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The genetics of hormone-induced cyclic AMP production and Phospholipid *N*-methylation in inbred strains of mice

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SUMMARY

Genetic variation in hormone-sensitive cyclic AMP production was investigated among inbred strains of mice. Significant strain differences were observed in β-adrenergic- and glucagon-stimulated adenylate cyclase activity. Comparable differences were also found in membrane methyltransferase I activity in these strains. Our results of studies using F_1 progeny of high and low strains suggest a dominance of high MT I activity over low MT I activity. Investigation of recombinant inbred lines between the high and low strains indicates that MT I activity is regulated by at least two major genes; *H-2*-congenic lines of several inbred strains were then used to identify an association between hormone-stimulated MT I activity and the mouse major histocompatibility complex.

1. INTRODUCTION

A wide variety of hormones and neurotransmitters are known to interact with specific cell surface receptors, thereby activating membrane-bound adenylate cyclase, resulting in an increase in intracellular cyclic AMP (Robison, Butcher & Sutherland, 1971). While the binding of ligands to various biological membranes has been quite well characterized, the subsequent molecular events leading to the activation of adenylate cyclase are still unclear. At least three components have been shown to comprise the hormone-sensitive adenylate cyclase system: (1) the hormone receptor, (2) a nucleotide regulatory unit, and (3) the catalytic unit, adenylate cyclase (Rodbell, 1980). The interactions of these three are modulated by the ligand (hormone) and a guanine nucleotide and involve the hydrolysis of GTP (Ross & Gillman, 1980; Ross, 1982). Although each of the three components has been solubilized in active form, the hormonal modulation of adenylate cyclase seems to require a relatively unperturbed membrane bilayer (Ross, 1982).

The specific lipid composition of the membrane appears to affect the adenylate cyclase system, as has been demonstrated by Cuatrecasas and co-workers (Cuatrecasas, 1974; Sayhoun *et al.* 1981). These investigators have shown in reconstitution

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experiments that the activity of the cyclase was stimulated by addition of phosphatidylcholine. Ross (1982) has also reported that the effects of phospholipids on the catalytic unit of this system and its coupling with the nucleotide regulatory component are specific for phosphatidylcholine. Furthermore, Ross (1982) suggests that the phosphatidylcholine is important primarily for the interaction of the two components rather than for the intrinsic activity of either.

Axelrod and co-workers (Hirata & Axelrod, 1978) have strongly implicated the process of membrane phospholipid *N*-methylation as a regulatory mechanism for β-adrenergic stimulation of adenylate kinase. Phospholipid *N*-methylation involves the sequential synthesis of phosphatidylcholine from phosphatidylethanolamine by two methyltransferases (MT I and MT II) which utilize S-adenosyl-L-methionine as the methyl donor. In certain systems, β-adrenergic agonist binding has been shown to increase methyltransferase activity and, conversely, increased membrane methylation appears to increase β-adrenergic stimulation of cyclic AMP production (Hirata & Axelrod, 1980). These effects have been postulated to be due to an increase in membrane fluidity resulting from the rapid translocation of phospholipids across the membrane. This increase in the fluidity, then, facilitates the coupling of the individual components of the adenylate cyclase system. The glucagon-stimulated adenylate cyclase activity has been shown to have a similar dependence on membrane phospholipid *N*-methylation (Castaño *et al.* 1980). Edidin and co-workers (Meruelo, 1974; Meruelo & Edidin, 1974; Lafuse & Edidin, 1978, 1980; Lafuse, Meruelo & Edidin, 1979) have reported an association of the mouse major histocompatibility complex, *H-2*, with the glucagon-stimulated production of cyclic AMP. From the work of Lafuse & Edidin (1978, 1980), it is evident that *H-2* affects glucagon-stimulated cyclic AMP production by exerting its effects at the level of the receptor. The mouse MHC has also been shown to influence liver MT I activity in inbred and congenic strains (Markovac & Erickson, 1984).

We have investigated the genetic variation in β-adrenergic and glucagon-stimulated cyclic AMP production with respect to the related membrane methylation activity among inbred strains of mice. We find significant strain differences in hormone-stimulated adenylate cyclase activity as well as in MT I activity. Our studies using *H-2* congenic strains also indicate an association of hormone-stimulated MT I activity with the mouse MHC. Furthermore, we find dominance of high MT I activity over low MT I activity and suggest that this activity is under the control of at least two major genes.

2. MATERIALS AND METHODS

(i) *Animals*

The A/J and C57BL/6J mice and their congenic strains were derived from mating pairs obtained from the Jackson Laboratory, Bar Harbor, ME. The recombinant inbred lines between the A/J and C57BL/6J strains were maintained by brother–sister matings of the original stocks (a gift from Dr Muriel Nesbitt, University of California, San Diego, La Jolla, CA).

(ii) *Preparation of hypatocytes*

Livers were rapidly excised from cervically dislocated animals and minced finely in ice-cold 1 mM-$NaHCO_3$, pH 7·4 buffer. The suspension was homogenized for five strokes at low speed with a Thomas Model 45 homogenizer and centrifuged for 1 min at 500 ***g***. The pellet was discarded and the supernatant recentrifuged for 10 min at 1500 ***g***. The resulting pellet was quick-frozen in a dry ice–ethanol bath and stored at −20 °C until use.

(iii) *Assay for determination of cyclic AMP*

Hepatocyte suspension (50–100 μg protein/ml) was preincubated with 50 μM-L-isoproterenol for 10 min at 37 °C. After addition of an equal volume of RPMI medium, the suspension was boiled for 15 min and centrifuged for 15 min at 1500 ***g***. The resulting supernatant was utilized for quantitation of cyclic AMP production by radioimmunoassay, as described by Frandsen & Krishna (1977). Cyclic AMP concentrations (pmol/μg protein) were calculated using the radioimmunoassay program of Duddles, Niswender & Midgley (1972). Protein content was determined by the method of Lowry *et al.* (1951).

(iv) *Assays for phospholipid methyltransferase*

The pellets were thawed by vigorous shaking in homogenization buffer, described by Castaño *et al.* (1980). Methyltransferase activities were determined essentially as described by Hirata *et al.* (1978). Briefly, the methylation rate of phosphatidylethanolamine (PE) by MT I was obtained by measuring the incorporation of radiolabel from S-adenosyl (methyl ^{3}H) L-methionine ([^{3}H]SAM) into phosphatidylmonomethylethanolamine (PMME). The reaction medium contained 10 mM $MgCl_2$, 0·1 mM sodium EDTA and 50 mM sodium acetate, pH 6·5. Ten μl (0·05–0·10 mg protein) of hepatocyte extract in 50 mM HEPES, 10 mM NaF, and 15 mM EGTA, pH 7·4, was added to a total volume of 50 μl. The assay was initiated by addition of 4 μM[^{3}H]SAM (15 Ci/mmol) and incubated for 30 min at 37 °C. The assay was terminated by addition of 3 ml chloroform/methanol/HCl (2/1/0·02, v/v) and 2 ml 0·1 M-KCl in 50% methanol. The tubes were shaken vigorously for 15 min and centrifuged for 10 min at 2000 ***g***. Following aspiration of the aqueous phase, the chloroform layer was rewashed with 2 ml 0·1 M KCl in 50% ethanol. One ml of the resulting chloroform phase was dried in a scintillation vial at 60 °C and counted with Instagel scintillation fluid. All the samples were assayed in duplicate and averaged.

MT II activity was assayed with 50 mM sodium borate buffer, pH 10, [^{3}H]SAM (500 mCi/mmol) and 100 μg PMME by the procedure described above.

Inhibition of transmethylation by S-adenosyl L-homocysteine was determined by its addition to the reaction mixture at a final concentration of 1 mM.

(v) *Visualization of products*

The chloroform phase was dried under nitrogen gas and the residue dissolved in 50 μl chloroform/methanol (2/1 v/v). The samples were applied to Silica Gel G chromatograph plates and developed in chloroform/methanol/water (75/25/4 v/v). Commerically available markers of PE, PMME, PDME and PC were co-chromatographed with the samples and the spots visualized by spraying with 0·06% Rhodamine 6G solution.

(vi) *Chemicals and reagents*

Chemicals for radioimmunoassay were obtained from Amersham, Arlington Hts, IL, as was the [^{3}H]SAM (5–15 Ci/mmol and 500 mCi/mmol). [^{3}H]SAM (5–15 Ci/mmol) was also obtained from ICN, Irvine, CA. S-adenosyl, L-homocysteine, L-isoproterenol, and the phospholipid markers were obtained from Sigma Chemical Co., St Louis, MO. All other reagents were obtained from Mallinckrodt, Paris., KY.

Table 1. *Isoproterenol-stimulated cyclic AMP production in mice*

Strain	*N*	Cyclic AMP* (pmol/μg protein)
A/J	4	0·45 ± 0·01
C57BL/6J	4	0·74 ± 0·02
AB6, BA6	4	0·59 ± 0·04

* Mean ± std. error.

Table 2. *Basal MT I activity in mice*

Strain	*N*	MT I* (fmol/μg protein)
A/J	6	9·13 ± 0·04
C57BL/6J	6	20·21 ± 0·27
AB6, BA6	6	20·98 ± 0·61

* Mean ± std. error.

3. RESULTS

Strain variation was found among inbred mice in β-adrenergic-stimulated cyclic AMP production. Upon preincubation with the β-adrenergic agonist L-isoproterenol, the A/J strain produced a lower intracellular level of cyclic AMP than did the C57BL/6J strain (0·45 ± 0·01 pmol/μg protein and 0·74 ± 0·02 pmol/μg protein, respectively), as shown in Table 1. Hepatocytes from the F_1 progeny of the high and low strains were tested, and produced a response intermediate to those of the parentals. There was no maternal or X-linked effect as no significant difference in isoproterenol-stimulated cyclic AMP production was found between the two classes of F_1s (AB6 vs B6A) (data not shown).

Membrane phospholipid *N*-methylation was determined as a function of incorporation of radiolabel from [^{3}H]SAM into phospholipid intermediates. Under the specified assay conditions, each methyltransferase was measured individually and

the reaction products visualized by thin-layer chromatography. No significant difference was found in the activity of MT II among the inbred strains tested. Strain variation was found, however, in MT I activity. These data, given in Table 2, indicate a twofold difference in basal MT I activity between the A/J and C57BL/6J strains (9·13 ± 0·04 fmol/μg protein and 20·21 ± 0·27 fmol/μg protein, respectively). All the F_1 progeny showed MT I activity levels comparable to those of the C57BL/6J parent. Recombinant inbred (RI) lines, representing F_2 combinations of parental alleles between the A/J and C57BL/6J strains, were subsequently examined, and the basal MT I activities obtained from these are shown in Table 3. All but one of the fourteen RI lines tested gave high MT I values, like the C57BL/6J parent. BA13, however, showed an activity similar to that of the A/J parent.

Table 3. *Methyltransferase I activity in recombinant inbred lines between A/J and C57BL/6J mice*

Strain	*N*	MT I activity* (fmol/μg protein)	*H-2* haplotype
A/J	6	9·13 ± 0·04	a
C57BL/6J	6	20·21 ± 0·27	b
AB1	4	20·49 ± 0·93	a
AB2	4	20·76 ± 0·17	a
AB4	4	19·86 ± 0·47	b
AB7	4	18·81 ± 0·49	a
AB12	4	19·14 ± 1·82	a
AB13	4	20·00 ± 0·18	a
AB15	4	24·24 ± 0·86	a
AB17	4	19·71 ± 0·85	a
BA1	4	20·15 ± 1·63	a
BA6	4	21·03 ± 0·18	a
BA11	4	19·63 ± 0·64	b
BA13	4	9·54 ± 0·36	b
BA14	4	22·92 ± 0·54	b
BA15	4	19·97 ± 0·26	b

* Mean ± std. error.

Hormone-stimulated MT I activity was then measured on hepatocyte membrane preparations which had been preincubated with 50 μm L-isoproterenol or 50 μm glucagon. The effects of hormonal stimulation on MT I activity in the A/J, C57BL/6J and F_1 hepatocytes are presented in Table 4. While both A/J and C57BL/6J showed increased activity upon hormonal stimulation, the MT I activity in the A/J hepatocytes increased significantly more (2–3-fold) with respect to basal level than did that in the C57BL/6J cells (approximately $1\frac{1}{3}$-fold). The F_1 progeny showed a response similar to that of the C57BL/6J parental strain with both isoproterenol and glucagon stimulation. Dose-response curves for MT I stimulation by each hormone indicate that both strains reach maximal activity at approximately 5×10^{-7} M concentration (Figs. 1 and 2).

H-2 congenic strains, which differ only in the portion of the genome containing the MHC, were tested for hormonal stimulation of MT I activity. These results are shown in Table 4 and indicate a difference in response between the B10.A and

Table 4. *Hormone-stimulated MT I activity in mice*

Strain	*N*	Basal MT I	Isoproterenol-stimulated MT I activity*	Fold stimulation	Glucagon-stimulated MT I activity*	Fold stimulation
A/J	3	9·13 ± 0·04	22·00 ± 1·51	2·41	26·55 ± 0·36	2·91
C57BL/6J	3	20·21 ± 0·27	25·23 ± 0·26	1·25	31·38 ± 0·70	1·55
F_1	6	20·98 ± 0·61	22·30 ± 0·38	1·06	29·58 ± 0·93	1·41
B10.A	3	8·39 ± 0·07	21·89 ± 0·15	2·61	27·10 ± 0·84	3·23
A.BY/Sn	3	20·13 ± 0·46	24·97 ± 0·14	1·24	30·69 ± 0·90	1·52

* Mean ± std. error.

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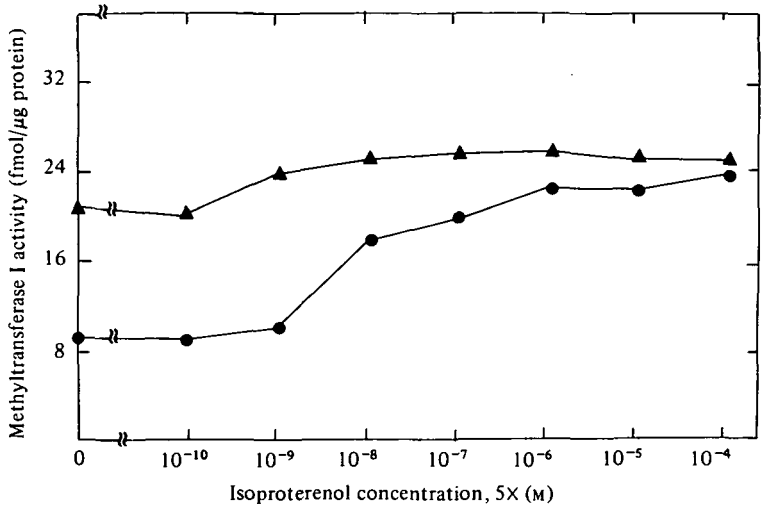

Figure 1. Dose–response curve for isoproterenol-stimulated MT I activity. ●——● A/J; ▲——▲ C57BL/6J; each point is the mean of two animals.

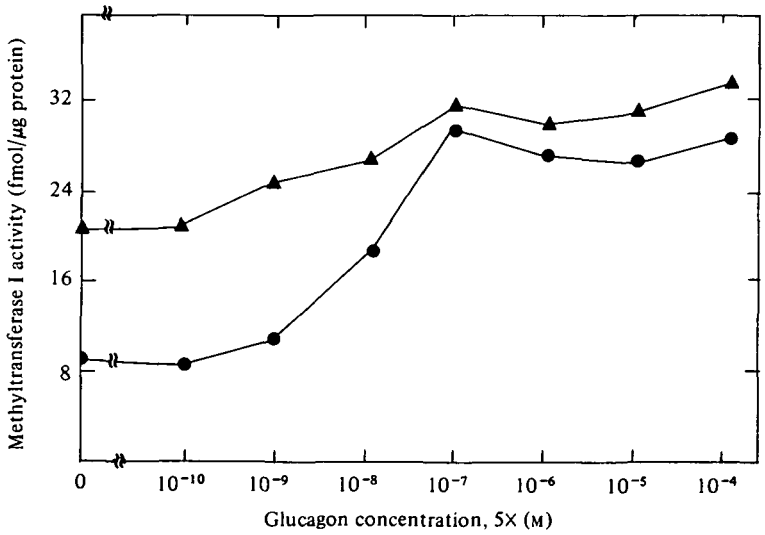

Figure 2. Dose–response curve for glucagon-stimulated MT I activity. ●——● A/J; ▲——▲ C57BL/6J; each point is the mean of two animals.

A.BY/Sn strains with respect to basal levels. B10.A, the congenic with the C57BL background and *H-2*^{a} haplotype, showed a much greater increase in MT I activity upon hormonal stimulation (like A/J, also *H-2*^{a}) than did A.BY/Sn, with the A background and *H-2*^{b} haplotype (like C57BL/6J, also *H-2*^{b}). All the strains tested increased their MT I activity to similar levels upon hormonal stimulation.

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4. DISCUSSION

Phosphatidylcholine synthesis by transmembrane methylation appears to be under the control of several hormones, including cathecholamines (Hirata & Axelrod, 1980), glucagon (Castaño *et al.* 1980), angiotensin and vasopressin (Alemany, Varela & Mato, 1981), which modulate the activation of adenylate cyclase. Not all receptor-mediated processes, however, interact with this series of methylation reactions. Stimulation of platelets, for example, with thrombin, prostaglandins or epinephrine, showed no effect on membrane methylation in these cells (Hirata & Axelrod, 1978). Phospholipid methylation was also found to be non-essential in amylase secretion and activation of adenylate cyclase by β-adrenergic agonist in the guinea pig and rat parotid gland (Padel, Unger & Soling, 1982). Recently insertion of purified β-adrenergic receptors from frog erythrocytes, guinea pig lung and hamster lung into receptor-deficient cells has been shown to result in the restoration of β-adrenergic responsiveness (Cerione *et al.* 1983). These investigations suggest that, in this system, the β-adrenergic binding polypeptide itself contains the necessary sites for mediating the activation of adenylate cyclase. Since β-adrenergic stimulation of adenylate cyclase has been shown to depend on transmembrane methylation in other systems, species- and tissue-specific differences may be involved in the extent of interaction between phospholipid methylation and receptor-mediated response.

In order to probe the interaction between hormone-sensitive adenylate cyclase and phospholipid *N*-methylation in hepatocyte membranes, we investigated the genetic variation in β-adrenergic-and glucagon-stimulated cyclic AMP production and transmethylation in inbred strains of mice. Significant differences were observed between A/J and C57BL/6J hepatocytes in isoproterenol stimulation of adenylate cyclase activity; the F_1 progeny of these two strains gave values intermediate to those of the parentals, suggesting a co-dominant effect.

When phospholipid *N*-methylation was previously measured in the A/J and C57BL/6J mice, significant differences were found in the basal levels of MT I (Markovac & Erickson, 1984), while no variation was detected in the activity of MT II. This genetic variation in MT I activity has been further explored. All F_1 animals showed high levels of enzyme activity, like the C57BL/6J parent. No sex-linked effect was apparent. These results suggest a dominance of high MT I activity over low MT I activity. The genetics of this trait were then investigated using recombinant inbred lines (RI) between the A/J and C57BL/6J strains. RI lines represent homoalleles of the F_2 generation and enable the study of biochemical variables which require more than one animal for experimentation (Bailey, 1971; Swank & Bailey, 1973). All but one of the 14 RI lines tested showed C57BL/6J-like MT I activity. This implies the existence of at least two major genes which regulate the activity of this enzyme. If two genes were involved in the regulation of MT I and low activity was only found with homozygosity for the alleles from A/J, one would expect to see one out of 4 RI lines exhibiting the recessive phenotype, i.e. that of the A/J parent. Three loci would predict 1/8; the 1/14 ratio observed is statistically compatible with either, or with more complex genetic explanations.

We have investigated the effects of hormonal stimulation on MT I activity in

A/J and C57BL/6J hepatocytes. While preincubation with either isoproterenol or glucagon increased MT I activity relative to basal levels in both strains, the effect was much more pronounced in the A/J strain. These mice showed a two- to three-fold stimulation while the C57BL/6J strain enzyme activity increased by approximately one-third. Furthermore both strains reached maximal stimulation of MT I at approximately 5×10^{-7} M hormone concentration. The F_1 progeny between the high and low strains again showed a C57BL/6J-like response to both hormones, suggesting a dominant effect. The codominance seen in the F_1 generation with respect to β-adrenergic stimulation of cyclic AMP production vs the dominance of the BL/6-like MT I response (both basal and stimulated levels) implies that while interaction exists between the cyclase and phospholipid methylation, there are separable genetic effects on both systems.

Genetic variation has been reported in intracellular levels of cyclic AMP among inbred strains of mice (Lafuse *et al.* 1979). Subsequently, this variation was found to segregate with the mouse major histocompatibility complex (MHC), *H-2*, haplotype (Lafuse & Edidin, 1978; Castaño *et al.* 1980). A/J ($H\text{-}2^a$) and C57BL/6J ($H\text{-}2^b$) strains were both found to have higher levels of liver cyclic AMP than did several other strains tested. The effects of the MHC were then found to be explained by differences in glucagon-stimulated adenylate cylase (Lafuse & Edidin, 1980). No difference associated with the *H-2* complex was found in basal, GMP–PNP-stimulated, or fluoride-stimulated adenylate cyclase. In the presence of glucagon, however, the strains with higher cyclic AMP levels reached half-maximal stimulation of adenylate cyclase at significantly lower hormone concentrations than did strains with lower cyclic AMP levels. This variation segregated consistently with *H-2* haplotype. A/J was not tested for glucagon-stimulated activity since several strains of the A origin seemed to exhibit background effects which apparently masked any *H-2*-associated differences. We previously observed an *H-2* effect on basal MT I activity among inbred and congenic strains (Markovac & Erickson, 1984). We now find an MHC association with both isoproterenol-and glucagon-stimulated MT I activity in the congenic strains B10.A and A.BY/Sn. B10.A and A/J, both $H\text{-}2^a$, showed a much greater increase in MT I activity upon hormonal stimulation than did the strains of $H\text{-}2^b$ haplotype, C57BL/6J and A.BY/Sn. Therefore, stimulated levels of methyltransferase activity do not reflect basal levels, and all the strains reach similar MT I levels after stimulation.

While the effect of *H-2* on methyltransferase activity was consistent and significant among inbred and congenic strains (Markovac & Erickson, 1984), investigation of this activity among recombinant inbred lines failed to suggest any correlation with the MHC. Our results indicate the actions of at least two major genes in the regulation of MT I activity. The putative recessive phenotype, BA13, was MHC-typed as $H\text{-}2^b$ (not $H\text{-}2^a$ like the putative recessive parent, A/J). This finding indicates that neither (or none) of these genes whose effects segregate in the A/J, C57BL/6J cross is associated with the MHC, barring the occurrence of a crossover. When the genetic background varies as it does in the RI lines, the effects of other genes seem to mask those of the *H-2* region. However, when the background is kept constant, as in the congenic strains, which differ only in that portion of the genome containing the *H-2* complex [3 to 10 centiMorgans (Klein

et al. 1982)], the MHC effect on MT I activity is detectable, consistent and highly significant. To further investigate the number of major genes involved in the regulation of MT I, additional strains could be tested. For example, the various RI lines, including BA13, could be backcrossed to the A/J parental strain and the progeny of these crosses be examined for MT I activity.

By utilizing these genetic variants, as well as those described with respect to variation in hormone-sensitive adenylate cyclase and methyltransferase activity, one can attempt to elucidate the interaction between membrane methylation and receptor-mediated cyclic AMP production.

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