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SHORT COMMUNICATION

Papaverine, a Potent Inhibitor of Respiration in C-6 Astrocytoma Cells

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SUMMARY

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Papaverine was used as an inhibitor of cyclic nucleotide phosphodiesterase in C-6 astrocytoma cells. That the diesterase was inhibited is suggested by the result that papaverine together with norepinephrine elevated the adenosine cyclic 3′,5′-monophosphate (cAMP) level more than did norepinephrine alone. Papaverine alone induced 3 times as much glycogen breakdown as did norepinephrine in 10 min, but did not cause cAMP elevation after this 10-min period. The adenine nucleotide content of these cells was analyzed with the purpose of accounting for the rapid glycogenolysis. Papaverine lowered creatine phosphate and ATP to 3% and 38%, respectively, of control values, whereas ADP and 5′-AMP were increased complementary to the ATP decline at the end of 10 min. Decreased creatine phosphate was apparent by 0.5 min after papaverine addition, whereas changes in adenine nucleotides occurred later. Conversion of phosphorylase *b* to phosphorylase *a* also occurred. Papaverine inhibited respiration in C-6 cells 50% at 5 μM and 90% at 0.5 mM, which accounted for the effect of papaverine on the phosphoryl energy carriers. These results demonstrate that the site of action of papaverine in C-6 astrocytoma cells is not limited to cyclic nucleotide phosphodiesterase. The data suggest that papaverine be used only with great caution as an inhibitor of this enzyme in cellular experimental systems.

Papaverine is an alkaloid derived from opium and has long been used to relax smooth muscle, particularly muscle in spasm (1). Recently papaverine has been shown to inhibit cyclic nucleotide phosphodiesterase in several tissues (2–7), and it has been proposed (3, 4) that this action of papaverine is related to the relaxation of smooth muscle. During experimentation in this laboratory in which the adenosine cyclic 3′,5′-monophosphate and glycogen contents of C-6 astrocytoma cells were measured, papaverine was employed as a phosphodiesterase inhibitor. Papaverine caused 3-fold more glycogenolysis than did norepinephrine after 10 min of incubation, although papaverine did not increase cAMP.[1] During analysis of adenine nucleotides of C-6 cell extracts, in search of an explanation for the glycogenolysis, marked reductions in the cellular high-energy phosphate stores were noted. It is

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[1] The abbreviation used is: cAMP, adenosine cyclic 3′,5′-monophosphate.

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TABLE 1

Effect of previous norepinephrine treatment on response of C-6 astrocytoma cells to norepinephrine and papaverine

Coverslip samples of cells were washed four times with 5 ml of Ham's F-10 medium and incubated for 3 hr in 3 ml of Ham's F-10 medium at 37° under an atmosphere of 5% CO_2–95% air in the presence or absence of 0.1 mM norepinephrine. Cells were then washed as before and incubated for 1 hr without hormone. One group of samples was fixed at this point in the procedure. The remaining samples were incubated for an additional 10 min with or without 0.1 mM norepinephrine and/or 0.5 mM papaverine. Incubations were terminated by transferring coverslip samples to beakers containing 1 ml of cold 5% trichloracetic acid. After 10 min the slips were transferred to 1 ml of sodium hydroxide to solubilize protein and glycogen. Values are means ± standard errors for the number of samples shown in parentheses.

	Incubation procedure				cAMP	Glycogen
	3-hr incubation with (+) or without (−) norepinephrine	1-hr incubation without norepinephrine	10-min incubation with (+) or without (−) drug			
			Norepinephrine	Papaverine		
					pmoles/mg protein	*nmoles/mg protein*
A	+	+	0	0	5.5 ± 1.7 (6)	44 ± 2.7 (6)
B	−	+	−	−	5.2 ± 0.8 (6)	48 ± 4.0 (6)
	−	+	+	−	1250 ± 91 (6)	30 ± 3.0 (6)
C	+	+	−	−	9.0 ± 3.7 (5)	43 ± 1.2 (6)
	+	+	−	+	8.7 ± 1.2 (6)	10 ± 0.6 (6)
	+	+	+	−	24 ± 1.2 (6)	34 ± 3.8 (6)
	+	+	+	+	122 ± 12 (6)	10 ± 1.6 (6)

the purpose of the present paper to describe these changes and to demonstrate that papaverine is a potent inhibitor of respiration in these cultured cells.

Unless indicated below, methods and materials were the same as described elsewhere (8).

A commercial oxygen electrode apparatus (Yellow Springs Instrument Company, oxygen electrode assembly, models 5331, 5093, and 5301) was modified for the present studies. The magnetic stirring bar of the chamber was replaced by a cylindrical Teflon bar made by filing the fins off a ¾-inch Spin Fin (Bel Art Products, F37125) to produce a cylindrical or disc-shaped stirring bar which was slightly smaller in diameter than the chamber. The electrode polarization and sensitivity were controlled as described by Estabrook (9). Finally, a cellophane membrane was placed between the usual Teflon membrane and the electrode tip to stabilize the electrode signal (10), displayed on a Honeywell Electronik 194 recorder. Routinely, the scale was expanded 5-fold, and zero was suppressed appropriately in order to display the oxygen signal. Samples of cells adherent to one side of round glass coverslips, 18 mm in diameter, were placed on top of the stirring disc. Frictional and/or capillary forces were sufficient to cause the coverslip to rotate with the stirring disc.

Papaverine was obtained as the hydrochloride from Eli Lilly and Company or as the free base from Sigma Chemical Company. Papaverine hydrochloride was dissolved in water to give a 30 mM solution; the free base was mixed with an equimolar amount of HCl. This amount of acid did not alter the pH of the incubation fluid.

The first observations of the present communication were incidental to ones on the effects of norepinephrine on C-6 astrocytoma cells (8). Cells were treated with norepinephrine for 3 hr and allowed to re-

gain control levels of cAMP and glycogen for 1 hr. Cells were then re-exposed to norepinephrine in the presence or absence of papaverine, and effects on cAMP and glycogen content of the cells were examined. The responses to norepinephrine (Table 1, A and B) were the same as those in experiments described elsewhere (8). Briefly, the cells elevated cAMP greatly when first exposed to norepinephrine but not when treated a second time, 4 hr later. A moderate decrease in glycogen content occurred in both instances after norepinephrine. Papaverine had no effect on the cAMP level in cells previously exposed to norepinephrine (Table 1, C), but papaverine together with norepinephrine caused cAMP to be elevated substantially more than did norepinephrine alone (Table 1, C). These results suggested that papaverine inhibited cyclic nucleotide phosphodiesterase in these cells. However, papaverine caused more extensive glycogenolysis than did norepinephrine (Table 1, C). In the presence of norepinephrine alone glycogen was decreased by 10 nmoles/mg of protein during 10 min of incubation, whereas in the presence of papaverine glycogen was decreased by 34 nmoles/mg. In the presence of both drugs glycogen breakdown was the same as with papaverine alone. Thus papaverine caused the glycogen content of the cells to decrease by 3.4 times as much as did norepinephrine.

Because cAMP was not increased after 10 min of incubation with papaverine, the levels of 5′-AMP in trichloracetic acid extracts from these cells were assayed to check for elevated 5′-AMP as the stimulant to glycogenolysis. Papaverine caused the 5′-AMP content to increase from 1 to 6 nmoles/mg of protein, and caused ADP to increase from 1 to 9 nmoles/mg. The drug caused the ATP content of the cells to decrease from 26 to 10 nmoles/mg of protein and led to almost complete depletion of creatine phosphate (from 27 to 1 nmole/mg of protein) during the 10-min incubation described in Table 1. An evaluation of the time course of the changes in these phosphoryl energy carriers is presented in Fig. 1. Coverslip samples of cells were incubated for periods of 0.5–10 min with or without 0.5 mM papaverine, and the cell extracts were analyzed for adenine nucleotides, creatine phosphate, glycogen, and protein. Results in Fig. 1 show the kinetics of changes in high-energy phosphate stores. Creatine phosphate was significantly depressed within 0.5 min after papaverine addition; by 2 min it was 75% decreased, and at 10 min it was 96% decreased. ATP was not decreased 0.5 min after papaverine addition but was decreased later, reaching approximately 40% of the initial value after 10 min. ADP rose during the first 5 min of the experiment, then appeared to level off at a concentration 3 times the control value. 5′-AMP remained

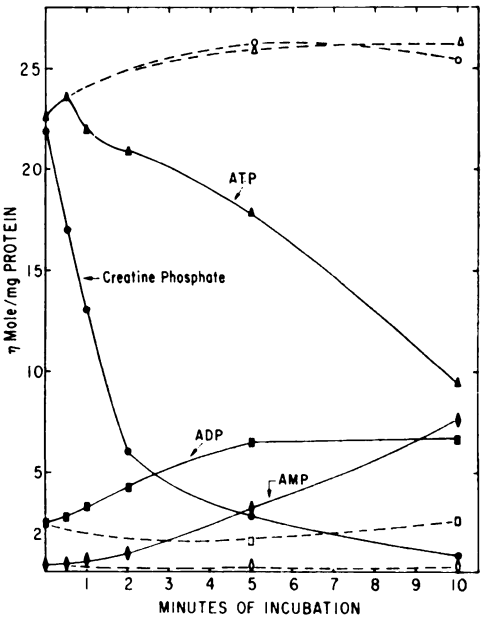

FIG. 1. *Effect of papaverine on adenine nucleotide and creatine phosphate content of C-6 astrocytoma cells*

All cells were incubated for 3 hr in the presence of norepinephrine, then washed and incubated for 1 hr without hormone as described in Table 1. Cells were then incubated for 0.5–10 min in the presence or absence of 0.5 mM papaverine as indicated. Incubations were terminated by transferring coverslip samples of cells to 5% trichloracetic acid. Data represent means from two experiments, each of which included triplicate incubation samples. ——, papaverine-treated samples; - - -, controls; ● and ○, creatine phosphate; ▲ and △, ATP; ■ and □, ADP; ◆ and ◇, AMP.

TABLE 2

Effect of papaverine on respiration by C-6 astrocytoma cells

Coverslip samples of cells were removed from growth medium (8), washed once with 5 ml of Ham's F-10 medium, and incubated for 10 min in 2 ml of this fluid in the oxygen electrode apparatus described in the text. The Ham's F-10 medium was equilibrated with 5% CO_2–95% air prior to the incubation. Papaverine was present for the entire incubation at the concentrations indicated. Data represent the results of two experiments, each of which included two or three replicate incubation samples.

Papaverine	Respiratory rate	Inhibition
M	*natoms oxygen/min/mg protein*	%
0	11.8	
1×10^{-7}	11.5	3
1×10^{-6}	10.0	15
5×10^{-6}	5.9	50
1×10^{-5}	4.0	66
1×10^{-4}	1.9	84
5×10^{-4}	1.1	90

constant for the first 0.5 min of incubation, then rose at an increasing rate during each subsequent interval, reaching 40 times the initial value by 10 min. cAMP was increased, from 4–6 pmoles/mg of protein in control samples, to 10–12 pmoles/mg, in papaverine-treated samples in these experiments (data not in figure).

The effect of papaverine on the phosphoryl energy carriers of C-6 cells has demonstrated a clear interaction of papaverine with the energy metabolism of these cells. The observed changes were expected to be coupled to a change in respiratory rate by one of three mechanisms. Papaverine might inhibit respiration, might stimulate respiration by uncoupling oxidative phosphorylation, or might stimulate respiration indirectly because of the increased ADP content of the cells. The effect of papaverine on respiration by C-6 cells adherent to coverslips was evaluated using the modified oxygen electrode apparatus described above. C-6 cells were incubated in the absence or presence of papaverine at concentrations from 0.1 to 500 μM (Table 2). Respiration was inhibited by papaverine in a dose-related manner. Fifty per cent inhibition of respiration occurred at 5 μM papaverine; 90% inhibition occurred at 0.5 mM papaverine, the dose used in previous experiments. This effect of papaverine accounts for the changes in phosphoryl energy stores of these cells.

The amount of glycogen breakdown during 10 min caused by papaverine in the experiments of Table 1 exceeded by a factor of 3 that initiated by norepinephrine. The time courses of the glycogenolysis caused by the two agents were therefore compared (Fig. 2). The rate of glycogen breakdown was nonlinear, as in past experiments (8). Papaverine indeed caused glycogenolysis to proceed more rapidly than did norepinephrine. During the first 5 min the average rate of glycogen breakdown was 4.5 nmoles/min/mg of protein in the presence of papaverine, and during the first 10-min interval it was 2.9

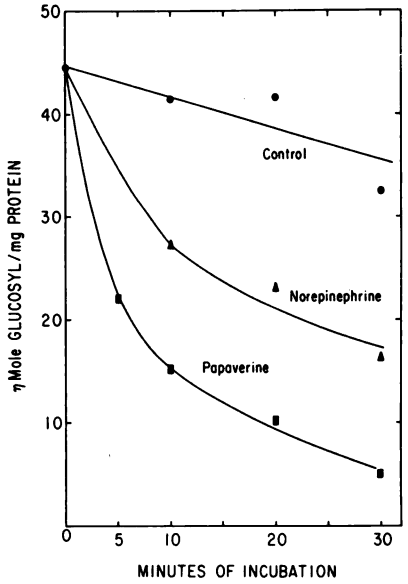

FIG. 2. *Effect of norepinephrine and papaverine on glycogen content of C-6 astrocytoma cells*

Coverslip samples of cells were removed from growth medium, washed, and incubated for 3 hr in the absence of norepinephrine as described in Table 1. Papaverine (0.5 mM) or norepinephrine (0.1 mM) was then added, and the cells were incubated for the times indicated. Incubations were terminated by transferring the coverslip samples to 5% trichloracetic acid. Data represent means from two experiments, each of which included duplicate incubation samples.

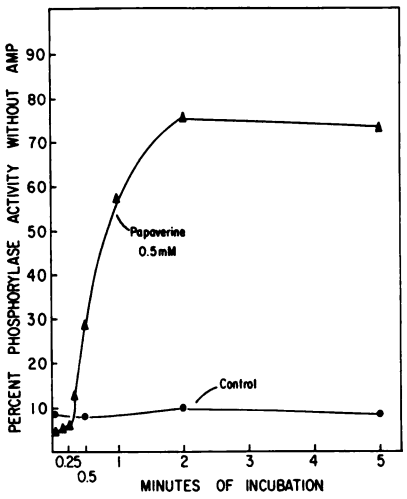

FIG. 3. *Effect of papaverine on phosphorylase a activity of C-6 astrocytoma cells*

Coverslip samples of cells were removed from growth medium, washed, and incubated for 3 hr in the absence of norepinephrine as described in Table 1. Papaverine (0.5 mM) was then added, and the cells were incubated for the times indicated. Incubations were terminated by freezing coverslip samples in liquid nitrogen (8). Cells were disrupted for phosphorylase assay by sonication (8). Data represent means from two experiments, each of which included duplicate incubation samples.

nmoles/min/mg. No 5-min data were obtained for norepinephrine, but during the first 10 min a smaller decrease occurred, representing an average rate of 1.7 nmoles/min/mg of protein. In the second and third 10-min intervals of the experiment the rate of glycogenolysis was not greatly different in the presence of the two agents; however, papaverine-treated samples contained smaller amounts of glycogen at these times because of the more extensive glycogenolysis during the first 10 min. It is concluded that papaverine causes more rapid glycogenolysis over-all than does norepinephrine. However, it must be acknowledged that glycogen breakdown may occur at least as rapidly in the presence of norepinephrine during certain time intervals, either because of more rapid phosphorylase activation (see below) or as a result of higher glycogen concentrations.

The effect of papaverine on the phosphorylase *b* and *a* content of the C-6 cells was assessed. Cells were incubated for intervals from 5 sec to 5 min; incubations were terminated by freezing coverslip samples of cells in liquid nitrogen. The percentage of total phosphorylase activity occurring in the absence of 5′-AMP was used as an index of the phosphorylase *a* activity of the cells. One minute after papaverine addition the enzyme was more than one-half converted to phosphorylase *a*, and by 2 min conversion was complete (Fig. 3). The enzyme retained approximately 25% dependence on added 5′-AMP, which is typical of phosphorylase *a* (11). Interestingly, a lag of approximately 15 sec occurred before a change in 5′-AMP dependence of the enzyme was apparent. This differed from the past observation of norepinephrine activation of phosphorylase, wherein phosphorylase was more than one-half converted to the *a* form during the first 15 sec of incubation (8). Also, the rate of conversion of the phosphorylase, once initiated, was slower than that seen previously with norepinephrine (8). Nonetheless, the phosphorylase *a* formation described in the present experiment is sufficiently rapid that the glycogenolysis caused by papaverine is attributed to phosphorylase *a* rather than to phosphorylase *b*.

The effects of papaverine described above suggest more than one site of action of this drug in C-6 astrocytoma cells. Papaverine potentiated the effect of norepinephrine on the cAMP level of these cells, suggesting a site of action at cyclic nucleotide phosphodiesterase as has been demonstrated for other tissues (2–7). Later experiments showed a clear effect of papaverine on respiration. Present knowledge of these effects does not indicate a common site of action for papaverine in causing the two effects.

The effects of papaverine in the present cells—inhibition of respiration and disruption of energy metabolism—signal caution in the use of this drug as an inhibitor of cyclic nucleotide phosphodiesterase in cellular experimental systems. The effect of papaverine on respiration is not limited to the present cells; inhibition of respiration in rat liver mitochondria has been observed.[2] Kisin (12) has reported that papaverine

[2] E. T. Browning, unpublished observations.

lowered the creatin phosphate level in cat heart and depressed oxidative phosphorylation. However, the effect of papaverine on respiration is probably not general among cyclic nucleotide phosphodiesterase inhibitors. In experiments similar to those reported here, 1-methyl-3-isobutylxanthine, a potent phosphodiesterase inhibitor, did not increase the 5′-AMP content of C-6 astrocytoma cells (8), suggesting that this inhibitor did not significantly disrupt the energy metabolism of these cells.

The hypothesis has been advanced that inhibition of cyclic nucleotide phosphodiesterase comprises the site of action of papaverine in vascular smooth muscle (3, 4). Although extrapolation from the present results to observations in smooth muscle is not justified, it may be pointed out that concentrations of papaverine which have been used to relax vascular smooth muscle maximally [290 μM (3, 13)] inhibit respiration of C-6 astrocytoma cells 90%; concentrations which produce a small degree of relaxation in vascular smooth muscle [12 μM (3, 13)] inhibit respiration of C-6 astrocytoma cells 70%.

An interesting feature of the experiments reported here is the observation that phosphorylase was converted from the *b* to the *a* form. That this conversion was not an artifact due to the increased 5′-AMP occurring in the cells is demonstrated by the fact that "phosphorylase conversion" was complete at 2 min, before 5′-AMP concentration increased significantly (Fig. 1). The cellular 5′-AMP present at 2 min after papaverine addition contributed only 30 nM 5′-AMP to the phosphorylase assay mixture. This 5′-AMP concentration is comparable to that contributed by 5′-AMP contaminating assay reagents (8) and is three orders of magnitude lower than that causing half-maximal stimulation of phosphorylase *b* in rabbit skeletal muscle (11).

The conversion of phosphorylase to the *a* form occurred over a somewhat longer time course than did the phosphorylase conversion caused by norepinephrine (8), and there was an approximately 15-sec lag between the time of papaverine addition and the beginning of the phosphorylase conversion. The phosphorylase kinase of skeletal muscle and brain may be converted to a form which is active at physiological pH by a cAMP-dependent phosphorylation. However, phosphorylase kinase also requires Ca^{++} whether the enzyme is in the nonactivated or the activated form (see ref. 8 for discussion). cAMP is not elevated by papaverine. It would appear either that the phosphorylase kinase becomes phosphorylated more slowly in the presence of the papaverine because of the low cAMP concentration, or that this enzyme is already in a partially phosphorylated state in both the absence and presence of papaverine and the rate-limiting factor in the activation of phosphorylase is the Ca^{++} concentration of the cytosol. Mitochondria normally contain Ca^{++}, which is accumulated by an energy-dependent mechanism (14). Upon inhibition of respiration by papaverine, Ca^{++} might be expected to exit from mitochondria and elevate the cytosolic Ca^{++} concentration, thereby stimulating phosphorylase kinase. Ca^{++}-loaded mitochondria are known to release Ca^{++} in the presence of uncouplers of oxidative phosphorylation (15).

The glycogenolysis which follows phosphorylase *a* formation caused by papaverine proceeds at a greater rate than occurs after phosphorylase *a* formation caused by norepinephrine. This point is discussed further elsewhere (8), with the conclusion that the increased inorganic phosphate concentration which is likely to occur in papaverine-treated cells may account for the increase in glycogenolytic flux. However, during the first minute or so after addition of norepinephrine or papaverine, it is quite possible that glycogen breakdown due to norepinephrine is more rapid than that due to papaverine. The phosphorylase *a* formation due to papaverine has not yet begun at 15 sec, when that due to norepinephrine is more than one-half complete (8); changes in adenine nucleotides by papaverine are not yet manifest (Fig. 2). Therefore significant glycogenolysis may occur earlier after norepinephrine than after papaverine addition. The resolution of this and other fine points of the relation of phosphorylase activation and glycogen breakdown in C-6 astrocytoma cells awaits more detailed investigation.

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