EFFECTS OF THEOPHYLLINE ON THE ACTIVATION AND NUCLEAR TRANSLOCATION OF THE HEPATIC GLUCOCORTICOID RECEPTOR AT LOW TEMPERATURE*

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SUMMARY: In vitro nuclear binding of the (^{3}H) dexamethasone-receptor complex from rat liver cytosol takes place at a slow rate when nuclei are incubated at low temperature. However, if theophylline is included during the incubation there is a threefold increase in the rate of nuclear binding. The activation by theophylline is independent of its known effect on cyclic AMP levels since the cyclic nucleotide has no effect on nuclear binding either in the absence or presence of theophylline. Activation ability is specific to methylxanthines and not to nucleoside derivatives. Theophylline may be acting directly on the (^{3}H) dexamethasone-receptor complex converting it to an active form.

INTRODUCTION

In the absence of the hormone which it binds, the glucocorticoid receptor is found only in the cytosol. The presence of hormone promotes translocation of the receptor molecule into the nucleus where it is presumed to bind to chromatin acceptor sites (1,2). In in vitro studies at low temperature, this translocation takes place very slowly. However, the rate of nuclear binding of the steroid-receptor complex is markedly increased by raising the temperature either prior to or during the nuclear incubation (1,3). In addition, it has been shown that the steroid-receptor complex can be activated to bind to nuclei at low temperature by increasing the ionic strength (1,3) or by the addition of calcium ions (4).

We have been investigating the possibility of direct binding of cyclic AMP to the glucocorticoid receptor of rat liver (5). In in vitro experiments theophylline has been used routinely to prevent the degradation of the added

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cyclic nucleotide. The following experiments suggest that theophylline may directly activate the hormone-receptor complex for nuclear translocation at low temperatures.

METHODS

Adrenalectomized male CD Sprague-Dawley rats (110 to 140 g; Charles River Breeding Labs) were used 7 to 14 days following surgery. Animals were killed by decapitation and the livers perfused *in situ* with cold 0.145 M NaCl through the portal vein. Livers were removed and homogenized in 1 volume of ice-cold homogenization medium composed of 0.25 M sucrose, 3mM $MgCl_2$, 50 mM tris-HCl, pH 7.55. The homogenate was centrifuged at 750 x g for 20 min and cytosol was prepared from this supernatant by a further centrifugation at 105,000 x g for 1 hr at 0-4°. After discarding the upper lipid layer, the cytosol was removed and incubated with (^{3}H) dexamethasone (New England Nuclear; 21.5 Ci/mmole) at a final concentration of 5×10^{-9} M in the presence or absence of an 800-fold excess of nonradioactive dexamethasone. After a 2 hour incubation at 0°, when steroid binding was complete, the specific macromolecular bound fraction was determined using the dextran-coated charcoal technique (6).

Nuclei were isolated by the method of Blobel and Potter (7) from the pellet obtained by low speed centrifugation of the liver homogenate. The washed nuclei were resuspended in homogenization buffer so that 1 ml contained the nuclei from 1 g of liver. Portions (1.5 ml) of the nuclear suspension were centrifuged (750 x g for 5 min) at 0° and the supernatant fractions discarded. Cytosol (0.8 ml) which had been preincubated with (^{3}H) dexamethasone was added, followed by homogenization buffer containing the same concentration of radioactive steroid, to a final volume of 3.2 ml. The nuclei were resuspended by gently stirring on a vortex mixer and the suspension incubated at 0 or 20° with periodic mixing. Following incubation, the nuclei were removed by centrifugation (750 x g for 5 min), washed twice with 1 ml of homogenization buffer, and then twice with 1 ml of 0.2% Triton X-100 in homogenization buffer. Finally, the nuclei were resuspended in 1 ml of buffer, and assayed for radioactivity

as previously described (8) and for DNA by the method of Burton (9). The specific nuclear binding of (^{3}H) dexamethasone was taken as the difference between the values in the presence and absence of nonradioactive steroid.

RESULTS AND DISCUSSION

When purified rat liver nuclei are incubated with (^{3}H) dexamethasone in a medium containing buffer very little specific nuclear binding of the glucocorticoid occurs. Even when the steroid is incubated with cytosol prior to incubation with nuclei the binding is low if the temperature and salt concentration are kept at a minimum (3,10). This is confirmed by the results presented in Table 1. Furthermore, it can be seen that if 5 mM theophylline is included during the time of the incubation there is a marked increase in the nuclear binding of the steroid. Cyclic AMP, both in the absence and presence of theophylline,

TABLE 1. Effect of theophylline and cyclic AMP on *in vitro* nuclear translocation of glucocorticoid receptor.

Rat liver cytosol and buffer were incubated with (^{3}H) dexamethasone in the presence of cyclic AMP and theophylline as indicated for 2 hours at 0°. These fractions were then incubated with nuclei for 1 hour at 0°. At the completion of the nuclear incubation, specific nuclear binding of (^{3}H) dexamethasone was determined as described in "Methods".

Medium	Additives	Specific nuclear binding pmoles/mg DNA Exp 1	Exp 2
Buffer	None	0.01*	0.01
Buffer	5 mM theophylline	0.01	0.01
Cytosol	None	0.06	0.04
Cytosol	0.17 μM cyclic AMP	0.06	0.04
Cytosol	5 mM theophylline	0.16	0.14
Cytosol	0.17 μM cyclic AMP + 5 mM theophylline	0.18	0.14

*This represents 330 dpm/200 μl nuclear suspension

had no effect on the degree of nuclear binding. The cyclic nucleotide was also shown to be ineffective at concentrations of 1 μM and 5 mM. It would appear then that the effect of theophylline is independent of its effects on cyclic-AMP levels This is not unreasonable as theophylline has been shown to influence markedly several metabolic processes by a mechanism unrelated to its effect of increasing the intracellular concentration of cyclic AMP (11,12).

Kalimi et al. (4) have shown that agents which are known to activate the rat liver glucocorticoid receptor also facilitate the binding of the glucocorticoid-receptor complex to DNA-cellulose. We have shown that theophylline significantly increases steroid-receptor complex binding to DNA-cellulose and consequently resembles the effects of other activating agents.

When the time-course of nuclear binding was measured, it was found that there is a slow but significant rate of nuclear binding of (^{3}H) dexamethasone in the presence of cytosol at 0^o. If theophylline was present throughout the incubation at a concentration of 5 mM this rate was increased approximately 3-fold. Although this rate of nuclear binding was lower than that seen when nuclei were incubated with the steroid in the presence of cytosol at 20^o, kinetic experiments indicate that maximal activation of nuclear binding by theophylline would be equivalent to that achieved by incubation at 20^o.

It can be seen from results presented in Table 2 that the effect can be observed with other methylxanthines. Aminophylline at the same concentration had a greater effect than theophylline. Caffeine, on the other hand, was less effective. Moreover, the effectiveness of each agent was independent of the commercial source of the methylxanthine and was maintained after recrystallization indicating that activation of the steroid receptor complex is not the result of a contaminant. The relative order of potency of these methylxanthines in effecting nuclear transfer of dexamethasone is the same as their ability to inhibit phosphodiesterase (13). However, when the potent phosphodiesterase inhibitors, papaverine, methylisobutylxanthine and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (LaRoche Ro-20-1724), were used they were shown to be ineffec-

TABLE 2. Specificity of the activation of the glucocorticoid receptor at low temperature

Hepatocyte nuclei were incubated at 0° for 1 hour with cytosol, which had been preincubated with (^{3}H) dexamethasone and 5 mM of the indicated methylxanthine. They were then washed and the specific nuclear binding of (^{3}H) dexamethasone was determined as in "Methods".

Additives	Specific nuclear binding pmoles/mg DNA
None	0.03†
Theophylline (Sigma)*	0.13
Theophylline (BDH)	0.14
Aminophylline (Sigma)	0.18
Caffeine (NBC)	0.06
Caffeine (Eastman)	0.06

*After filtration through Norit A charcoal while hot and subsequent recrystallization, theophylline gave essentially the same results.

†This value represents 1090 dpm/200 µl nuclear suspension

tive in activating the glucocorticoid receptor. This finding strengthens the argument that the theophylline effect is independent of its ability to inhibit phosphodiesterase.

Theophylline has no effect on nuclear binding of (^{3}H) dexamethasone when the nuclei are incubated in a buffer medium (Table 1). This indicates that the effect of theophylline is likely to be on the glucocorticoid receptor complex. Alternatively, it could be having an effect on the nuclei since the methylxanthine is present during the nuclear incubation. To test this possibility we preincubated nuclei for 1 hour with buffer containing 5 mM theophylline prior to incubation with cytosol prelabeled with (^{3}H) dexamethasone. These nuclei bound (^{3}H) dexamethasone to the same extent as untreated nuclei. We have also shown that the theophylline can be removed, by subjecting the cytosol to

chromatography on Sephadex G-25 prior to nuclear incubation, while retaining the increase in nuclear binding. From these experiments it is concluded that theophylline is having a direct effect on the cytosol fraction and having no influence on the nuclei.

In preliminary studies using (^{14}C) theophylline (Amersham/Searle; 16.6 mCi/mmole) we have shown that theophylline is not translocated to the nucleus either in the presence or absence of dexamethasone. This implies that theophylline does not bind permanently to the receptor but rather brings about an irreversible conformational change in the receptor molecule thereby enhancing its translocation to the nucleus.

The extent of activation is dependent on the concentration of theophylline used and is far from being maximal at a concentration of 5 mM (Figure 1). In fact, if these data are plotted on a double reciprocal plot, the concentration of theophylline required for half maximal effect on nuclear binding is 20 mM. As mentioned earlier a "Vmax" value indicates that it is theoretically possible to activate the receptor at 0^{o} by theophylline to the same extent as obtained by incubation at 20^{o}. This further supports the contention that the effect of theophylline may be directly on the receptor and is independent of its effect on cyclic-AMP as concentrations of methylxanthine derivative well below this level are maximally effective in inhibiting phosphodiesterase (13).

Theophylline has been shown to have no effect on the time-course of cytosol binding of (^{3}H) dexamethasone to the receptor at 0^{o}. However, preliminary experiments suggest that the apparent affinity of cytosol binding of the steroid to the receptor is increased in the presence of theophylline. These results support the idea that the methylxanthine does not have an effect on dexamethasone binding to the cytosol receptor but rather, activates the dexamethasone-receptor complex. Thus we propose the following scheme:

$$S + R \rightleftarrows SR \rightarrow SR^{*} \rightarrow \text{nuclear transfer}$$

theophylline effect

where S denotes steroid, R the specific glucocorticoid receptor protein, SR

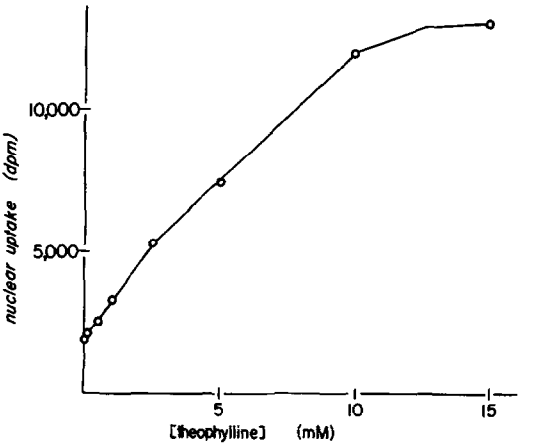

Figure 1. *Effect of theophylline on the nuclear binding of* (^{3}H) *dexamethasone* at low temperature. Rat liver cytosol was incubated with (^{3}H) dexamethasone in the presence of theophylline at the concentration indicated for 2 hours at 0°. The cytosol was then incubated with nuclei for 1 hour at 0° and the nuclear binding of (3H) dexamethasone determined.

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the complex between the steroid and the receptor and SR* the activated complex. In further support of this proposal it was observed that in the presence, but not the absence of theophylline the degree of cytosol binding of (^{3}H) dexamethasone was underestimated using the charcoal absorption technique (6) when compared with that obtained by gel filtration. Higgins et al. (14) have shown this to be a property of the glucocorticoid receptor of HTC cells after activation. They suggested that, in contrast to the inactive steroid-receptor complex, the activated complex is either dissociated or adsorbed by charcoal.

Recently, Kalimi et al. (4) have demonstrated that thermal activation leads to a conversion of the steroid-receptor complex to a form with a more acidic isoelectric point. In addition, they have shown that there is no change in sedimentation behavior of the complex as it undergoes thermal activation. On the other hand, the presence of calcium ions, which activates the glucocorticoid receptor at low temperature, was shown by sedimentation analysis in sucrose density gradients to induce aggregation of the steroid-receptor complex. Whether or not theophylline causes similar changes in the physical properties of the

receptor is currently under investigation.

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