# EVIDENCE AGAINST A REQUIREMENT FOR PHOSPHOLIPID METHYLATION IN ADENYLATE CYCLASE ACTIVATION BY HORMONES

## Methyltransferase inhibitors do not impair cyclic AMP accumulation induced by glucagon or β-adrenergic agents in rat hepatocytes

J.-S. SCHANCHE, D. ØGREID[†], S. O. DØSKELAND[†], M. REFSNES[+], T. E. SAND[+], P. M. UELAND and T. CHRISTOFFERSEN[+,*]

*Department of Pharmacology, MFH-bygget, Haukeland Sykehus, [†]Department of Anatomy, Årstadveien 19, University of Bergen, N 5000 Bergen* and *[+]Institute of Pharmacology, University of Oslo, P. O. Box 1057, Blindern, Oslo 3, Norway*

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### 1. Introduction

Phospholipid methylation has been implicated in the transduction through plasma membranes of a variety of receptor-mediated signals [1], including activation of adenylate cyclase by isoprenaline [2,3], lectin-induced formation of arachidonic acid, $Ca^{2+}$-influx and mitogenesis [4,5], antigen-stimulated histamine release [4,6], and response to chemoattractants [1,7]. Transmethylation is one of the 2 pathways for the formation of phosphatidylcholine, proceeding by successive steps of enzymatic transfer of methyl-groups to phosphatidylethanolamine from the donor *S*-adenosylmethionine (AdoMet), to yield the final product via mono- and di-methylated intermediates [8–11]. The evidence for the hypothesis that this transmethylation is involved in linking receptor signals to cellular responses is derived from experiments showing that phospholipid methylation led to increased membrane fluidity [12], that the various kinds of cell activation were associated with methylation of phosphatidylethanolamine [1–6], and that some of these processes could be blocked by the use of methyltransferase inhibitors [4–6,13]. Still the causal relationship between these phenomena may be questioned [14,15].

In the case of adenylate cyclase and phospholipid methylation, conflicting results exist. Data from reticulocytes may indicate that interaction between the β-adrenergic receptor and the agonist promotes receptor–adenylate cyclase coupling via phospholipid transmethylation [2]. In contrast, results from liver indicate no effect of AdoMet-induced phospholipid methylation on adenylate cyclase [16], while the existence of cyclic AMP-dependent phospholipid methyltransferase in rat liver microsomes has been reported [17]. Furthermore, in lymphocytes transmethylation inhibitors were found to enhance, rather than diminish, cyclic AMP responses to a variety of agents [18].

We show here that in isolated hepatocytes the phospholipid transmethylation can be inhibited almost completely (>95%), by pretreatment with $c^3$Ado and Hcy, without any impairment of the cyclic AMP response to glucagon, adrenaline or isoprenaline. These results indicate that neither an increase nor a sustained high rate of phospholipid methylation is essential for the full responsiveness of the adenylate cyclase to glucagon or β-adrenergic agents.

*Abbreviations:* AdoMet, *S*-adenosyl-L-methionine; Hcy, homocysteine; AdoHcy, *S*-adenosyl-L-homocysteine; $c^3$Ado, 3-deazaadenosine; $c^3$AdoHcy, *S*-3-deazaadenosyl-L-homocysteine; PuoHcy, a purine nucleoside analogue of AdoHcy; MIX, 1-methyl-3-isobutylxanthine; Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid

* To whom correspondence should be addressed

### 2. Experimental

L-[*methyl*-$^3$H]Methionine (75 Ci/mmol) was from the Radiochemical Centre, Amersham. Other chemi-

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cals and radioisotopes were from the sources in [19–21].

Male Wistar rats (~200 g) fed ad libitum were used. In some animals 70% hepatectomy [22] was done to induce liver regeneration. Hepatocytes were isolated by either of 2 modifications [19,23] of an in vitro collagenase perfusion technique [24].

The hepatocytes were suspended in Krebs-Ringer solution with 25 mM Hepes (pH 7.4), containing 10 mM glucose and 1% bovine serum albumin [25]. Incubations were performed using 0.8–2.0 ml cell suspension (1–5 × $10^6$ cells/ml), under continuous shaking at 37°C. During a preincubation period of 40 min some of the incubates were exposed to the following agents, singly or in combination: $c^3$Ado, Hcy, and 50 μM unlabelled l-methionine of L-[*methyl*-$^3$H]methionine (62.5 mCi/mmol). Other additons and length of subsequent incubations were as indicated. Unless otherwise specified, the incubations were terminated by quickly spinning the cells down (1000 × *g*, 20 s) before homogenizing them in the appropriate extraction solutions for further analyses (see below).

### 2.1. *Determination of [$^3$H]*methyl *incorporation into phospholipids, and identification of radioactive phospholipids*

Pellets from cell suspensions that had been exposed to [*methyl*-$^3$H]methionine were homogenized in 1.2 M HCl, collected on glass-fiber filters, and washed and extracted as in [21] for determination of total phospholipid radioactivity. The butanol extract was analyzed by thin-layer chromatography [21], and >90% of the radioactive material chromatographed as phosphatidylcholine/lysophosphatidylcholine.

### 2.2. *Determination of cellular content of AdoMet, AdoHcy and $c^3$AdoHcy*

Cell pellets containing 2–3 × $10^6$ hepatocytes were extracted with 5% sulfosalicylic acid. The extracts were analyzed by high-pressure liquid chromatography on a Partisil SCX column, eluted isocratically, using 200–600 mM ammonium formate (pH 3–4) as the mobile phase.

### 2.3. *Assay of cyclic AMP and adenylate cyclase*

Pellets from the incubates (or in some cases full cell suspensions) were homogenized in either 0.3 M trichloroacetic acid or 0.6 M perchloric acid. The trichloroacetic acid extracts were neutralized with $CaCO_3$ [26], and acetylated and assayed for cyclic AMP by radioimmunoassay [27]. The extracts in perchloric acid were mixed with a half volume of 1.08 M KOH/0.9 M $KHCO_3$, centrifuged and analyzed by a protein binding assay [28]. Adenylate cyclase activity was measured in lysates of hepatocytes as in [29], using 0.2 mM [α-$^{32}$P]ATP and Dowex 50/alumina double columns.

## 3. Results

### 3.1. *Perturbation of phospholipid methylation*

$c^3$Ado perturbs various transmethylation reactions in the intact cells, by increasing the cellular content of the endogeneous inhibitor, AdoHcy, and by serving as a metabolic precursor for the Ado-Hcy analogue, $c^3$AdoHcy [30]. The supply of exogeneous Hcy further enhances the accumulation of AdoHcy (see below). Treatment of hepatocytes with 0.3 mM $c^3$Ado reduced the rate of phospholipid methylation by ~97%. The inhibition was also observed in the presence of glucagon, with or without MIX (fig.1). $c^3$Ado increased (by ~70%) the specific activity of AdoMet (not shown). No significant effect of glucagon on the uninhibited (i.e., no $c^3$Ado) rate of incorporation of [$^3$H]methyl groups into phospholipids was noted in our experiments (fig.1). The ratio AdoMet/PuoHcy

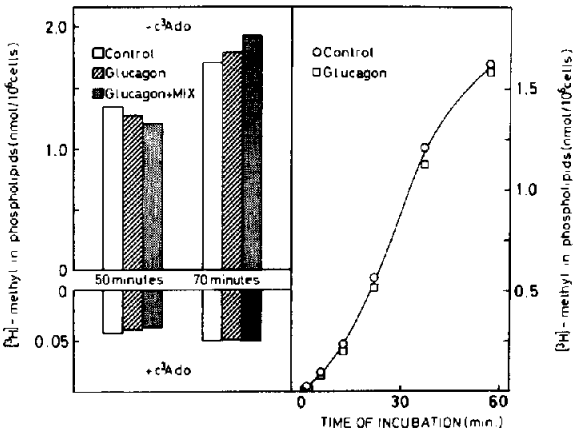


Fig.1. Phospholipid methylation in rat hepatocytes. Left: incorporation of [$^3$H]methyl-groups from [$^3$H]methionine into phospholipids of hepatocytes incubated for 40 min with radioactive methionine in the absence and presence of $c^3$Ado (0.3 mM). At this time point, portions of the cell suspension were supplemented with glucagon (0.1 μM), glucagon plus MIX (0.5 mM) or no addition (control), and further incubated with these agents. The total incubation time is shown on the figure. Right: time course of phospholipid methylation in the absence (control) and presence of glucagon (0.1 μM).

Table 1
Effect of precursors for transmethylation inhibitors on hepatocyte *S*-adenosylmethionine (pmol/$10^6$ cells) and methylation index (*MI*)

| Addition during preincubation | AdoMet | AdoHcy | $c^3$AdoHcy | *MI* |
|---|---|---|---|---|
| No inhibitor | 390 | 68 | – | 6 |
| 3-Deazaadenosine (0.3 mM) | 860 | 590 | 1200 | 0.5 |
| 3-Deazaadenosine + D,L-homocysteine (0.35 mM) | 770 | 750 | 2200 | 0.25 |

Incubation time was 40 min. *MI* = *S*-adenosylmethionine/ total purine derivatives of homocysteine

(i.e., the ratio between AdoMet and all purine nucleoside analogues of Hcy), termed methylation index (*MI*), has been stated to reflect the transmethylating capacity of the cell [30,31]. The cellular content of AdoMet and AdoHcy in the untreated hepatocytes was ~390 and 70 pmol/$10^6$ cells, respectively, corresponding to *MI* ≈ 6. Preincubation of the cells with 0.3 mM $c^3$Ado for 40 min led to a massive accumulation of AdoHcy and $c^3$AdoHcy, and only a moderate increase of AdoMet, with *MI* dropping to ~0.5 (table 1). A further decrease (to 0.25) was observed when $c^3$Ado was combined with 0.5 mM Hcy. Prolonged incubation led to even lower *MI*-values (not shown). Although these data indicate that Hcy decreases further the methylating capacity of $c^3$Ado-treated cells, and might enhance the pronounced inhibition of phospholipid methylation that $c^3$Ado exerts when administered alone (fig.1), measurements of the methylated phospholipids after combination of the 2 inhibitors were not carried out because Hcy may affect the disposition of extracellular [$^3$H]methionine.

### 3.2. *Influence of methyltransferase inhibitors on hepatocyte cyclic AMP levels*

Hepatocytes exposed for 40 min to $c^3$Ado, alone or in combination with Hcy, showed significantly elevated levels of cyclic AMP (25 and 50% above basal, respectively). The attenuation of this effect when the phosphodiesterase inhibitor MIX was present (fig.2A) suggest that $c^3$Ado and Hcy increased the cyclic AMP level by inhibiting the breakdown rather than enhancing the synthesis.

### 3.3. *Influence of methyltransferase inhibitors on the cyclic AMP accumulation and adenylate cyclase activation induced by glucagon and β-adrenergic agents*

Preincubation of hepatocytes with $c^3$Ado, or $c^3$Ado plus Hcy, did not reduce the cyclic AMP response to glucagon (fig.2,3). The time course of the cyclic AMP accumulation (fig.2) showed that at early stages no

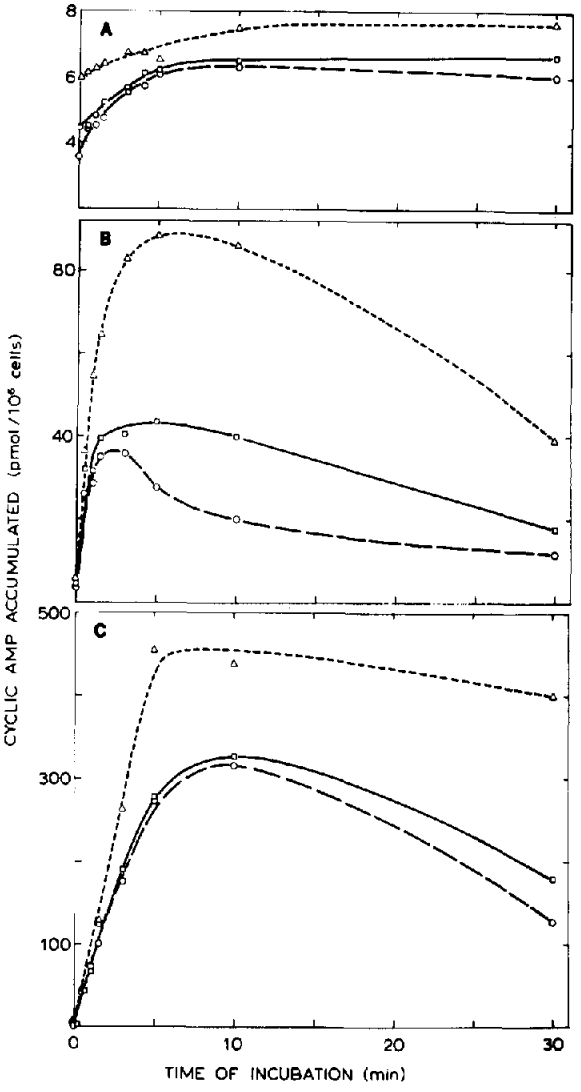


Fig.2. Effect of methyltransferase inhibitors on the time course of cyclic AMP accumulation in rat hepatocytes. (A) The effect of MIX (0.5 mM): (—○—) control cells; (—□—) cells preincubated (40 min) with $c^3$Ado (0.3 mM); (- -△- -) cells preincubated with $c^3$Ado + Hcy (0.35 mM). (B) The effect of glucagon (2 nM). Symbols as above. (C) The effect of glucagon + MIX. Concentrations and symbols as above.

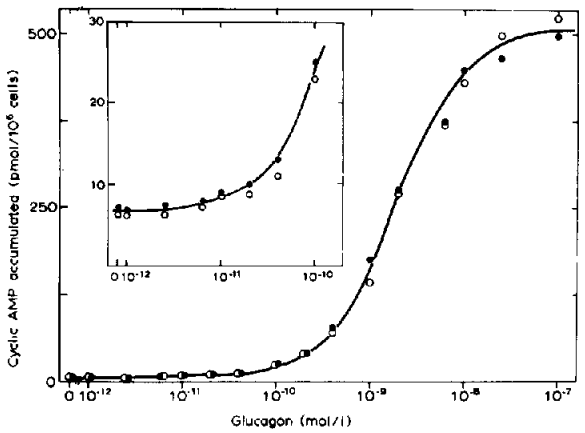


Fig.3. Effect of methyltransferase inhibitors on the cyclic AMP content of hepatocytes incubated with varying concentrations of glucagon. Untreated cells (–○–) and cells preincubated (40 min) with 0.3 mM $c^3$Ado (–●–) were incubated for 5 min with glucagon in the presence of 0.5 mM MIX. Inset shows the response to very low concentrations of glucagon.

significant difference existed between control cells and those treated with methyltransferase inhibitors, but increasing length of incubation with the hormone led to considerably enhanced accumulation in the $c^3$Ado/Hcy-treated cells, probably due to phosphodiesterase inhibition.

Since in rat hepatocytes maximal cyclic AMP formation appears to require occupation of only a small fraction of the glucagon receptors [25], blockage of a phospholipid methylation-dependent receptor–cyclase coupling theoretically would lead to a right-shifted dose–response curve, without altering the maximal response. However, in cells incubated with hormone for 5 min (fig.3) or 90 s (not shown) no difference in dose–response curves for glucagon between control cells and hepatocytes treated with $c^3$Ado for 40 min was seen (fig.3).

Because the hypothesis that phospholipid methylation promotes receptor–cyclase coupling was based on studies with β-adrenergic receptor activation [1–3], the effects of adrenaline and isoprenaline were also examined. We used the observation that adrenergic responsiveness, which is normally very low in rat liver, increases during regeneration [32]. It was found that in hepatocytes isolated 48 h after 70% hepatectomy the strong response to adrenaline was not diminished by preincubation with methyltransferase inhibitors (table 2), and the initial time course or dose–response curve for cyclic AMP accumulation after isoprenaline (fig.4) were not altered. Again, in prolonged incubations $c^3$Ado plus Hcy amplified the response to isoprenaline.

To exclude the possibility that a minor inhibition of cyclic AMP formation by transmethylation inhibitors might be masked by their inhibition of the breakdown, hepatocytes (preincubated as described for the measurement of cyclic AMP accumulation) were quickly lysed and assayed for hormone-sensitive adenylate cyclase activity. No reduction in the treated cells was found (table 3).

Table 2
Effect of methyltransferase inhibitors on hormone-induced accumulation of cyclic AMP (pmol/$10^6$ cells) in hepatocytes from regenerating rat liver

| Addition during preincubation | Basal | Glucagon (1.4 μM) | Adrenaline (50 μM) |
|---|---|---|---|
| No inhibitor | 9.8 ± 0.6 | 212.5 ± 18.9 | 180.8 ± 9.5 |
| 3-Deazaadenosine (0.3 mM) | 12.9 ± 3.1 | 203.0 ± 25.0 | 167.8 ± 20.5 |
| D,L-Homocysteine (0.35 mM) | – | 235.5 ± 29.1 | 194.5 ± 23.2 |
| 3-Deazaadenosine + D,L-homocysteine | – | 227.8 ± 11.5 | 183.8 ± 17.5 |

Preincubation time with or without inhibitors was 40 min. The cells were exposed to hormones for 90 s in the presence of 0.5 mM MIX before the assay of cyclic AMP. The results are mean ± SEM of 4 replicates

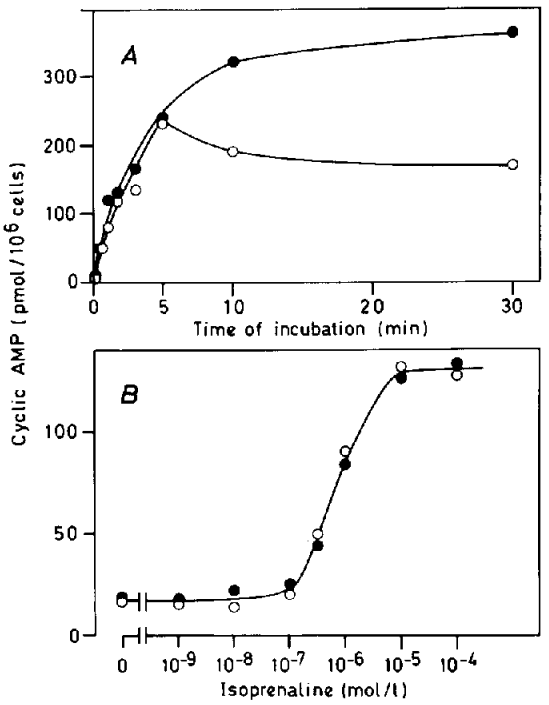


Fig.4. Effect of methyltransferase inhibitors on the cyclic AMP response of hepatocytes (from regenerating rat liver) to isoprenaline. (A) Time course of cyclic AMP accumulation induced by 50 μM isoprenaline: (–○–) control cells; (–●–) cells preincubated (40 min) with $c^3$Ado (0.3 mM) + Hcy (0.35 mM). (B) Dose–response relationship for the cyclic AMP accumulation after 90 s exposure to varying concentrations of isoprenaline: (–○–) control cells; (–●–) cells preincubated (40 min) with $c^3$Ado (0.3 mM). MIX was present (0.5 mM) during all incubations after the pretreatment with methyltransferase inhibitors.

Table 3
Effect of methyltransferase inhibitors on adenylate cyclase activity (pmol cyclic AMP . $min^{-1}$ . mg $protein^{-1}$) in hepatocytes from regenerating rat liver

| Addition during preincubation | Basal | Glucagon (14 μM) | Isoprenaline (50 μM) |
|---|---|---|---|
| No inhibitor | 3.9 | 25.2 | 10.6 |
| 3-Deazaadenosine (0.3 mM) | 4.9 | 28.7 | 12.7 |
| 3-Deazaadenosine + D,L-homocysteine (0.35 mM) | 4.2 | 28.8 | 13.1 |

Hepatocytes were preincubated with or without methyltransferase inhibitors for 40 min. After quickly spinning the cells down, they were lysed and assayed for adenylate cyclase activity. The results are mean of 2 expt

## 4. Discussion

Here, inhibition of phospholipid methylation in isolated rat hepatocytes did not affect the time course or the dose–effect relationship for the cyclic AMP response to glucagon or β-adrenergic agents. The results strongly disfavour the concept that the transmethylation is essential for receptor–adenylate cyclase coupling. Although the experimental conditions were not directly comparable, this is not in accordance with the data obtained in rat reticulocytes [2] which partly formed the basis for the hypothesis of the role of phospholipid methylation [1]. In those cells it was found that pretreatment with AdoMet increased isoprenaline-sensitive adenylate cyclase activity and that the stimulatory action of isoprenaline on phosphatidylethanolamine methylation [2] could not be mimicked by 8-Br-cyclic AMP, cholera toxin or NaF [2], indicating that the effect was exerted by β-receptors independently of cyclic AMP. These results are, however, compatible with experiments on rat liver membranes, where exposure to AdoMet, which enhanced the formation of methylated phospholipids, did not modify the basal, NaF-, GTP- or glucagon-sensitive adenylate cyclase activity [16]. Furthermore, the fact that in our experiments the stimulation of the cyclic AMP formation after β-adrenergic agents as well as after glucagon was unaltered, despite almost complete inhibition of phospholipid methylation, indicated that at least in these cells transmethylation is not selectively required for the β-receptor-mediated activation of the adenylate cyclase.

Phospholipid methylation has been reported to increase the number of β-receptors in reticulocytes [13]. Thus, although we found that the cyclic AMP response to isoprenaline was not diminished after incubation with transmethylation inhibitors for up to 70 min, the possibility that even longer pretreatment might have revealed impairment of the adrenergic effect was not ruled out. Neither can we exclude the possibility that hepatocytes from regenerating liver, which have strongly enhanced adrenaline-responsive cyclic AMP formation [32] and increased number of β-receptors (unpublished), have become less dependent on transmethylation for the β-adrenergic activation. Further studies are required to decide if phospholipid methylation might be involved in the increased adrenaline responsiveness of the hepatocyte adenylate cyclase that is observed in a variety of conditions [32–34].

The report that both glucagon and cyclic AMP increase the phospholipid methyltransferase activity (determined in broken cell preparations) of hepatocytes [35] (although not confirmed under these conditions) provides support for the concept [1] that hormones may stimulate phospholipid methylation, but indicate that this is secondary to, rather than a trigger of, the adenylate cyclase activation.

The observation that $c^3$Ado plus Hcy exerted long-term amplification of the cyclic AMP responses in the hepatocytes may support the data from lymphocytes [18] and oocytes [36] that agents that inhibit methyltransferase can influence the cyclic AMP system by mechanisms not necessarily involving AdoMet-dependent phospholipid methylation.

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