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DRUG-INDUCED PORPHYRIN BIOSYNTHESIS—XVIII. EFFECT OF MODULATION OF INTRACELLULAR CYCLIC AMP LEVELS ON DRUG-INDUCED PORPHYRIN BIOSYNTHESIS IN CHICK EMBRYO LIVER CELLS MAINTAINED IN SERUM-FREE MEDIUM*

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Abstract—Chick embryo liver cells were maintained in serum-free Waymouth medium containing insulin and thyroxine. Several agents which are reported to increase intracellular adenosine 3′:5′-monophosphate (cyclic AMP) levels, viz. glucagon, sodium fluoride, cyclic AMP or its dibutyryl derivative, 3-isobutyl-1-methylxanthine and papaverine, enhanced allylisopropylacetamide (AIA)-induced porphyrin biosynthesis. The enhancement is particularly apparent at low doses of AIA. On the other hand, agents which are reported to decrease intracellular cyclic AMP levels, viz. alloxan and imidazole, diminished the ability of AIA to induce porphyrin accumulation. Adenosine, which is reported to inhibit cyclic AMP-dependent protein kinases, diminished the ability of AIA to induce porphyrin accumulation.

Adenosine 3′:5′-monophosphate (cyclic AMP) and its dibutyryl derivative, although not essential for allylisopropylacetamide (AIA)-induced porphyrin biosynthesis, have been shown to enhance the accumulation of porphyrins by AIA in chick embryo liver cells maintained in serum-free medium [1]. 3-Isobutyl-1-methylxanthine (MIX), a potent cyclic nucleotide phosphodiesterase inhibitor, exerted an effect similar to cyclic AMP on AIA-induced porphyrin biosynthesis [1]. It was tentatively concluded that cyclic AMP is not a second messenger to AIA in this system, but that elevated levels of this cyclic nucleotide enhance AIA-induced porphyrin biosynthesis [1]. The objective of the present study was to examine further the role of cyclic AMP in AIA-induced porphyrin biosynthesis by determining the effect of modulation of hepatic intracellular cyclic AMP levels in chick embryo liver cell culture. The agents chosen to modulate cyclic AMP levels were those previously employed to determine the role of cyclic AMP in other biological processes [2], viz. exogenous cyclic AMP, glucagon, sodium fluoride (NaF), alloxan, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), imidazole, MIX, papaverine and adenosine.

METHODS

Chemicals. Allylisopropylacetamide was a gift from Hoffmann–La Roche, Montreal, Quebec. Adenosine 3′:5′-monophosphate (cyclic AMP), L-thyroxine sodium pentahydrate (T_4), insulin (bovine pancreas, 24 I.U./mg), glucagon (bovine and porcine pancreas), sodium fluoride, 5,5′-dithio bis(2-nitrobenzoic acid), alloxan, papaverine, imidazole and adenosine were obtained from Sigma Chemical Co., St. Louis, MO. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Waymouth MD 705/1 medium was obtained from Grand Island Biological Co., Grand Island, NY.

Cell culture technique. The details of the cell culture technique have been described previously [3–5]. The cells were maintained in 60 mm diameter disposable plastic Petri dishes (Falcon Plastic) containing 5 ml of Waymouth MD 705/1 medium supplemented with 60 mg penicillin G, 100 mg streptomycin sulfate, 1.0 mg insulin and 1.0 mg T_4/litre. In the experiments shown in Figs. 2 and 3, insulin was omitted from the medium. After an initial incubation period of 24 hr, the medium was discarded, replaced with fresh medium and re-incubated for a further 24 hr. T_4 (1.0 mg) was dissolved in 0.01 N NaOH (2 ml) prior to addition to 1 litre of medium and insulin (1.0 mg) was dissolved directly in 1 litre of medium. Cyclic AMP was dissolved in medium (10 μl.). For addition to cells adenosine, alloxan and NaF were dissolved in sterile distilled water (10 μl.); DTNB, MIX, papaverine and AIA were dissolved in 95% redistilled ethanol (10 μl.); and low doses of theophylline or aminophylline, theobromine, caffeine and xanthine were dissolved and higher doses were suspended in 95% ethanol (10 μl.). All compounds except AIA were present during both 24-hr incubation periods; AIA was added to cells only during the second 24-hr period of incubation.

Assay of porphyrins. Porphyrins were assayed as described previously [6].

Results are expressed as ng porphyrins/mg of protein.

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RESULTS AND DISCUSSION

In a previous study [1], it was shown that the effect of cyclic AMP on AIA-induced porphyrin biosynthesis depends upon the concentration of cyclic AMP. The response of chick embryo liver cells to increasing doses of AIA is shown in Fig. 1. The addition of 100 μg/ml of cyclic AMP resulted in a significant enhancement of AIA-induced porphyrin biosynthesis at all doses of AIA (Fig. 1), the enhancement being greatest at lower doses of AIA (3–10 μg/ml). In a previous study [1], it was shown that the minimum concentration of cyclic AMP (8.5 nmoles/ml) needed for enhancement of AIA-induced porphyrin accumulaton was higher than the level reported in chicken blood (0.23 nmole/ml). However, since the cyclic nucleotide is rapidly metabolized by hepatocytes, it is likely that actual concentrations in the cell culture system may be close to physiological levels. It is of interest that a similar effect on AIA-induced porphyrin biosynthesis was observed when dibutyryl cyclic AMP (10 μg/ml) was substituted for cyclic AMP (100 μg/ml). In the absence of AIA, cyclic AMP (100 μg/ml) had no effect on porphyrin levels while dibutyryl cyclic AMP (30–100 μg/ml) exerted a slight effect. Tomita *et al.* [7], using an organ culture of chick embryo liver, found dibutyryl cyclic AMP to be without effect on AIA-induced δ-aminolevulinic acid (ALA) synthetase activity. However, the effect of cyclic AMP was studied only at a single dose of AIA (40 μg/ml) and it is possible that the enhancing effect was missed, particularly since the greatest enhancing effects are seen at low doses (3–10 μg/ml) of AIA in our experiments. In our previous studies [1], dibutyryl cyclic AMP exerted no enhancing effects when tested with higher doses of AIA (30, 100 and 300 μg/ml). Sassa and Kappas [8], using chick embryo liver cells cultured in serum-free medium, also reported no effect of cyclic AMP derivatives on AIA-induced porphyrin biosynthesis. However, in their study, the cyclic nucleotides were only added in the second 24-hr incubation period, at lower concentrations (approximately 5 ng/ml) than used in our study, and a very high dose of AIA (100 μg/ml) was used. Therefore, as with Tomita *et al.* [7], the enhancing effect of cyclic AMP would have been missed.

Glucagon (3 ng/ml or 10 ng/ml) exerted a marked stimulatory effect on AIA-induced porphyrin biosynthesis (Fig. 2) in chick embryo liver cells maintained in serum-free medium supplemented with T_4 but no other hormones. The response of chick embryo liver cells to increasing doses of glucagon in the presence and absence of AIA (3 μg/ml) in serum-free medium containing T_4 is shown in Fig. 3. Glucagon was without effect on

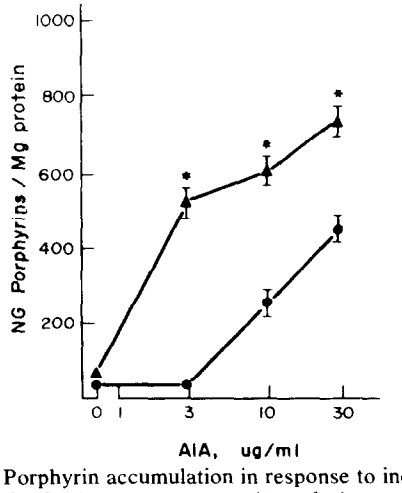

Fig. 1. Porphyrin accumulation in response to increasing doses of AIA in the presence (▲) and absence (●) of cyclic AMP (100 μg/ml). Asterisks indicate a significant difference (0.05) in porphyrin accumulation between cells maintained in the presence of cyclic AMP and those maintained in its absence at the same dose of AIA. Each point represents the mean of eight to sixteen determinations ±S. E. M.

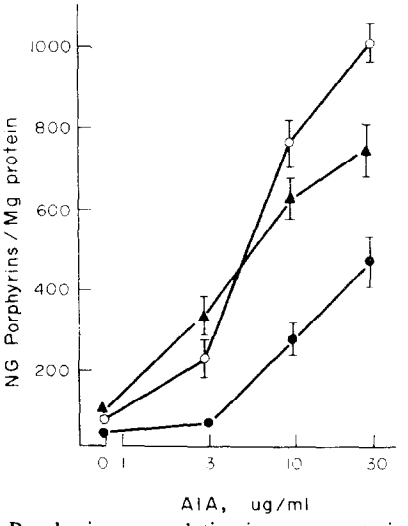

Fig. 2. Porphyrin accumulation in response to increasing doses of AIA in the presence of 3 ng/ml (○) or 10 ng/ml (▲) of glucagon and in its absence (●). Each point represents the mean of eight to sixteen determinations ±S. E. M. The points in the upper two curves (○) and (▲) at 3, 10 and 30 μg/ml of AIA are significantly different (0.05) to the corresponding points in the lower curve (●).

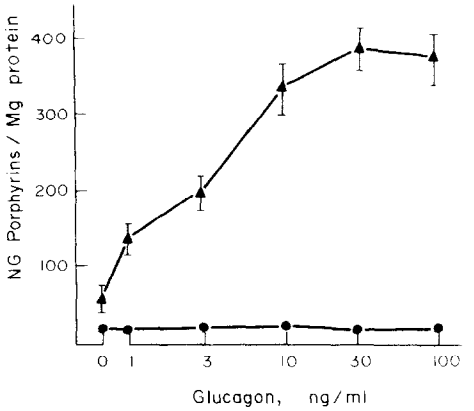

Fig. 3. Porphyrin accumulation in response to increasing doses of glucagon in the presence of 3 μg/ml of AIA (▲) and in its absence (●). Each point represents the mean of eight determinations ±S. E. M. All the points in the upper curve (▲) are significantly different (0.05) to those in the lower curve (●).

porphyrin biosynthesis in the absence of AIA but markedly enhanced the ability of AIA to induce porphyrin accumulation. In this respect, the action of glucagon resembles that of cyclic AMP. The half-maximal effect of glucagon occurred at 3 ng/ml and a significant effect was observed with 1 ng/ml. Studies have shown that glucagon stimulates the *de novo* synthesis of a variety of hepatic enzymes [9] and that its action is due to its ability to stimulate hepatic adenylate cyclase with a concomitant increase in intracellular cyclic AMP levels [10–13]. The portal concentration of glucagon in rats is reported to be 0.25 ng/ml [14]. The apparently lower sensitivity of chick embryo hepatocytes to glucagon may be due to proteases leaking from damaged cells or carried over from the collagenase-hyaluronidase procedure used to dissociate the liver in our experiments [15]. In our cells, the sensitivity to glucagon was similar to that reported for isolated rat liver cells [13] and isolated perfused rat liver [16]. The fact that the sensitivity observed in isolated chick embryo liver cells is similar to that observed in the intact rat liver suggests that the membrane glucagon receptors (adenylate cyclase) of our isolated cells are reasonably well preserved. Tomita *et al.* [7], using an organ culture system, and Sassa and Kappas [8], using chick embryo liver cell culture, were unable to detect a glucagon effect. However, both groups of workers restricted their study to a single, high dose of AIA. Moreover, glucagon was added only in the second incubation period in contrast to our studies where it was added in both 24-hr periods. In addition, insulin was omitted from our cell culture medium when glucagon effects were demonstrated, while it was included in the medium used by Sassa and Kappas [8]. Clearly, in order to demonstrate hormonal effects, it is necessary to investigate a wide range of doses of AIA (or other porphyrin-inducing drugs) as well as a range of hormone concentrations.

NaF is reported to activate adenylate cyclase [17–19]. Therefore, it was anticipated that NaF, like glucagon, would enhance AIA-induced porphyrin biosynthesis. The addition of 20 μg/ml of NaF resulted, as anticipated, in a significant enhancement of AIA-induced porphyrin biosynthesis at all doses of AIA (Fig. 4). The enhancement was, however, not as great as previously observed with either cyclic AMP or glucagon (Figs. 1–3). The effect of higher doses was to completely inhibit AIA-induced porphyrin biosynthesis. A possible reason for this finding is that NaF inhibits many magnesium-requiring enzymes [20] and, therefore, may inhibit an enzyme required for optimal activity of the heme biosynthetic pathway.

Alloxan and DTNB have been shown to inhibit adenylate cyclase [21] and it was anticipated that these compounds would lower intracellular cyclic AMP levels and diminish AIA-induced porphyrin biosynthesis. As anticipated, the addition of alloxan (20 μg/ml) significantly inhibited porphyrin production by AIA, while it was without effect alone (Fig. 5). The addition of either 10 μg/ml or 100 μg/ml of DTNB (Fig. 6) significantly enhanced AIA-induced porphyrin biosynthesis at lower doses of AIA (3–10 μg/ml). This result was not anticipated and it is possible that DTNB may have several properties in addition to its ability to inhibit adenylate cyclase.

The next series of experiments was carried out with MIX, a potent inhibitor of cyclic nucleotide phosphodiesterase [22–25]. At low doses (1–30 μg/ml) MIX was devoid of porphyrin-inducing activity (Fig. 7), while at higher doses (100–300 μg/ml), it enhanced porphyrin production significantly over control values (Fig. 7). The addition of MIX (30 μg/ml) resulted in a significant enhancement of porphyrin production at all doses of AIA (Fig. 8); the enhancement was greatest at lower doses of AIA (3–10 μg/ml). In this series of

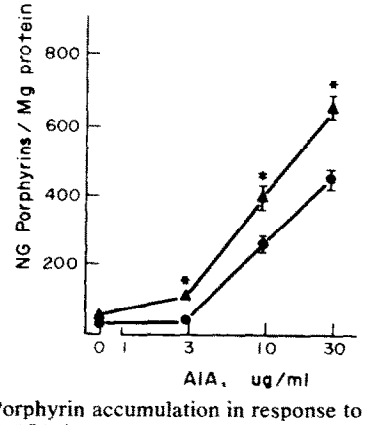

Fig. 4. Porphyrin accumulation in response to increasing doses of AIA in the presence (▲) and absence (●) of NaF (20 μg/ml). Asterisks indicate a significant difference (0.05) in porphyrin accumulation between cells maintained in the presence of NaF and those maintained in its absence at the same dose of AIA. Each point represents the mean of four to sixteen determinations ±S. E. M.

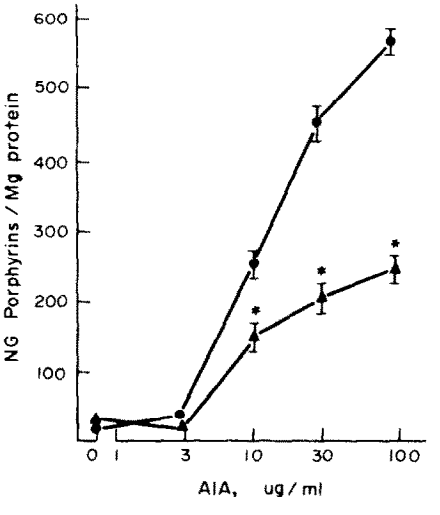

Fig. 5. Porphyrin accumulation in response to increasing doses of AIA in the presence (▲) and absence (●) of alloxan (200 μg/ml). Asterisks indicate a significant difference (0.05) in porphyrin accumulation between cells maintained in the presence of alloxan and those maintained in its absence at the same dose of AIA. Each point represents the mean of eight to sixteen determinations ±S. E. M.

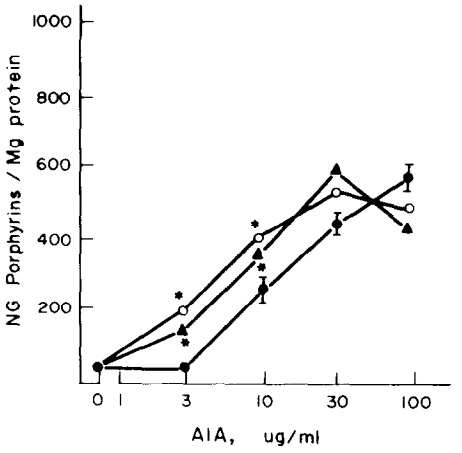

Fig. 6. Porphyrin accumulation in response to increasing doses of AIA in the presence of 10 μg/ml (○) or 100 μg/ml (▲) of DTNB and in its absence (●). Asterisks indicate a significant difference (0.05) in porphyrin accumulation between cells maintained in the presence of DTNB and those maintained in its absence at the same dose of AIA. Each point represents the mean of eight to twelve determinations ±S. E. M.

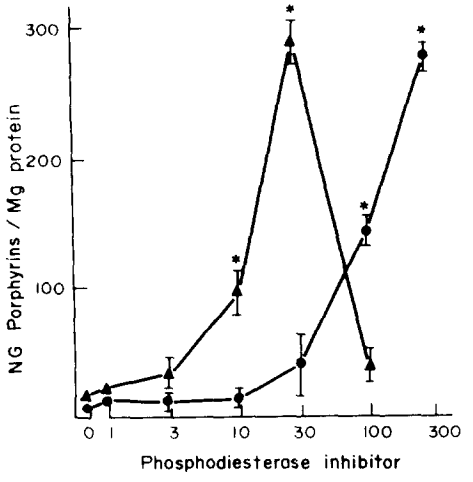

Fig. 7. Porphyrin accumulation in response to increasing doses of MIX (●) and papaverine (▲). Asterisks indicate points that are significantly different (0.05) from control. Each point represents the mean of eight to twelve determinations ±S. E. M.

experiments, MIX was present in both 24-hr incubation periods of the liver cells. This resulted in a greater enhancement of AIA-induced porphyrin accumulation than was observed previously [1] when MIX was present only in the second incubation period. A series of xanthine derivatives, viz. theophylline or aminophylline (3–300 μg/ml), theobromine (10–300 μg/ml), caffeine (3–300 μg/ml) and xanthine (10–300 μg/ml) were examined for their effect on porphyrin biosynthesis. In contrast to results obtained with MIX, these compounds were unable to induce porphyrin biosynthesis and had no effect on AIA-induced porphyrin biosynthesis. It was concluded that the activity of MIX, in contrast to the inactivity of other methylxanthines, was probably due to its

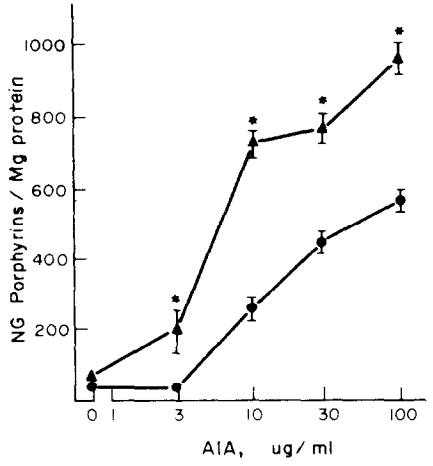

Fig. 8. Porphyrin accumulation in response to increasing doses of AIA in the presence (▲) and absence (●) of MIX (30 μg/ml). Asterisks indicate a significant difference (0.05) in porphyrin accumulation between cells maintained in the presence of MIX and those maintained in its absence at the same dose of AIA. Each point represents the mean of eight to twelve determinations ±S. E. M.

higher lipophilicity and, therefore, its greater penetrability into hepatocytes. However, methylxanthines possess a variety of additional properties [22, 23] and it is possible that the effect of MIX may be related to one or several of its other pharmacological properties.

Papaverine is a phosphodiesterase inhibitor which has been reported to be 10–1000 times more potent than methylxanthines [24]. Papaverine was able to induce porphyrin biosynthesis at doses as low as 10 μg/ml (Fig. 7). A dose of 3.0 μg/ml was chosen for subsequent experiments since it was insufficient to induce porphyrin accumulation alone. The addition of papaverine (3.0 μg/ml) to the medium resulted in a significant enhancement

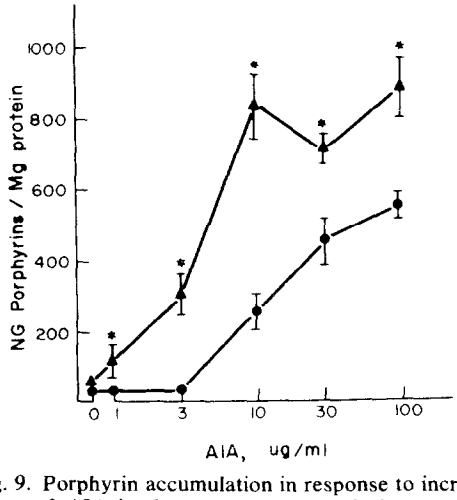

Fig. 9. Porphyrin accumulation in response to increasing doses of AIA in the presence (▲) and absence (●) of papaverine (3 μg/ml). Asterisks indicate a significant difference (0.05) in porphyrin accumulation between cells maintained in the presence of papaverine and those maintained in its absence at the same dose of AIA. Each point represents the mean of eight to sixteen determinations ±S. E. M.

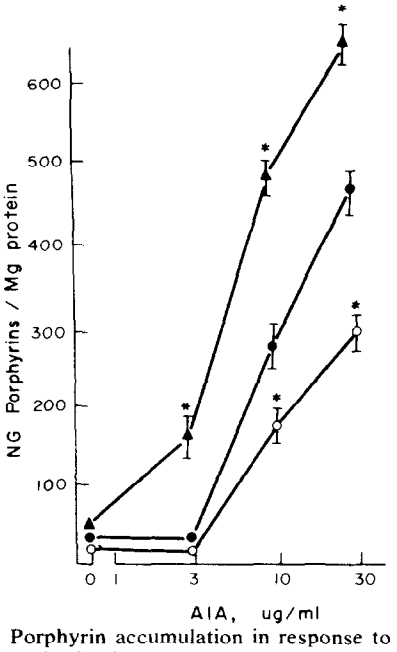

Fig. 10. Porphyrin accumulation in response to increasing doses of AIA in the presence of 200 μg/ml (○) or 500 μg/ml (▲) of imidazole and in its absence (●). Asterisks indicate a significant difference (0.05) in porphyrin accumulation between cells maintained in the presence of imidazole and in its absence at the same dose of AIA. Each point represents the mean of eight to sixteen determinations ±S. E. M.

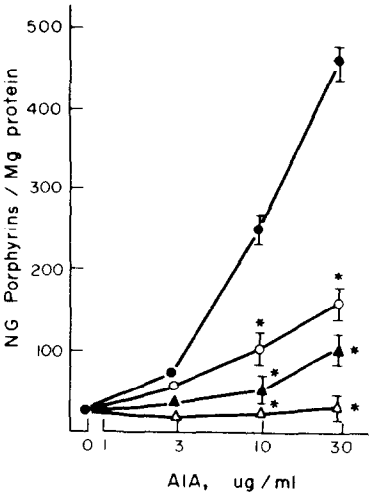

Fig. 11. Porphyrin accumulation in response to increasing doses of AIA in the presence of 10 μg/ml (○), 30 μg/ml (▲) and 100 μg/ml of (△) adenosine and in its absence (●). Asterisks indicate a significant difference (0.05) in porphyrin accumulation between cells maintained in the presence of adenosine and in its absence at the same dose of AIA. Each point represents the mean of eight to sixteen determinations ±S. E. M.

of AIA-induced porphyrin biosynthesis (Fig. 9), the effect being greatest at lower doses of AIA. While it is possible that the effects of papaverine are unrelated to phosphodiesterase inhibition, the fact that papaverine and MIX, compounds unrelated chemically, exert effects similar to those of cyclic AMP and dibutyryl cyclic AMP strengthens the possibility that these two agents are exerting effects on porphyrin biosynthesis by inhibition of phosphodiesterases [23]. Other explanations are possible, however, for the combined effects of AIA and MIX or papaverine, and a definitive interpretation of these results will be facilitated by measurement of intracellular levels of cyclic AMP.

Imidazole stimulates the cyclic nucleotide phosphodiesterase [26] and it was anticipated that it would lower cyclic AMP levels and inhibit AIA-induced porphyrin biosynthesis. The addition of imidazole (200 μg/ml) resulted in an inhibition of AIA-induced porphyrin biosynthesis (Fig. 10) at higher doses of AIA (10–30 μg/ml). However, raising the dose of imidazole to 500 μg/ml resulted in an enhancement of AIA-induced porphyrin accumulation (Fig. 10). It is of interest that imidazole has been reported to inhibit cyclic nucleotide phosphodiesterase in some tissues at higher doses [24]. Interpretation of these results will be facilitated by measurement of intracellular cyclic AMP levels.

Adenosine stimulates the accumulation of cyclic AMP in brain slices [27], platelets [28] and a variety of cultured cells [29, 30]. However, adenosine has been reported to inhibit protein kinases in many tissues [31]. The addition of 10 and 30 μg/ml of adenosine resulted in a significant inhibition of AIA-induced porphyrin biosynthesis (Fig. 11). Increasing the concentration of adenosine from 30 μg/ml to 100 μg/ml resulted in greater inhibition of porphyrin biosynthesis (Fig. 11). However, at 100 μg/ml adenosine appeared to exert toxic effects on the cells as judged by the protein determination on cells attached to the Petri dish. These results suggest that, in our system, adenosine inhibits cyclic AMP-dependent protein kinase.

The results of our study are summarized in Table 1. Agents which have been reported to increase intracellular cyclic AMP levels (exogenous cyclic AMP, glucagon, NaF, MIX and papaverine) enhanced AIA-induced porphyrin biosynthesis. While MIX and papaverine induced porphyrin accumulation in the absence of AIA, their enhancing effect was observed at concentrations which were ineffective by themselves. Those agents which decrease hepatic cyclic AMP levels (alloxan and imidazole) had an inhibitory effect on AIA-induced porphyrin accumulation. Adenosine, which is reported to inhibit protein kinases had an inhibitory effect on AIA-induced porphyrin biosynthesis, suggesting that the effect of cyclic AMP in our system, as in most systems [32], is mediated through a protein kinase. That the inhibitory effect of alloxan, imidazole and adenosine (10 and 30 μg/ml) on AIA-induced porphyrin biosynthesis was not due to death of the chick liver cells is clear from the following consideration: death of the cells would have caused detachment of cells from the Petri dishes [6], a phenomenon which was not observed. A toxic effect of these agents would have been recognized by a decreased number of cells adhering to the dish. An estimate of the cells adhering to the dish was obtained from the protein content. No decrease in cell content per dish was observed with alloxan, imidazole or

Table 1. Effect of various agents which modulate intracellular cyclic AMP levels on AIA-induced porphyrin biosynthesis

Agent and mechanism of action*	Concn used (μg/ml)	Intracellular cyclic AMP levels	Effect on AIA-induced porphyrin biosynthesis	
			Anticipated	Observed
Cyclic AMP	100	Increase	Increase	Increase
Stimulator of adenylate cyclase				
NaF	20	Increase	Increase	Increase
	200			Decrease
Glucagon	0.001–0.10	Increase	Increase	Increase
Inhibitors of adenylate cyclase				
Alloxan	200	Decrease	Decrease	Decrease
DTNB	10–100	Decrease	Decrease	Increase
Inhibitor of phosphodiesterase				
MIX	30	Increase	Increase	Increase
Papaverine	3	Increase	Increase	Increase
Theophylline	3–300	Increase	Increase	No effect
Theobromine	10–300	Increase	Increase	No effect
Caffeine	3–300	Increase	Increase	No effect
Stimulator of phosphodiesterase				
Imidazole	200	Decrease	Decrease	Decrease
	500			Increase
Inhibitor of cyclic AMP-dependent protein kinase				
Adenosine	10–100		Decrease	Decrease

*See Ref. 2.

adenosine (10 and 30 μg/ml); a decrease was, however, noted with the highest dose of adenosine (100 μg/ml). In summary, the present data support the concept that elevation of intracellular cyclic AMP in chick embryo liver cells in culture enhances AIA-induced porphyrin biosynthesis, while lowering intracellular cyclic AMP levels diminishes AIA-induced porphyrin biosynthesis. It is of importance to compare these results with those obtained in other systems. Edwards and Elliott [32], who used rat liver cells in suspension, showed that AIA-induced ALA synthetase activity was dependent on the presence in the medium of cyclic AMP or its dibutyryl derivative. In contrast, investigators who studied the effect of cyclic AMP or its dibutyryl derivative on AIA-induced hepatic ALA synthetase activity in the rat, reported no effect, an inhibition of activity, or enhancement of activity [33, 34]. Moreover, the phosphodiesterase inhibitor theophylline has been shown to inhibit AIA-induction of ALA synthetase in chick embryo and rat liver [35]. Thus, the results in the chick embryo and rat liver cells cannot be uncritically extrapolated to the *in vivo* situation.

Wicks [9] has concluded that cyclic AMP has the capacity to influence protein synthesis at both transcriptional and translational levels in liver. Its ability to increase protein synthesis is presumably due to its capacity to promote phosphorylation of various cellular components by a cyclic AMP-dependent protein kinase [9, 36]. Porphyrin-inducing drugs such as AIA are believed to act by increasing the levels of ALA synthetase-specific messenger RNA [37, 38]. Therefore, a possible explanation for the enhancement of AIA-induced porphyrin biosynthesis at low doses of AIA by cyclic AMP and various compounds which act to increase intracellular cyclic AMP is that cyclic AMP activates cellular machinery devoted to protein synthesis and provides one or more factors (e.g. increased template activity, ribosomes, etc.) which may be limiting in the synthesis of ALA synthetase. At high doses of AIA these factors would presumably be synthesized in optimum amounts in the absence of cyclic AMP.

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