ci/mmol), Amersham-Searle (Arlington Heights, IL); AG1X2 resin, Biorad Labs (Richmond, CA); Scinti-

verse, dithiothreitol and reduced glutathione, Fisher Scientific (Pittsburgh, PA). All reagents were dissolved at appropriate dilutions in 0.1 M NaCl. Diamide used in direct assays of lymphocyte phosphodiesterase was dissolved in 50 mM Tris-HCl, 5 mM $MgCl_2$ (pH 7.5) (buffer). The melting point of the commercially obtained diamide was 110—112°C as shown by Crawford and Rapp [10].

Preparation of lymphocytes. Human peripheral blood lymphocytes were purified from heparinized peripheral blood by dextran sedimentation and density gradient centrifugation on Ficoll/Hypaque gradients as previously described [11]. Following three centrifugations at 140 × *g* for 7 min in a Beckman-J6 centrifuge, the resulting preparation was 90—95% small lymphocytes, 5—10% monocytes and contained less than ten platelets/100 nucleated cells. Monocytes were identified following latex particle ingestion (2.02 μm polyvinyl toluene, Dow Chemical Co.) as previously described [12]. Cells containing one or more particles were scored as positive. Platelets were enumerated under phase contrast microscopy. Cell viability of the lymphocyte preparations was always greater than 90% as assayed by trypan blue dye exclusion. None of the agents tested induced more than 5% cell death during the course of incubation.

Lymphocyte incubations. Lymphocytes were suspended in Gey's buffer [13] which had been previously warmed to 37°C and bubbled with 95% O_2/5% CO_2 to bring the pH 7.35. Lymphocytes were added to tubes containing diamide and/or phytohemagglutinin or concanavalin A and incubated without shaking at 37°C in an atmosphere of 95% O_2/5% CO_2 for varying periods of time. Incubations were terminated by centrifuging the cells as described below.

Measurement of intracellular cyclic AMP. $5.0 \cdot 10^6$ (in 0.5 ml) lymphocytes, incubated as described above, were centrifuged at 2500 × *g* for 2 min in a Beckman-J6 centrifuge. The supernatant was rapidly decanted and the cell pellets were snap frozen in a solid CO_2/C_2H_5OH bath. Cyclic AMP was extracted from the cell pellets as previously described [14] and cyclic AMP was measured by the radioimmunoassay of Steiner et al. [15]. Control experiments indicated that none of the reagents used affected the radioimmunoassay for cyclic AMP.

Phosphodiesterase assay. Lymphocytes incubated as described above or unincubated were sedimented at 2100 × *g* in a Beckman-J6 centrifuge and washed once in cold 50 mM Tris-HCl/5 mM $MgCl_2$ (pH 7.5) (buffer). The lymphocytes were resuspended in cold buffer at $100 \cdot 10^6$/ml. All subsequent steps were carried out at 4°C. The suspension was disrupted by sonication (6—10-s bursts at 35% of low output) using a Biosonics III sonicator, and the homogenate was centrifuged at 16 000 × *g* for 30 min. Phosphodiesterase activity was assayed in the supernatant. Phosphodiesterase was assayed by the method of Thompson and Appleman [16] as modified by d'Armiento et al. [17]. The reaction mixture contained, in a total volume of 0.1 ml: buffer, 100 000—200 000 cpm cyclic [^{3}H]AMP and various concentrations of unlabeled cyclic AMP or 100 000—200 000 cpm cyclic [^{3}H]GMP and various concentrations of unlabeled cyclic GMP, and 10 μl of the 16 000 × *g* lymphocyte supernatant (10—30 μg/protein). The reaction was initiated at 37°C for 10 min. The initial reaction was terminated by boiling for 2 min. 10 μl of a 0.5 mg/ml solution of 5′-nucleotidase was added to each tube and the tubes were incubated for 30 min at 37°C. The second reaction was terminated by the addition of 0.5 ml of

a 1 : 3 slurry of AG1X2 resin suspended in water. Following agitation and centrifugation at 2200 × *g*, radioactivity in 0.2 ml of the supernatant was determined in 2.0 ml of Scintiverse in a Searle Mark III scintillation counter. Studies from our laboratory have demonstrated that when performed in this manner phosphodiesterase activity is linear with both time and protein concentration and provides accurate kinetic analysis of phosphodiesterase activity [18]. In control experiments less than 15% of adenosine or 10% of guanosine was nonspecifically bound to the resin and the fraction of nucleotide which bound was constant from $1 \cdot 10^{-3}$ to $1 \cdot 10^{-11}$ M. Moreover when phosphodiesterase activity is extracted from human peripheral blood lymphocytes as described here the phosphodiesterase activity measured at high cyclic AMP concentrations and the cyclic GMP phosphodiesterase activity reflect hydrolytic activity intrinsic to lymphocytes and are not the result of platelet or monocyte contamination. Protein was measured by the method of Lowry et al. [19]. Kinetic analysis was performed using the Lineweaver-Burk analysis [20] and the K_i for diamide was determined by the method of Dixon [21].

Results

Effect of diamide on intracellular cyclic AMP

In the absence of mitogenic lectins diamide at concentrations between $5 \cdot 10^{-3}$ M and $1 \cdot 10^{-4}$ M markedly increased intracellular cyclic AMP levels (Fig. 1). At concentrations above $5 \cdot 10^{-3}$ M the effect was variable. In some instances there was little effect of diamide while in other experiments there was a modest decrease in intracellular cyclic AMP levels (Compare Fig. 1, Tables I and II). Concentrations of $5 \cdot 10^{-5}$ M or below had no effect in unstimulated lymphocytes. In contrast, diamide at concentrations of $5 \cdot 10^{-4}$ M or higher consistently decreased intracellular cyclic AMP in lectin-stimulated lymphocytes.

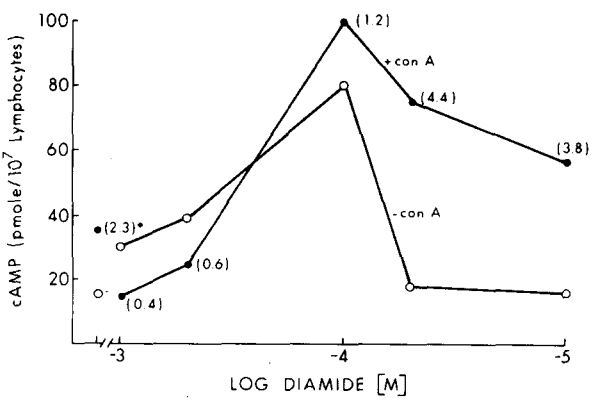

Fig. 1. Dose-response of diamide effect on intracellular cyclic AMP in control and concanavalin A-stimulated lymphocytes. Lymphocytes were incubated at $2.5 \cdot 10^6$/ml in Gey's buffer with varying concentrations of diamide in the presence (•) or absence (○) of 30 μg/ml concanavalin A (Con A) for 30 min at 37°C. The reaction was terminated by centrifugation and cyclic AMP assayed. Results are the mean of triplicate determinations performed on lymphocytes from a single donor. Similar results were seen in three other experiments. Numbers in parenthesis represent stimulation ratios (concanavalin A ± diamide/control ± diamide).

Fig. 1 shows a diamide dose-response curve in lymphocytes incubated with 30 μg/ml concanavalin A. Concanavalin A alone gave a 2.3-fold increase in intracellular cyclic AMP. $5 \cdot 10^{-4}$ M diamide decreased the level seen by 33% and $1 \cdot 10^{-3}$ M diamide by 66%. In both instances these levels were less than comparable incubations in the absence of lectin. Similar results were seen with phytohemagglutinin. Concentrations of diamide of $1 \cdot 10^{-4}$ M or less had the opposite effect — there was marked augmentation at each of the 3 concentrations tested and even at $1 \cdot 10^{-5}$ M there was a 55% increase over the concanavalin A (alone) control.

The inhibitory effect is not dependent on the time of incubation as shown in Table I. $5 \cdot 10^{-4}$ M diamide inhibited the increase in cyclic AMP in lymphocytes incubated in the presence or absence of concanavalin A for 15, 30 or 60 min. As with concanavalin A a similar effect of $5 \cdot 10^{-4}$ M diamide was seen in lymphocytes incubated with phytohemagglutinin for varying periods of time. The effect was also seen at differing concentrations of phytohemagglutinin and concanavalin A. Table II shows the results of one experiment. Concanavalin A and phytohemagglutinin both increased intracellular cyclic AMP in lymphocytes with phytohemagglutinin being the more potent stimulus in this experiment. However, $5 \cdot 10^{-4}$ M diamide was equally effective in inhibiting the rise in intracellular cyclic AMP when expressed on a percent basis.

Table II also demonstrates the effect of $1 \cdot 10^{-5}$ M diamide in phytohemagglutinin and concanavalin A-stimulated lymphocytes. There was potentiation of the increase in cyclic AMP with both lectins. The percent increase was greater for concanavalin A, the weaker stimulator, than phytohemagglutinin. The absolute increases in pmol/10^7 cells were similar: 20.8 and 19.6 for 3 and 30 μg/ml concanavalin A, and 14.4 and 33.2 for 2 and 20 μg/ml phytohemagglutinin.

Effect of diamide on phosphodiesterase in intact lymphocytes

The data presented above are consistent with inhibition of lymphocyte cyclic AMP phosphodiesterase at low concentrations which would result in elevated levels of intracellular cyclic AMP in both control and lectin-stimulated

TABLE I

Lymphocytes ($2.5 \cdot 10^6$) in 0.5 ml of Gey's buffer were incubated with or without 30 μg/ml concanavalin A in the presence or absence of $5 \cdot 10^{-4}$ M diamide for 15, 30 or 60 min at 37°C. The reaction was terminated by centrifugation and cyclic AMP was assayed. Results shown are mean ± S.E. for triplicate determinations performed on lymphocytes from a single donor. Similar results were seen in three other experiments. Numbers in parentheses are stimulation ratios (concanavalin A/control).

	Cyclic AMP (pmol/10^7 cells)		
	15 min	30 min	60 min
Control	42.2 ± 3.1	25.6 ± 0.7	16.8 ± 0.7
Diamide ($5 \cdot 10^{-4}$ M)	15.6 ± 0.3 (2.0)	13.6 ± 1.5	11.8 ± 0.3
Concanavalin A (30 μg/ml)	84.8 ± 4.7 (2.0)	57.8 ± 4.9 (2.2)	44.8 ± 3.4 (2.7)
Concanavalin A (30 μg/ml) + diamide ($5 \cdot 10^{-4}$ M)	11.2 ± 0.4	8.6 ± 0.2	8.8 ± 0.1

TABLE II

Lymphocytes ($2.5 \cdot 10^6$) in 0.5 ml Gey's buffer were incubated at 37°C for 30 min with the lectins shown in the presence or absence of $5 \cdot 10^{-4}$ M diamide or $1 \cdot 10^{-5}$ M diamide. The reaction was terminated by centrifugation and cyclic AMP was assayed. Results shown are the means ± S.E. of triplicate determinations for lymphocytes from a single donor. Similar results were seen in three other experiments. Numbers in parentheses represent the stimulation ratio (experimental/control or experimental + diamide/control + diamide).

	Control	Diamide ($5 \cdot 10^{-4}$ M)	% of control	Diamide ($1 \cdot 10^{-5}$ M)	% no diamide
Control	7.9 ± 0.1	8.6 ± 0.2	109	7.9 ± 0.2	100
Concanavalin A					
3 μg/ml	12.8 ± 0.2 (1.6)	6.9 ± 0.1 (0.8)	54	33.6 ± 3.0 (4.2)	262
30 μg/ml	17.7 ± 0.7 (2.2)	12.1 ± 1.0 (1.4)	68	37.3 ± 2.3 (4.7)	210
Phytohemagglutinin					
2 μg/ml	70.8 ± 6.6 (9.0)	44.9 ± 2.9 (5.2)	63	85.2 ± 1.3 (10.8)	120
20 μg/ml	82.0 ± 6.4 (10.4)	56.0 ± 6.1 (6.5)	68	115.2 ± 6.5 (14.6)	140

lymphocytes. To examine this question directly, lymphocytes were incubated with varying concentrations of diamide for 30 min and phosphodiesterase extracted and assayed. In lymphocytes disrupted by sonication 95% of the phosphodiesterase activity is not sedimentable at 16 000 × *g*. Phosphodiesterase activity in the 16 000 × *g* supernatant exhibits two apparent K_m values for cyclic AMP hydrolysis, an apparent low K_m of approximately 2.0 μM and an apparent high K_m of 40—75 μM and one single hydrolytic activity for cyclic GMP with an apparent K_m of 6—25 μM [18]. For this reason we examined the effect of diamide on phosphodiesterase activity in the 16 000 × *g* supernatant from sonicated lymphocytes. When the diamide dose-response curve was examined there was a slight, but significant, decrease in phosphodiesterase activity measured at 2 μM cyclic AMP and $1 \cdot 10^{-6}$ M diamide and a progressive inhibition of phosphodiesterase activity with a decrease to 42% of control levels at $1 \cdot 10^{-3}$ M (data not shown). Table III shows the effect of $1 \cdot 10^{-3}$ M

TABLE III

Lymphocytes ($20 \cdot 10^6$/ml) in Gey's buffer were incubated with $1 \cdot 10^{-3}$ M diamide, $1 \cdot 10^{-2}$ M glutathione or both for 30 min at 37°C. The cells were washed twice, resuspended in buffer and disrupted. Results shown are the mean of triplicate determinations performed on lymphocyte from a single donor.

Addition	Phosphodiesterase (pmol/min per mg)		
	Cyclic AMP ($5 \cdot 10^{-5}$ M)	Cyclic AMP ($2 \cdot 10^{-6}$ M)	Cyclic GMP ($2 \cdot 10^{-5}$ M)
0.1 M NaCl (control)	848	280	369
Diamide ($1 \cdot 10^{-3}$ M)	428	120	147
Glutathione ($1 \cdot 10^{-2}$ M)	1006	269	419
Diamide ($1 \cdot 10^{-3}$ M + glutathione ($1 \cdot 10^{-2}$ M)	1068	259	413

diamide on phosphodiesterase activity assayed at 50 μM and 2 μM cyclic AMP and 20 μM cyclic GMP. At these substrate concentrations the predominant enzyme activities being examined are those with apparent high K_m cyclic AMP, low K_m cyclic AMP and cyclic GMP, respectively. There was marked suppression of phosphodiesterase activity at each substrate concentration. Glutathione in molar excess completely blocked the effect of diamide indicating that in intact diazene moiety was necessary for diamide's action.

The diamide effect was rapid with maximum suppression seen in lymphocytes incubated with $1 \cdot 10^{-3}$ M diamide for 5 min (data not shown).

When lymphocytes were incubated with $1 \cdot 10^{-3}$ M diamide for 15 min, washed and resuspended in warm Gey's buffer, the recovery of phosphodiesterase activity differed depending on the nucleotide and concentration tested. When phosphodiesterase was assayed at 50 μM cyclic AMP or 20 μM cyclic GMP there was a gradual recovery of phosphodiesterase activity over a 2 h period (Table IV). In contrast the phosphodiesterase activity assayed at 2 μM cyclic AMP showed no recovery of activity over 120 min (Table IV).

Effect of diamide on phosphodiesterase isolated from lymphocytes

The effect of various concentrations of diamide on phosphodiesterase activity in the 16 000 × *g* supernatant from lymphocytes was examined at substrate concentrations of 2.0 μM cyclic AMP and 50 μM cyclic AMP and 10—50 μM cyclic GMP. Fig. 2a demonstrates the effect of diamide on cyclic AMP hydrolysis. When tested at 2.0 μM cyclic AMP, diamide demonstrated significant inhibition of phosphodiesterase activity at concentrations from $1 \cdot 10^{-2}$ to $1 \cdot 10^{-4}$ M with no effect at lower concentrations. In five experiments the inhibition of 10.0 mM diamide ranged from 40 to 70%. When the concentration of cyclic AMP was raised to 50 μM another pattern emerged. $1 \cdot 10^{-2}$ M diamide was slightly inhibitory while at $1 \cdot 10^{-4}$ M there was marked enhancement of phosphodiesterase activity. As can be seen in Fig. 2a the effect of $1 \cdot 10^{-4}$ M diamide on cyclic AMP hydrolysis could be blocked by $1 \cdot 10^{-4}$ M dithiothreitol suggesting that the effect of diamide was related to its sulfhydryl activity. Similar blockage of diamide effect of cyclic GMP phosphodiesterase was also seen (not shown). However, at higher diamide concentrations dithio-

TABLE IV

Lymphocytes ($20 \cdot 10^6$/ml) in Gey's buffer were incubated with $1 \cdot 10^{-3}$ M diamide or control for 15 min at 37°C. The cells were washed twice with warm Gey's buffer, resuspended at $20 \cdot 10^6$/ml and incubated in the absence of diamide. At times indicated the cells were washed twice in Gey's buffer, resuspended in buffer and disrupted. Control levels for phosphodiesterase measured at 120 min were 197.9 pmol/min per mg, 65.4 pmol/min per mg and 239.9 pmol/min per mg for $5 \cdot 10^{-5}$ M cyclic AMP, $2 \cdot 10^{-6}$ M cyclic AMP, and $2 \cdot 10^{-5}$ M cyclic GMP, respectively.

Time after removal of diamide (min)	% of phosphodiesterase activity		
	Cyclic AMP ($5 \cdot 10^{-5}$ M)	Cyclic AMP ($2 \cdot 10^{-6}$ M)	Cyclic GMP ($2 \cdot 10^{-5}$ M)
0	10	45	39
60	31	47	47
120	65	42	87

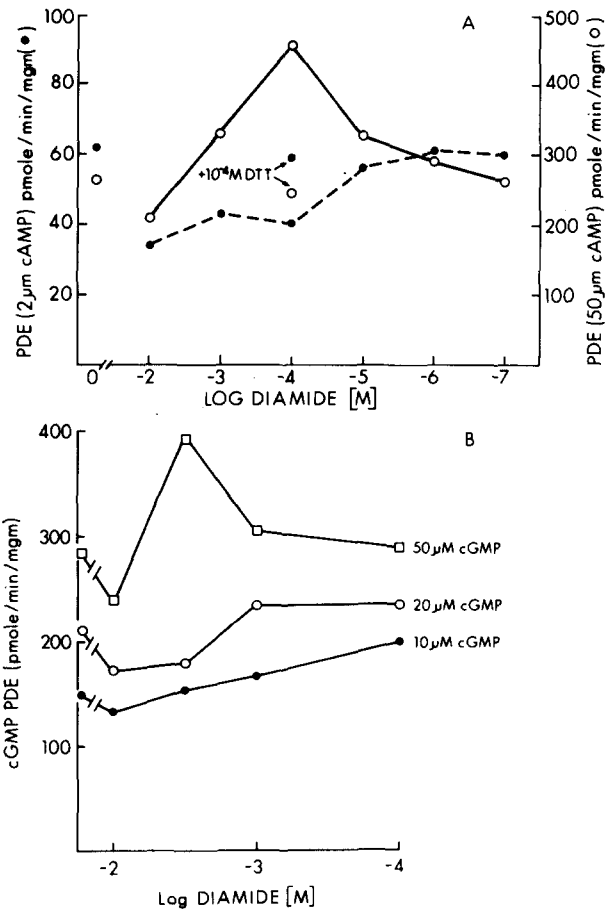

Fig. 2. (A) Effect of diamide on cyclic AMP phosphodiesterase (PDE). The 16 000 × *g* supernatant from sonicated lymphocytes was incubated with 2 μM cyclic AMP or 50 μM cyclic AMP in the presence of varying concentrations of diamide. Points marked $+10^{-4}$ M dithiothreitol (DTT) indicate incubation of diamide with 10^{-4} M dithiothreitol prior to the addition of 16 000 × *g* supernatant. Each point represents the mean of triplicate observations performed on lymphocytes from a single donor. Each experiment was performed four times with similar results. (B) Effect of diamide on cyclic GMP phosphodiesterase. The 16 000 × *g* supernatant from sonicated lymphocytes was incubated with 50, 20 or 10 μM cyclic GMP in the presence of varying concentrations of diamide and phosphodiesterase activity was measured. Each point represents the mean of triplicate observations performed on lymphocytes from a single donor. Each experiment was performed four times with similar results.

threitol in equimolar or greater concentrations was not completely inhibitory which may indicate a non-sulfhydryl effect of diamide.

Fig. 2b demonstrates the dose-response curves for the effect of diamide on cyclic GMP hydrolysis measured at three substrate concentrations. $1 \cdot 10^{-2}$ M diamide inhibited cyclic GMP hydrolysis at each substrate concentration tested. Lower concentrations of diamide enhanced phosphodiesterase activity at 10 and 20 μM cyclic GMP. The most marked effect was seen at 50 μM cyclic GMP; $5 \cdot 10^{-3}$ M diamide markedly augmented phosphodiesterase activity with no effect at lower concentrations.

To further examine the effects of diamide, phosphodiesterase activity was measured at cyclic AMP concentrations from 0.6 μM to 100 μM in the presence and absence of $1 \cdot 10^{-2}$ M diamide. The Lineweaver-Burk plot demonstrated two apparent K_m values of 2.8 μM and 50 μM. 10 mM diamide induced a 40% decrease in the V of the low-K_m phosphodiesterase with no change in the K_m indicative of classical non-competitive inhibition (not shown). The effect of diamide on the low-K_m activity was further evaluated by examining the effect of various concentrations of diamide on cyclic AMP phosphodiesterase activity measured at low substrate concentrations. When these data were analyzed by the method of Dixon non-competitive inhibition with a K_i of 1.3—2.5 mM (four experiments) was demonstrated. Similar experiments were also done on cyclic GMP hydrolysis at low substrate concentrations and high diamide concentrations. Diamide was a non-competitive inhibitor of cyclic GMP phosphodiesterase activity with K_i ranging from 3.3 to 10 mM.

The effect of diamide on cyclic AMP phosphodiesterase was further examined using cyclic AMP concentrations from 25 to 200 μM. Lineweaver-Burk analysis demonstrated an increase in the V and a decrease in the apparent K_m in the presence of $1 \cdot 10^{-4}$ M diamide. This type of stimulation is indicative of 'uncompetitive' action and suggests a three component reaction [22] (see below). A similar type of enhancement was seen with high cyclic GMP concentrations (data not shown).

Discussion

Although originally synthesized as a specific glutathione oxidant [1], diamide has been demonstrated to have additional activities which are consistent with the oxidation of intracellular protein sulfhydryl groups [3—5] or the formation of protein-glutathione sulfhydryl mixed disulfides [2]. In the present communication two new actions of diamide on cyclic nucleotide phosphodiesterase are described. First, when phosphodiesterase extracted from lymphocytes was incubated with diamide there was non-competitive inhibition of both low-K_m cyclic AMP and cyclic GMP phosphodiesterase activity. In this action diamide appears similar to a number of other sulfhydryl-active reagents [23,24]. Dithiothreitol was able to completely reverse the inhibition seen at $1 \cdot 10^{-4}$ M diamide but was not as effective at higher diamide concentrations. This is similar to the inability of dithiothreitol to reverse the effects of other sulfhydryl reagents and suggests an action which may be unrelated to sulfhydryl groups [9]. Second, diamide augmented both high-K_m cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity assayed at high substrate concentrations. This effect resembles the action of imidazole originally described by Butcher and Sutherland [25]. Like diamide, imidazole was effective only at high substrate concentrations. Since imidazole is only effective in the presence of the calcium-dependent phosphodiesterase activator [26] this suggests that human peripheral blood lymphocytes contain a similar phosphodiesterase activator [27]. The kinetic analysis of diamide enhancement is consistent with this analysis. The Lineweaver-Burk analysis indicates an uncompetitive interaction which is seen when there is a three component reaction [22].

When intact lymphocytes were incubated with diamide, washed, and phosphodiesterase extracted and assayed, the effect of diamide differed significantly from its direct effect on lymphocyte phosphodiesterase. First treatment of intact lymphocytes with diamide inhibited phosphodiesterase activity at lower concentrations than the direct effect on phosphodiesterase. There was slight, but statistically significant, inhibition at $1 \cdot 10^{-6}$ M as opposed to little effect of diamide at concentrations less than $1 \cdot 10^{-4}$ M when added directly to the phosphodiesterase assay. This difference was not due to differences in the time necessary for inactivation of phosphodiesterase since preincubation of phosphodiesterase with diamide prior to addition of substrate did not affect the results. Diamide reacts better with glutathione sulfhydryl than with protein sulfhydryl groups [28] and thus would be expected to form glutathione protein mixed disulfides more efficiently than protein-protein mixed disulfides. One possibility for the observed results may be that glutathione, present in millimolar concentrations in lymphocytes [29], may actually augment the effect of diamide by forming glutathione-phosphodiesterase mixed disulfides. In the phosphodiesterase assay glutathione is present in much lower concentrations, due to dilution, and this may reduce the effect of diamide. Indeed in preliminary experiments additions of small amounts of glutathione to the reaction mixture augmented diamide inhibition of phosphodiesterase providing support for this concept.

When intact lymphocytes were incubated with diamide and then disrupted, augmentation of phosphodiesterase was never observed, irrespective of the concentration of diamide or of cyclic AMP or cyclic GMP used in the assay. This suggests that in the intact lymphocyte phosphodiesterase activity may be insensitive to the activator similarly to that described for mouse lymphocytes [30]. However, following the disruption procedure used here the phosphodiesterase may become sensitive to the activator allowing the effect of diamide to be seen.

Diamide also appears to react differently with different lymphocyte phosphodiesterases. When lymphocytes were incubated with $1 \cdot 10^{-3}$ M diamide and washed, the phosphodiesterase activity seen at 50 μM and 20 μM cyclic GMP is partially reversible over 120 min. In contrast the inhibition of the cyclic AMP phosphodiesterase at low substrate concentrations remained constant over the same period. This suggests that sulfhydryl oxidation of phosphodiesterase is responsible for cyclic AMP hydrolysis at high substrate concentrations and cyclic GMP hydrolysis is readily reduced by cellular reductases while the low-K_m phosphodiesterase is not. Whether this represents differences in the structure of the individual phosphodiesterase or the interaction of diamide with the phosphodiesterase, for example formation of glutathione-protein disulfides in one case and protein-protein disulfides in the other, awaits purification and characterization of the individual enzymes.

The effect of diamide on whole cell cyclic AMP levels in mitogen-stimulated lymphocytes appears to be that at concentrations at which diamide is an effective inhibitor of mitogenesis ($2 \cdot 10^{-4}$ M [6]) the predominant effect of diamide is inhibition of the mitogen-induced increase in lymphocyte cyclic AMP. The fall in cyclic AMP in lymphocytes incubated with lectins and diamide may be due to inhibition of adenylate cyclase or to augmentation of phosphodiesterase. Several considerations suggest that adenylate cyclase inhibi-

tion is the controlling factor. First, data to be presented elsewhere indicates that diamide, at concentrations of $2 \cdot 10^{-4}$ M or higher, inhibits both basal and mitogen-stimulated adenylate cyclase activity. Second, augmentation of phosphodiesterase is only demonstrable at high cyclic nucleotide levels which are in excess of the levels which can be generated in the intact lymphocyte. Finally, there was no augmentation of phosphodiesterase activity in intact lymphocytes incubated with diamide, presumably because phosphodiesterase in the intact lymphocyte is fully activated [28].

The effect of diamide at lower concentrations is also of interest. At $1 \cdot 10^{-5}$ M diamide has no inhibitory effect on lymphocyte activation [6]. However, at this concentration there was augmentation of the rise in cyclic AMP seen with both phytohemagglutinin and concanavalin A, presumably due to phosphodiesterase inhibition. It has generally been accepted that non-mitogenic agents which raise intracellular cyclic AMP are inhibitors of the activation process [7]. Diamide appears to be unique in its lack of inhibition. However, as we have pointed out previously [31], cyclic AMP appears to be compartmentalized in the lymphocyte and it is possible that diamide does not alter cyclic AMP levels in the appropriate compartment.

Although diamide has many diverse actions which made assessment of its actual role in the inhibition of lymphocyte transformation difficult, several pertinent actions of diamide are of interest. First, inhibition and augmentation of phosphodiesterase activity must be added to the list of diamide's actions. Second, the marked inhibition of the lectin-induced increase in intracellular cyclic AMP suggests this may be responsible for the early inhibition of lymphocyte activation, and third, this study suggests that all non-mitogenic agents which increase intracellular cyclic AMP are not a priori inhibitors of lymphocyte activation.

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