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Catecholamine-Induced Release of the Folic Acid Analogue, Methotrexate, from Rat Hepatocytes in Suspension

An *Alpha*-Adrenergic Phenomenon

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SUMMARY

Studies were undertaken to explore the mechanism(s) for release of the folic acid analogue, methotrexate, from freshly isolated rat hepatocytes in suspension. When cells are at steady state with exchangeable intracellular methotrexate, net efflux of methotrexate is induced by 10 μM epinephrine. This net efflux of methotrexate induced by epinephrine is markedly potentiated by 3-isobutyl-1-methylxanthine at concentrations which do not, alone, result in net loss of methotrexate from the cells. Epinephrine (in the presence of isobutyl methylxanthine) is the most potent of the catecholamines tested in inducing methotrexate efflux; equimolar norepinephrine or phenylephrine are less effective, and isoproterenol is essentially ineffective. This order of potency for the catecholamines suggests an *alpha*-adrenergic-mediated exit of methotrexate from these cells. This is further supported by the observations that the *alpha* antagonists phenoxybenzamine and prazosin significantly depress methotrexate efflux induced by epinephrine plus isobutyl methylxanthine, whereas the *beta* antagonists propranolol and dichloroisoproterenol have no effect on induction of drug exit. Incubation of hepatocytes with the calcium-chelating agent ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid reduces or eliminates efflux of methotrexate induced by epinephrine or epinephrine plus isobutyl methylxanthine, consistent with calcium involvement in *alpha*-adrenergic phenomena. Similarly, arginine vasopressin effects methotrexate release by a calcium-dependent mechanism. This calcium-dependent efflux of methotrexate from hepatocytes induced by *alpha*-adrenergic stimuli *in vitro* may represent a "secretory" phenomenon which modulates release of this antifolate into the capillary sinusoid or bile canaliculus when the hepatocyte is in its usual spatial orientation within the liver lobule.

INTRODUCTION

Hepatocytes transport a variety of endogenous and exogenous compounds across their heterogeneous membrane surfaces during uptake of these compounds into liver from blood as well as during secretion back into blood sinusoid or bile canaliculus (1, 2). Although *net* uptake of a variety of compounds into liver and *net* secretion from liver into bile *in vivo* can be readily quantitated, studies with isolated hepatocytes have provided an opportunity for the accurate quantitation of bidirectional fluxes of compounds across hepatocyte membranes (3, 4). However, these studies have focused largely on the characteristics of drug influx and net accumulation, and little information is available regarding mechanisms for control of the exit of compounds from liver cells.

Studies from this laboratory have characterized the membrane transport and intracellular disposition of the folic acid analogue, MTX,[2] in freshly isolated rat hepatocytes (5). This drug enters hepatocytes via two mediated influx routes. Once in the cell, intracellular drug is bound to dihydrofolate reductase, converted to polyglutamate derivatives, and distributed between "freely exchangeable" and "less readily exchangeable" compartments (5), the latter presumably reflecting drug bound to dihydrofolate reductase and other sites within the cell. Recently we reported an energy-dependent efflux of both freely exchangeable and less readily exchangeable intracellular MTX from freshly isolated hepatocytes in suspension induced by Bt_2cAMP or IBMX (6) and raised

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[2] The abbreviations used are: MTX, methotrexate; 4-amino-N^{10}-methylpteroylglutamic acid; Bt_2cAMP, dibutyryl cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine; K-H buffer, Krebs-Henseleit buffer; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide.

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the possibility that these observations *in vitro* may be the corollary of MTX "secretion" into the bile canaliculus and/or the hepatic sinusoid when the hepatocyte is in its usual spatial orientation in intact liver *in vivo*. Although the induction of MTX efflux by these agents suggested a cyclic nucleotide-mediated phenomenon, the absence of efflux after exposure of hepatocytes to glucagon or isoproterenol, agents which increase cellular cyclic AMP (7, 8), suggested the possibility that these effects might be independent of cellular cyclic AMP.

In this paper we report on further studies that explore MTX efflux from hepatocytes and present data which suggest that the net exit of MTX from isolated hepatocytes is mediated by an *alpha*-adrenergic mechanism. These observations raise the possibility that MTX secretion from hepatocytes *in vivo* may be an *alpha*-adrenergic-modulated event. Furthermore, since there is evidence that MTX and bile salts share common transport routes in hepatocytes (9), this phenomenon may have relevance to control of hepatic bile salt secretion.

MATERIALS AND METHODS

Preparation of Hepatocytes. Hepatocytes in suspension were prepared by a modification of the method of Berry and Friend (10), which increases cell yield and viability. The livers of male Sprague-Dawley rats (175–275 g) were perfused (at a rate of 20 ml/min) via the portal vein as follows: (a) perfusion of 100 ml of calcium-free K-H buffer (11) containing 1 mM EGTA—to waste; (b) perfusion of 100 ml of calcium-free K-H buffer—to waste; and (c) a 20-min recirculating perfusion (225 ml total volume) of K-H buffer containing 2 mM $CaCl_2$ and 0.045% collagenase. All of the above solutions were maintained at pH of ~7.4 with 95% O_2/5% CO_2 at 37° in an incubation bath. The liver was excised, minced, and suspended in 40 ml of K-H buffer containing 2 mM $CaCl_2$, 0.25% gelatin, and 0.0056% collagenase and shaken gently by hand for 5 min in a 37° water bath to free dispersed cells from the connective tissue matrix. The cell suspension was filtered through a double layer of cheesecloth to remove undigested tissue, and the suspension was centrifuged at 50–60 × *g* for 2 min. The pelleted hepatocytes were resuspended and washed three times in 35 ml of K-H buffer containing 2 mM Ca^{2+} and 0.25% gelatin.

For preparation of "calcium-depleted" hepatocytes, calcium was excluded from the solution added to the minced liver and during incubation. EGTA (1 mM) was included in the washing steps as well as in the incubation solution in some experiments (12).

For measurement of MTX uptake and release, the cells were suspended at 37°, pH 7.4, in K-H buffer containing 2 mM $CaCl_2$, 0.25% gelatin, and 10 mM sodium ascorbate to prevent degradation of the catecholamines and continuously gassed with a mixture of 95% O_2/5% CO_2. After incubation with [^{3}H]MTX and the various test drugs, portions of the cell suspensions were pipetted into 10 ml of ice-cold 0.9% NaCl solution to terminate the reaction. The suspension was then centrifuged at 300 × *g* for 30 sec to pellet the cells, following which the cells were washed and centrifuged twice more. The pellet was then placed on polyethylene tares, dried overnight at 75°, and weighed. Intracellular ^{3}H was determined by liquid scintillation spectroscopy after dissolving the pellet in 250 μl of 1 N KOH, followed by neutralization with 250 μl of 1 N HCl and the addition of Beckman Readi-Solv.

Chemicals. [3′,5′,9-^{3}H]MTX, with an initial specific activity of 30 Ci/mole, was obtained from Amersham/Searle Corporation (Arlington Heights, Ill.) and purified by DEAE-cellulose chromatography (13). The catecholamines, (−)-phenylephrine HCl, (−)-norepinephrine bitartrate, (−)-epinephrine bitartrate, (−)-isoproterenol bitartrate, the blocking agents, (±)-propranolol HCl, dichloroisoproterenol HCl and yohimbine HCl, Bt_2cAMP, IBMX, cyclic AMP, dibutyryl cyclic GMP, theophylline, arginine vasopressin, and collagenase were obtained from Sigma Chemical Company (St. Louis, Mo.). Angiotensin II was obtained from Ciba-Geigy Corporation (Summit, N.J.). Phenoxybenzamine, kindly provided by Smith Kline & French Laboratories (Philadelphia, Pa.), was dissolved in ethanol and the solution was discarded after 2 weeks. Prazosin HCl was kindly provided by Pfizer Laboratories (New York, N. Y.). The catecholamines were dissolved and frozen in 10 mM sodium ascorbate, the nucleotide derivatives in water; and the IBMX, theophylline, and A23187 in DMSO. All other chemicals were reagent-grade.

RESULTS

Effects of epinephrine on net MTX efflux from hepatocytes in the presence or absence of IBMX. Previous studies from this laboratory established that Bt_2cAMP and IBMX (at concentrations of 2.5 mM) induce the net loss of both freely exchangeable and less readily exchangeable MTX from isolated hepatocytes (6). Although a portion of intracellular ^{3}H represents MTX polyglutamate derivatives, ^{3}H that leaves the cells under these conditions represents the monoglutamate alone. Figure 1 (*upper* and *lower panels*) demonstrates that 10 μM epinephrine induces the net efflux of a small, but significant, quantity of intracellular drug (9.7 ± 2.5% in 18 experiments; $p < 0.005$) in the absence as well as the presence of DMSO.[3] A 100 μM concentration of epinephrine is only slightly more effective than 10 μM epinephrine in inducing MTX efflux; raising the epinephrine concentration to 500 μM results in an additional loss of 20% of intracellular ^{3}H (Fig. 1, *upper panel*). Figure 1 (*lower panel*) demonstrates that the induction of ^{3}H release by epinephrine is potentiated by 50 μM IBMX. However, 0.5 mM IBMX produces maximal potentiation of release by epinephrine (39.2 ± 3.5% of intracellular ^{3}H based upon 14 experiments) without, alone, causing net MTX efflux[4] and was routinely used in the following studies.

Effects of other catecholamines on net MTX efflux. The ability of a variety of other catecholamines to induce MTX efflux in the presence of 0.5 mM IBMX was eval-

[3] The vehicle DMSO, used to solubilize the IBMX, does not alone potentiate the effect of epinephrine (compare *upper* and *lower panels* of Fig. 1).

[4] At a concentration of 0.5 mM, IBMX alone terminates net MTX uptake, but there is no net loss of MTX from the cells (data not shown). Potentiation of epinephrine-induced efflux of MTX was also observed utilizing 0.5 mM theophylline, although this drug was less effective than IBMX.

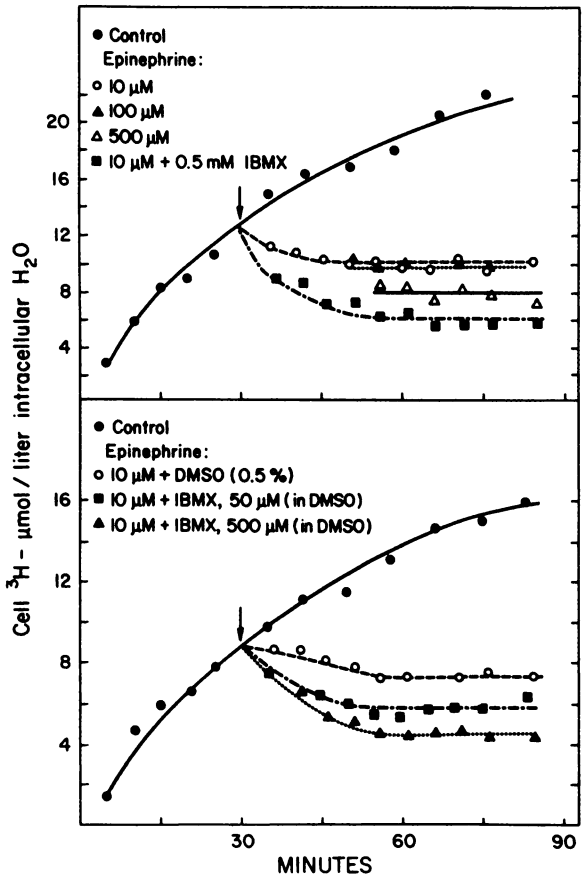

FIG. 1. *Induction of MTX release by epinephrine: effect of concentration and potentiation by IBMX*

Hepatocytes incubated with 1 μM [^{3}H]MTX for 30 min were exposed to (*upper panel*) epinephrine at 10 μM (○), 100 μM (▲), 500 μM (△), or 10 μM in the presence of 0.5 mM IBMX in DMSO (■) or (*lower panel*) 10 μM epinephrine plus 0.5% DMSO (○); 10 μM epinephrine plus 50 μM IBMX (in 0.5% DMSO (■); or 10 μM epinephrine plus 500 μM IBMX (in 0.5% DMSO) (▲). The level of intracellular drug was monitored for an additional 60 min.

uated (Table 1). Epinephrine was the most potent catecholamine tested, with an over-all order of effectiveness of epinephrine > norepinephrine > phenylephrine ≫ isoproterenol. Thus, the net efflux of MTX induced by catecholamines follows the hierarchy of potency of *alpha*-adrenergic effectors (14).

Blockade of MTX efflux by alpha-antagonists. A number of experimental observations provide further evidence for an *alpha*-adrenergic basis for this efflux phenomenon. Figure 2 describes an experiment in which cells were first incubated with MTX; *alpha*- or *beta*-blocking agents were then added, followed 2 min later by epinephrine plus IBMX. The induction of net MTX efflux from hepatocytes by epinephrine plus IBMX was not reduced by the *beta*-antagonist propranolol at a concentration of 25 μM.[5] In contrast, the irreversible *alpha*-antagonist, phenoxybenzamine, at a concentration of 25 μM reduced the subsequent epinephrine plus IBMX-induced net loss of MTX from hepatocytes by 80.3 ± 2.5% (based upon four separate experiments). Inhibition by phenoxybenzamine of MTX efflux induced by epinephrine plus IBMX

[5] Dichloroisoproterenol, like propranolol, was without effect in the induction of [^{3}H]MTX efflux by epinephrine plus IBMX.

TABLE 1

Effectiveness of various catecholamines in induction of ^{3}H efflux after incubation of cells with [^{3}H]MTX

Hepatocytes incubated for 30 min with 1 μM [^{3}H]MTX were exposed to various catecholamines (at 10 μM concentration) in the presence of 0.5 mM IBMX, and the level of intracellular drug was monitored for an additional 60 min. Loss of intracellular ^{3}H is reported as a percentage decrease of the total intracellular drug level. Values in parentheses indicate the number of replicate experiments performed on different days.

Treatment	% Intracellular ^{3}H released
10 μM epinephrine + 0.5 mM IBMX	37.73 ± 4.72 (6)
10 μM norepinephrine + 0.5 mM IBMX	30.42 ± 3.91 (5)
10 μM phenylephrine + 0.5 mM IBMX	21.63 ± 4.57 (5)
10 μM isoproterenol + 0.5 mM IBMX	0.038 ± 5.10 (6)

suggests an $alpha_1$-mediated phenomenon (15). This was confirmed by utilizing the specific $alpha_1$- and $alpha_2$-antagonists prazosin and yohimbine, respectively (15, 16). Table 2 indicates that yohimbine was modestly effective in inhibiting MTX efflux induced by epinephrine plus IBMX, whereas prazosin was essentially equipotent with phenoxybenzamine as an adrenergic inhibitor—consistent with induction of MTX release occurring via an $alpha_1$-mediated pathway.

Calcium dependency of MTX efflux induced by adrenergic agents: effects of vasopressin. There is evidence that *alpha*-adrenergic effectors act by the mobilization of intracellular or extracellular calcium (12, 17–19). To evaluate the effect of reduction in cell calcium on the net loss of MTX induced by epinephrine plus IBMX, hepatocytes were washed and incubated in calcium-free buffer. Accumulation of both exchangeable and less readily exchangeable drug was minimally affected by this treatment. However, the effect of epinephrine plus IBMX was markedly reduced; there was loss of only 13.2 ± 5.6 of intracellular MTX after exposure of cells to these agents in the absence of calcium (based upon seven experiments performed on separate days). This is in contrast to the net loss of 39% of intracellular drug in the presence of calcium (see above). In some experiments (Fig. 3), epinephrine plus IBMX was completely ineffective in inducing drug efflux in the absence of calcium, although net drug uptake was terminated.

Vasopressin has been demonstrated to mimic the *alpha*-adrenergic induction of hepatic glycogenolysis (17), presumably by altering transmembrane calcium flux. Table 3 demonstrates that vasopressin, at a concentration of 1×10^{-8} M, induced significant ^{3}H release from hepatocytes which had accumulated [^{3}H]MTX. The percentage of intracellular ^{3}H released was less than that induced by epinephrine plus IBMX, and increasing the vasopressin dose up to 1×10^{-6} M only minimally increased its effectiveness. Angiotensin II, like vasopressin, exerts *alpha*-adrenergic-like effects on hepatic glycogenolysis (17). However, Fig. 4 indicates that angiotensin inhibits net MTX uptake in an apparently concentration-independent manner but does not induce net loss of intracellular ^{3}H.

The importance of calcium in the adrenergic induction of methotrexate is indicated further in Fig. 5. In these

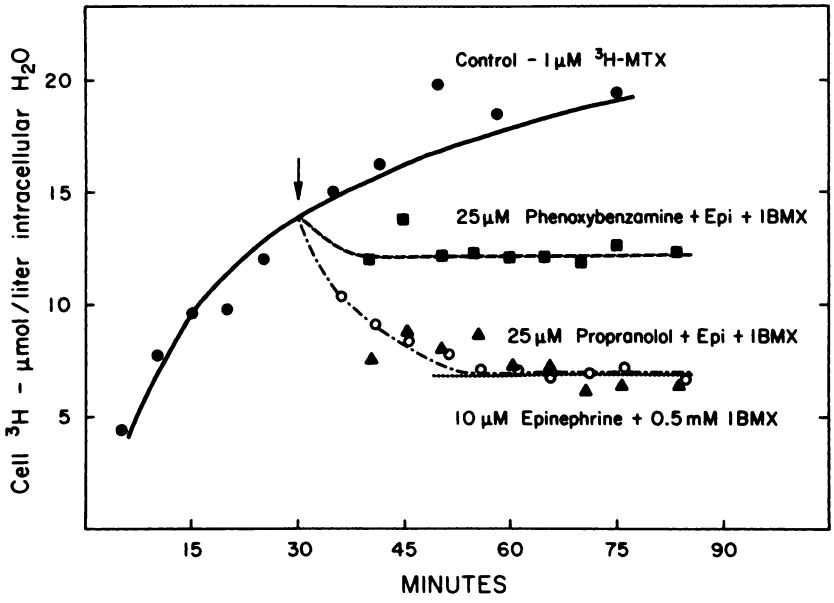

FIG. 2. *Effect of adrenergic antagonists on induction of MTX release by epinephrine plus IBMX*

Hepatocytes incubated with 1 μM [³H]MTX for 30 min were exposed to 10 μM epinephrine (*Epi*) plus 0.5 mM IBMX (○), 25 μM phenoxybenzamine followed 2 min later by 10 μM epinephrine plus 0.5 mM IBMX (■), or 25 μM propranolol followed 2 min later by 10 μM epinephrine plus 0.5 mM IBMX (▲). The level of intracellular drug was monitored for an additional 55 min.

experiments, hepatocytes washed and incubated in calcium-free medium (in the presence of 1 mM EGTA) were exposed to either 10 μM epinephrine, 0.5 μM vasopressin, or 0.5 mM IBMX. In the absence of calcium, epinephrine was without effect on net MTX transport or release. This should be compared with the significant induction of methotrexate release by epinephrine in the presence of calcium shown in Fig. 1. In the absence of calcium, vasopressin inhibited methotrexate uptake, but did not induce the release of antifolate, as described in Table 3. The effect of 0.5 mM IBMX alone, i.e., termination of net drug uptake, was not altered in the absence of calcium, indicating a calcium-independent effect.

Calcium independence of MTX release induced by Bt_2cAMP and IMBX. The apparent absence of a requirement for extracellular calcium in the termination of net MTX uptake by IBMX led to a reexamination of the importance of calcium in the induction of methotrexate release by Bt_2cAMP and IBMX reported previously (6). Table 4 presents the percentage of intracellular ³H released from cells loaded with [³H]MTX by Bt_2cAMP and IBMX in the presence or absence of calcium. The induction of ³H release by IBMX is clearly independent of

TABLE 2

Alpha-antagonist inhibition of epinephrine plus IBMX-induced efflux of MTX

Hepatocytes incubated with 1 μM [³H]MTX for 30 min were exposed to 10 μM epinephrine plus 0.5 mM IBMX alone or with prior exposure to the *alpha*-antagonists. The level of intracellular ³H was monitored for an additional 60 min. The percentage inhibition was calculated from the ratio between differences in intracellular ³H after exposure to epinephrine plus IBMX in the presence and absence of antagonist and the difference in cellular ³H before and after exposure to epinephrine plus IBMX ± standard error: [intracellular ³H (antagonist + epinephrine + IBMX) − intracellular ³H (epinephrine + IBMX)]/[intracellular ³H (initial) − intracellular ³H (epinephrine + IBMX)] × 100. Values in parentheses indicate number of individual experiments performed.

Alpha-antagonist	% Inhibition
Phenoxybenzamine (25 μM)	70.3 ± 10.3 (3)
Prazosin (25 μM)	62.8 ± 7.5 (4)
Yohimbine (25 μM)	28.8 ± 14.7 (4)

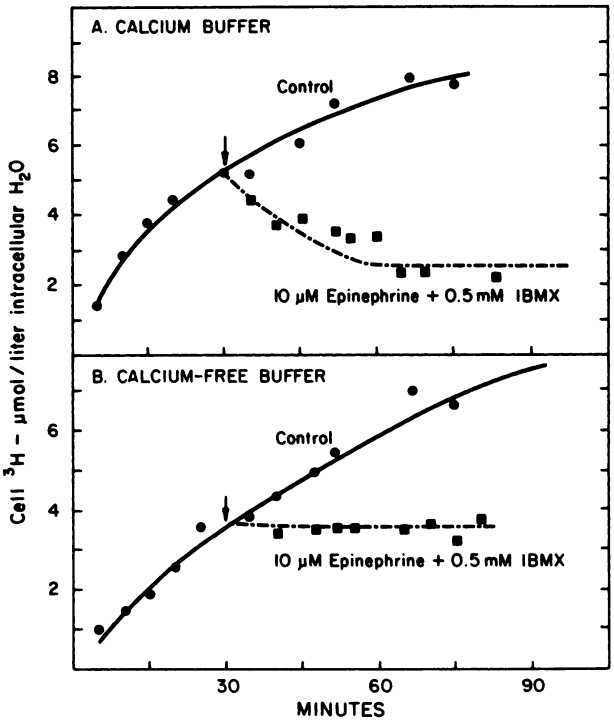

FIG. 3. *Contribution of calcium in induction of MTX release by epinephrine plus IBMX*

Hepatocytes washed in buffer containing 2 mM $CaCl_2$ (*upper panel*) or buffer with calcium excluded and 1 mM EGTA added (*lower panel*) were incubated with 1 μM [³H]MTX for 30 min before exposure to 10 μM epinephrine plus 0.5 mM IBMX. The level of intracellular drug was monitored for an additional 60 min.

TABLE 3

Percentage of intracellular ^3H released by vasopressin

Hepatocytes incubated with 1 μM [^{3}H]MTX for 30 min were exposed to various concentrations of vasopressin, and the level of intracellular ^3H was monitored for an additional 60 min. The percentage of intracellular ^3H lost is expressed (± standard error). Values in parentheses indicate the number of experiments performed on separate days.

Vasopressin concentration	*% Release*
1×10^{-9} M	5.1 ± 3.4 (3)
1×10^{-8} M	20.6 ± 5.5 (3)
1×10^{-7} M	24.4 ± 3.2 (4)
1×10^{-6} M	28.1 ± 6.0 (3)

extracellular calcium, whereas the induction of methotrexate release by Bt_2cAMP is only modestly attenuated in a calcium-free buffer.

Further evidence that induction of MTX release by Bt_2cAMP is independent of the release induced by alpha-adrenergic agents. The relative insensitivity to calcium of MTX release induced by Bt_2cAMP or IBMX suggests a separate mechanism of action for these agents and the adrenergic compounds (whose activities are clearly calcium-dependent). Further evidence for this independence of action is presented in the studies described in Fig. 6. In these studies, 10 μM epinephrine and 0.5 mM Bt_2cAMP induced an equivalent loss of cell ^3H from hepatocytes incubated with [^{3}H]MTX. When these agents were added together, the loss of ^3H was the sum of the independent effects of these compounds, consistent with induction of antifolate release occurring via independent mechanisms.

Effect of A23187, butyrate, ascorbate, or cyclic GMP. Additional studies which verified the importance of calcium in adrenergic induction of MTX release involved the calcium ionophore A23187. Figure 7 demonstrates that 5 μM A23187 induces ^3H release upon addition to a hepatocyte suspension at steady state with exchangeable [^{3}H]MTX. A23187 was also effective at a concentration of 2 μM (data not shown). Figure 7 also indicates that dibutyryl cyclic GMP is effective in inducing [^{3}H]MTX efflux. The minimal perturbations of [^{3}H]MTX uptake

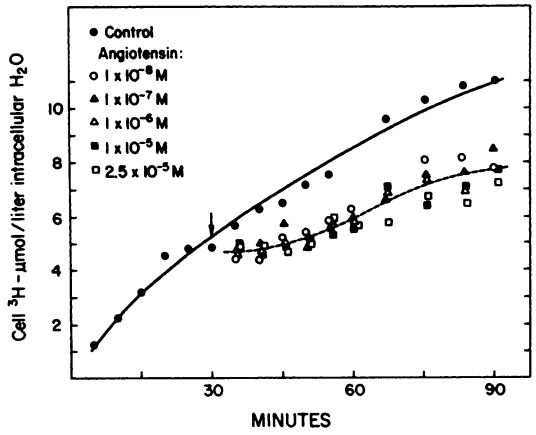

FIG. 4. *Exposure of hepatocytes containing MTX to angiotensin*

Hepatocytes incubated with 1 μM [^{3}H]MTX for 30 min were exposed to various concentrations of angiotensin II. The level of intracellular ^3H was monitored for an additional 60 min.

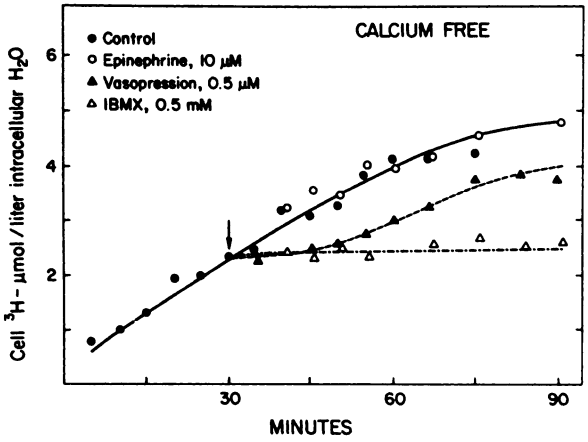

FIG. 5. *Lack of induction of MTX release by vasopressin or epinephrine in the absence of calcium*

Hepatocytes washed and resuspended in calcium-free buffer containing 1 mM EGTA were incubated for 30 min with 1 μM [^{3}H]MTX before exposure to 10 μM epinephrine (○), 0.5 mM vasopressin (▲), or 0.5 mM IBMX (△).

by 2.5 mM sodium butyrate or 10 mM ascorbate, respectively, indicate that the effects of the dibutyryl derivatives are not artifacts induced by breakdown to free butyrate and that the effects of the adrenergic agents are not a function of changes induced by the ascorbate in the incubation medium.

DISCUSSION

These studies demonstrate the induction of net efflux of the folic acid analogue, MTX, from freshly isolated rat hepatocytes in suspension by concentrations of catecholamines (and vasopressin) which have been shown to modulate various biosynthetic and degradative processes in liver (17–20). These data represent the first report of an adrenergic-induced efflux phenomenon in hepatocytes that is not associated with a biosynthetic or degradative process (e.g., glycogenolysis). Secretion of various hormones, like glucagon and adrenocorticotropin, has likewise been reported to be under adrenergic control (21, 22). Because of the likelihood that this induction of net loss of MTX from isolated cells may be a corollary of MTX secretion into the bile canaliculus and/or hepatic sinusoid when the hepatocyte is oriented in the liver lobule, these observations suggest the possibility of an

TABLE 4

Effect of Ca^{2+} on induction of [^{3}H]MTX release by Bt_2cAMP or IBMX

Hepatocytes washed and incubated in either K-H buffer containing 2 mM Ca^{2+} (control) or K-H buffer in the absence of calcium and the presence of 1 mM EGTA (Ca^{2+}-free) were allowed to accumulate [^{3}H]MTX (at an extracellular concentration of 1 μM) for 30 min before exposure to Bt_2cAMP or IBMX. Intracellular ^3H was monitored for an additional 60 min. The percentage of ^3H released is expressed as mean ± standard error. Values in parentheses indicate the number of experiments performed on separate days.

Treatment	% ^{3}H released	
	Control	Ca^{2+}-free
Bt_2cAMP (2.5 mM)	42.4 ± 6.8 (4)	34.5 ± 1.5 (6)
IBMX (2.5 mM)	35.6 ± 4.7 (3)	38.1 ± 1.4 (3)

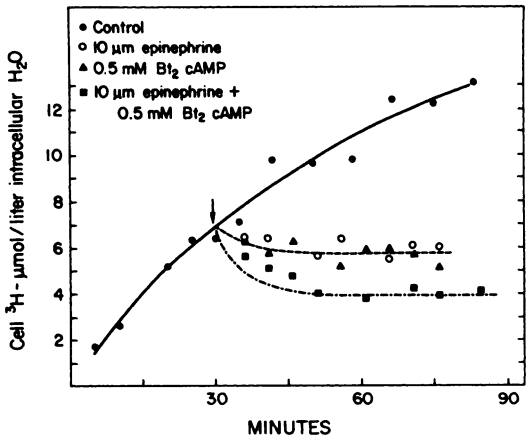

FIG. 6. *Induction of MTX release by epinephrine in the absence or presence of Bt_2cAMP*

Hepatocytes incubated for 30 min with 1 μM [^{3}H]MTX were exposed to 0.5 mM Bt_2cAMP (▲), 10 μM epinephrine (○), or 10 μM epinephrine plus 0.5 mM Bt_2cAMP (■).

adrenergic-controlled "secretory" phenomenon in liver *in vivo*.

The data further suggest that the induction of the net loss of MTX from hepatocytes by catecholamines has the characteristics of an *alpha*-adrenergic-mediated phenomenon. This suggestion is based upon the following observations: (a) the order of potency of the catecholamines in the induction of net MTX efflux is epinephrine > norepinephrine > phenylephrine ≫ isoproterenol, following the hierarchy of the *alpha*-adrenergic effectors (14); (b) efflux induced by epinephrine plus IBMX is markedly inhibited by the *alpha* blockers phenoxybenzamine and prazosin (15, 16), whereas the *beta* blockers dichloroisoproterenol and propranolol (23) are without effect; and (c) the induction of net MTX efflux by catecholamines is, at least in part, calcium-dependent, consistent with reports that a reorganization of intracellular calcium (via intracellular organellar or transmembrane calcium flux) is a basic component in the response to *alpha*-adrenergic stimuli (17–19).

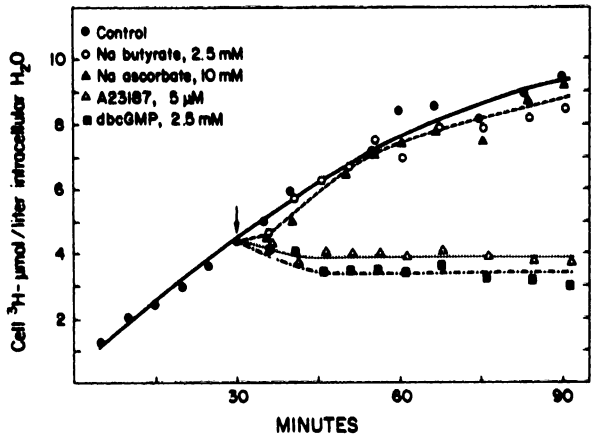

FIG. 7. *Exposure of hepatocytes containing MTX to butyrate, ascorbate, A23187, or Bt_2cGMP*

Hepatocytes incubated with 1 μM [^{3}H]MTX (in an ascorbate-free buffer) for 30 min were exposed to 2.5 mM sodium butyrate (○), 10 mM sodium ascorbate (▲), 5 μM A23187 (△), or 2.5 mM dibutyryl cyclic GMP (*dbcGMP*) (■).

The modest inhibition by yohimbine of epinephrine plus IBMX-induced release of MTX in the face of potent inhibition by phenoxybenzamine and prazosin suggests that catecholamine induction of MTX release takes place via an $alpha_1$-adrenergic mechanism. This is further consistent with studies indicating that the adrenergic receptors in the rat hepatocyte are primarily of the $alpha_1$ subtype (24).

Finally, induction of MTX release by vasopressin, which mimics *alpha*-agonists in control of hepatic carbohydrate metabolism, further supports the *alpha*-adrenergic nature of these phenomena. However, the lack of induction of MTX release by angiotensin II is not readily explained; this may indicate some lack of sensitivity of the putative angiotensin receptor under our experimental conditions.

The importance of calcium in the mediation of *alpha*-adrenergic release of MTX from rat hepatocytes is evident in these studies. The effectiveness of epinephrine, epinephrine plus IBMX, and vasopressin is markedly reduced in a calcium-free environment. A great deal of controversy exists in the literature on the relative contributions of calcium from intracellular pools versus extracellular calcium in *alpha*-adrenergic phenomena (18, 25, 26). The present experiments do not distinguish between the importance of intracellular and extracellular calcium in the mediation of these effects, as intracellular as well as extracellular calcium may be depleted by washing in the presence of 1 mM EGTA (12). The observations that MTX release induced by Bt_2cAMP or IBMX is relatively calcium-independent whereas that for the adrenergic agents is markedly calcium-dependent, and the additive effects of Bt_2cAMP and epinephrine in the induction of MTX release, indicate that these agents act via independent mechanisms. This is not unlike the control of hepatic glycogenolysis, where adrenergic agents act via a calcium-dependent *alpha*-adrenergic pathway and glucagon acts via a calcium-independent, cyclic AMP-dependent pathway (27). However, our studies do not indicate that cyclic AMP is the mediator of the effects of Bt_2cAMP or IBMX in induction of MTX efflux.

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