STUDIES ON THE MECHANISM OF ACTION OF CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE ON STEROID HYDROXYLATIONS IN ADRENAL HOMOGENATES

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Cyclic 3',5'-adenosine monophosphate (cyclic 3',5'-AMP) has recently been shown to stimulate selectively steroid C-11β hydroxylase activity in rat adrenal homogenates fortified with glucose-6-phosphate (G-6-P) and $NADP^+$ (Roberts, Creange and Fowler, 1964). This stimulation resulted in increased formation of corticosterone (B) from exogenous 11-deoxycorticosterone (DOC) or progesterone and also augmented production of 11β-hydroxyprogesterone from the latter substrate. The effect of the cyclic nucleotide was presumed to be mediated via stimulation of α-glucan phosphorylase, which in turn led to enhanced production of G-6-P from glycogen and a concomitant increase in NADPH generation (Haynes, 1958). However, if cyclic 3',5'-AMP stimulated steroid 11β-hydroxylation in adrenal homogenates only by this mechanism, its effects on steroid hydroxylations should be accentuated under conditions where G-6-P concentrations were limiting and eliminated in the presence of maximally effective amounts of this hexose phosphate. The experiments reported below demonstrate that this is not the case and also provide evidence that the corticosteroidogenic effect of the cyclic nucleotide in adrenal homogenates is not dependent upon enhanced NADPH generation.

EXPERIMENTAL

Adrenal glands were subjected to a preliminary incubation for 60 min in bicarbonate buffer, then homogenized as previously described

(Roberts et al., 1964). Aliquots of the homogenate (0.4 ml equivalent to 30 mg adrenal tissue) were incubated 15 min in 2 ml bicarbonate buffer containing 0.2 μmoles (0.05 μC) ^{14}C-progesterone and other additives as noted below. Activities of the steroid hydroxylase systems were estimated from the percentage recoveries of added radioactivity found in the various progesterone metabolites (Roberts et al., 1964). Table 1 reveals that both components of the NADPH-generating system, or NADPH itself, must be added for utilization of exogenous progesterone to occur in homogenates of adrenals subjected to a preliminary incubation (see also Haynes and Berthet, 1957). Cyclic 3',5'-AMP stimulated conversion of DOC to B, 11β-hydroxyprogesterone formation, overall steroid C-11β hydroxylation, and total steroid hydroxylations in the presence of both G-6-P and NADP, but not when either substance was lacking. However, it will be noted that overall C-21 hydroxylase activity was actually decreased, so that measurements of α-ketolic steroids only (as with blue tetrazolium) would not reveal the stimulatory effect of the cyclic nucleotide under these circumstances. The effect of cyclic 3',5'-AMP was also obtained when G-1-P (2.5 mM or greater) was substituted for G-6-P. Glycogen was ineffective as a substrate for NADPH generation in adrenal homogenates (see also, Haynes and Berthet, 1957), even when cyclic 3',5'-AMP was added. These findings suggested that NADPH generation from endogenous sources did not take place either in the presence or absence of cyclic 3',5'-AMP and that the action of the cyclic nucleotide was not dependent upon endogenous production of hexose phosphates. Further support for this conclusion was obtained when the amount of $NADP^+$ added was held constant (1 μmole) but G-6-P concentration was varied. Even when G-6-P was present in an amount (10 μmoles) which was well in excess of that required for maximal activities of steroid C-11β and C-21 hydroxylase systems in the presence of 1 μmole of $NADP^+$, cyclic 3',5'-AMP still selectively stimulated C-11β hydroxylase activity and enhanced total

Table 1

The effects of NADPH, components of the NADPH-generating system, and cyclic 3',5'-AMP on steroid hydroxylations in rat adrenal homogenates†

Additives	No. of samples	C-11β hydroxylase	C-21 hydroxylase	Total hydroxylase	DOC	B	11β-hydroxy progesterone
G-6-P	4	0	0	0	0	0	0
G-6-P, 3',5'-AMP	4	0	0	0	0	0	0
$NADP^+$	3	0	0	0	0	0	0
$NADP^+$, 3',5'-AMP	3	0	0	0	0	0	0
G-6-P, $NADP^+$	24	16.4 ± 1.0	77.9 ± 3.0	94.3 ± 3.3	62.5 ± 2.7	15.4 ± 1.0	1.0 ± 0.4
G-6-P, $NADP^+$, 3',5'-AMP	23	71.9 ± 4.8	58.8 ± 2.5	131.6 ± 5.9	11.2 ± 2.1	47.6 ± 2.8	24.3 ± 2.7
*G-6-P, $NADP^+$	5	12.9 ± 0.8	71.7 ± 1.3	84.6 ± 0.9	58.8 ± 2.2	12.9 ± 0.8	0
*G-6-P, $NADP^+$, 3',5'-AMP	5	80.0 ± 5.8	49.5 ± 3.8	129.5 ± 9.4	2.9 ± 0.7	46.6 ± 3.7	33.4 ± 1.9
NADPH	11	12.5 ± 1.5	86.1 ± 3.9	98.5 ± 3.9	73.6 ± 4.4	12.5 ± 1.5	0
NADPH, 3',5'-AMP	7	78.7 ± 7.5	62.1 ± 4.0	140.8 ± 8.6	12.1 ± 3.4	50.0 ± 3.5	28.7 ± 7.9

†G-6-P (1 μmole), $NADP^+$ (1 μmole), NADPH (1 μmole) and cyclic 3',5'-AMP (8 μmoles) were added except in experiments marked (*) where 10 μmoles G-6-P were present in a final volume of 2 ml. Incubation was carried out for 15 min. Results are expressed as μmoles (x 10^2) of steroid converted or formed/100 mg adrenal/hr. The means ± S.E.M. are shown.

steroid hydroxylations (Table 1). The possibility that cyclic 3',5'-AMP stimulated corticosteroidogenesis via activation of G-6-P dehydrogenase seemed to be ruled out by the observation that addition of the enzyme in maximally effective amounts did not prevent the action of the nucleotide.

Cyclic 3',5'-AMP also selectively stimulated C-11β hydroxylase activity in the presence of preformed NADPH even when the reduced cofactor was present in a concentration (0.5 mM) which was maximally effective for steroid hydroxylations over the 15-min incubation period (Table 1). The effect of cyclic 3',5'-AMP was not eliminated at levels of NADPH which were 4 times this concentration. Addition of G-6-P in these instances did not enhance the action of the reduced cofactor nor eradicate the response to the cyclic nucleotide.

DISCUSSION

The present data would appear to be incompatible with the concept that cyclic 3',5'-AMP stimulates steroid hydroxylation in the adrenal cortex solely by enhancing glycogen phosphorylation and NADPH generation (Haynes, 1958). Thus, exogenous NADPH or NADPH-generating system was required for the action of the cyclic nucleotide in rat adrenal homogenates prepared from tissues which had been subjected to a preliminary incubation in the absence of added glucose. Concentrations of NADPH, G-6-P, or G-6-P dehydrogenase which were maximally active for steroid hydroxylation in the absence of cyclic 3',5'-AMP did not eliminate the effect of cyclic 3',5'-AMP. Therefore, increased production of G-6-P via glycogen phosphorylation or stimulation of NADPH generation by oxidation of G-6-P or other substrates did not seem to be involved. In this connection, cyclic 3',5'-AMP appears to have no effect on phosphoglucomutase or G-6-P dehydrogenase activities (Haynes, 1958; McKerns, 1964).

It is possible that the mechanism of action of cyclic 3',5'-AMP differs in the intact cell where adequate concentrations of glycogen,

α-glucan phosphorylase, phosphoglucomutase and enzymes of the hexose monophosphate shunt may be present. However, Koritz (1962) has provided evidence that the action of the cyclic nucleotide on corticosteroidogenesis in rat adrenal sections involves mechanisms other than glycogen breakdown. He suggested that cyclic 3',5'-AMP may function to increase the level of corticosteroid precursors. A similar mechanism had earlier been proposed as a partial explanation for the action of ACTH (Stone and Hechter, 1954; Koritz and Péron, 1958). Provision of steroid substrates from endogenous precursors cannot explain the present data, especially since the effect of the cyclic nucleotide was enhanced as the concentration of substrate was increased (Roberts et al., 1964). The stimulatory effect of cyclic 3',5'-AMP on steroid C-11β hydroxylations was accompanied by a depression of C-21 hydroxylase activity under some circumstances (Table 1). However, this was not a ubiquitous finding. In addition, where inhibition occurred, the stimulation of C-11β hydroxylase activity was much greater than the inhibition of C-21 hydroxylation. Furthermore, total steroid hydroxylations and C-11β hydroxylation of DOC were also stimulated by the cyclic nucleotide. Therefore, enhancement of C-11β hydroxylation by cyclic 3',5'-AMP was not a consequence of the depression of C-21 hydroxylase activity. The present results strongly suggest that cyclic 3',5'-AMP can selectively stimulate C-11β hydroxylations in the adrenal cortex by a mechanism which is independent of glycogen phosphorylation, NADPH generation, and endogenous corticosteroid precursors.

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