IN VITRO EFFECTS OF THEOPHYLLINE AND AMINOGLUTHETIMIDE UPON BASAL AND ACTH INDUCED cAMP LEVELS AND STEROID OUTPUT BY THE NORMAL HUMAN ADRENAL GLAND

By
Kenneth V. Honn and Walter Chavin

ABSTRACT

The *in vitro* effects of theophylline and aminogluthetimide upon basal and ACTH stimulated cAMP, cortisol and aldosterone responses of normal human adrenocortical tissue were evaluated. Theophylline increased basal cAMP levels and cortisol output, however, basal aldosterone output was depressed. Theophylline in concert with ACTH depressed cortisol and aldosterone output. Aminogluthetimide alone did not affect basal cAMP levels, however, the normal cAMP response to ACTH was delayed in aminogluthetimide pre-treated adrenals. Aminogluthetimide also depressed basal and ACTH stimulated cortisol and aldosterone output with the latter being more sensitive. The findings indicate that both theophylline and aminogluthetimide produce effects upon the adrenal in addition to inhibition of phosphodiesterase and cholesterol side-chain cleavage, respectively. Further, theophylline depression of ACTH stimulated steroid output may be helpful in understanding the interplay between a number of factors in the control of adrenal steroid biosynthesis and release.

Numerous inhibitory drugs have been employed to help elucidate the mechanism of ACTH action upon adrenocortical tissue. Two commonly used drugs in this regard are theophylline, a phosphodiesterase inhibitor (*Halkerston 1975*), and aminogluthetimide, a competitive inhibitor of adrenal cholesterol side chain cleavage activity (*Kahnt & Neher 1966*).
Among the criteria discussed by Robison et al. (1971) in defining the role of cAMP in hormone action, it is clear that a phosphodiesterase inhibitor should act synergistically with the hormone thereby potentiating the physiological response. In this regard, a considerable discrepancy exists with respect to theophylline action in the adrenal. Theophylline has been reported to both potentiate and depress ACTH stimulated steroidogenesis (Halkerston 1975) so that the effect of this inhibitor upon the human adrenal cannot be generalized from studies in other species.

Aminogluthetimide, originally used as an anticonvulsant, is currently undergoing evaluation as a chemotherapeutic agent for various malignancies including those of the adrenal (Orth & Liddle 1971; Lipton & Santen 1974; Sanford et al. 1976). Therefore, as these two drugs have both important research and clinical applications, their effects upon basal and ACTH stimulated cAMP levels and steroid output by the human adrenal in vitro were evaluated.

MATERIALS AND METHODS

Reagents

The following materials were utilized in this study: chromatographically purified porcine ACTH (150 IU/mg; Schwarz/Mann); bovine serum albumin, Fraction V (Pentex); aminogluthetimide (CIBA Pharmaceutical Co.); theophylline (Schwarz/Mann). The radioimmunoassay reagents were: cAMP antiserum (rabbit), [125I]succinyl cAMP-TME, and goat anti-rabbit antiserum (Collaborative Res. Inc.); cortisol antiserum (sheep), [125I]cortisol-TME; aldosterone antiserum (sheep) (Bio-Ria), [1,2,6,7(n)-3H]-aldosterone (Amersham/Scarlé) and rabbit anti-sheep antiserum (Bio-Ria).

Adrenal glands

Six adrenal glands were obtained at surgery from women with breast carcinoma. These patients received no previous antimitotic or X-ray therapy. Adrenals from 3 female patients aged 59–64 years (60.5–107.7 kg) were immediately placed in cold (0–4°C) Krebs-Ringer bicarbonate buffer, KRBGA (pH 7.4, 200 mg glucose/100 ml, 0.50% (w/v) serum albumin fraction V). Pathologic examination showed no evidence of metastases in any of the adrenals utilized. The glands were trimmed free of adherent tissue, bisected, demedullated, diced (2 × 3 mm) and pre-incubated (37°C) in KRBGA for 45 min.

Experimental procedures

Following pre-incubation, the adrenal dice were incubated (1 ml KRBGA; 37°C; 95% O2 + 5% CO2) in a Dubnoff metabolic shaker for 1–32 min. The dice were exposed to ACTH (100 mIU/ml), aminogluthetimide (10 µg/ml), theophylline (10 mm) or KRBGA alone. Further, dice were pre-treated (4 min) in ACTH (100 mIU/ml), aminogluthetimide, or theophylline followed by transfer to ACTH (100 mIU/ml) plus the appropriate test substance (theophylline or aminogluthetimide). Adrenal incubates were quenched in liquid nitrogen at given intervals. Adrenal tissue and incubation
medium were analyzed separately. The tissue was processed and analyzed for cAMP (radioimmunoassay) as previously described (Honn & Chavin 1975a). Cortisol and aldosterone secretion into the incubation medium were quantitated by modification of the radioimmunoassay procedures of Foster & Dunn (1974) and McKenzie & Clements (1974), respectively. These modifications employed the second antibody technique. The lower limits of detection with these procedures were 195 fm cAMP, 39 pg cortisol and 78 pg aldosterone. The total protein content of the adrenal incubates were determined (Honn & Chavin 1975b) and the data expressed as pm cAMP or ng steroid/mg protein, X ± SEM or % control. A minimum of four replicate incubations were used per datum point. Data were analyzed by analysis of variance and Student’s t-test for unpaired observations. Differences were accepted as significant when \( P < 0.05 \).

**RESULTS**

Incubation of adrenals in KRBGA revealed a low basal level of cAMP throughout the period studied (Fig. 1). In addition, continuing basal level of steroid output by the control adrenal tissue resulted in an accumulation of cortisol and aldosterone in the incubation medium (Fig. 2, Table 1). ACTH (100 mIU/ml) stimulated significant cortisol (Fig. 3) and aldosterone (Table 1) output by human adrenal tissue.

Theophylline significantly increased the cAMP level at 1 min and maintained an elevated cAMP level throughout the period studied (Fig. 1). Cortisol output in response to theophylline was elevated early (2 min) but was first significantly increased above the control level at 8 min and continued to increase throughout the period studied (Fig. 2). Pre-treatment with ACTH followed by ACTH + theophylline elevated cortisol output above that evoked by
**Fig. 2.**

*In vitro* temporal (1–32 min) cortisol responses of human adrenocortical tissue to theophylline (10 mM) compared to basal cortisol output in KRBGA vehicle. Vertical bar indicates *SEM* except when smaller than the symbol.

Theophylline (Fig. 2) but the elevation was significantly less than the cortisol response to 100 mIU/ml ACTH at 32 min (Fig. 3). Pre-treatment with theophylline followed by transfer to theophylline + ACTH resulted in cortisol levels significantly lower than the response to either substance alone but not significantly different from that of the control groups (Fig. 3).

Theophylline significantly depressed basal adrenal aldosterone output at the 4 min interval (Table 1). Although aldosterone output by the theophylline treated adrenal tissue continued at the longer time intervals such remained

**Table 1.**

*In vitro* temporal effect of theophylline (10 mM) upon basal and ACTH (100 mIU/ml) stimulated aldosterone output (ng/mg protein; X ± SEM) by human adrenocortical dice.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>ACTH</th>
<th>Theophylline</th>
<th>ACTH pre-treatment followed by ACTH + theophylline</th>
<th>Theophylline followed by pre-treatment ACTH + theophylline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7 ± 0.60</td>
<td>2.8 ± 0.45</td>
<td>1.2 ± 0.20</td>
<td>1.9 ± 0.37</td>
<td>1.2 ± 0.21</td>
</tr>
<tr>
<td>2</td>
<td>2.4 ± 0.25</td>
<td>3.6 ± 0.35</td>
<td>1.7 ± 0.42</td>
<td>3.2 ± 0.25</td>
<td>1.5 ± 0.10</td>
</tr>
<tr>
<td>4</td>
<td>4.2 ± 0.50</td>
<td>5.0 ± 0.50</td>
<td>2.0 ± 0.28</td>
<td>3.4 ± 0.33</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>8</td>
<td>4.6 ± 0.78</td>
<td>5.8 ± 0.30</td>
<td>3.2 ± 0.30</td>
<td>4.1 ± 0.59</td>
<td>2.9 ± 0.60</td>
</tr>
<tr>
<td>16</td>
<td>4.8 ± 0.47</td>
<td>6.3 ± 0.37</td>
<td>3.4 ± 0.50</td>
<td>4.5 ± 0.36</td>
<td>3.3 ± 0.36</td>
</tr>
<tr>
<td>32</td>
<td>5.3 ± 0.28</td>
<td>8.3 ± 1.10</td>
<td>3.7 ± 0.33</td>
<td>5.0 ± 1.0</td>
<td>4.0 ± 0.50</td>
</tr>
</tbody>
</table>

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Effects of pre-treatment (4 min) in theophylline (10 mM) or ACTH (100 mIU/ml) followed by transfer to ACTH + theophylline upon subsequent cortisol production by human adrenocortical tissue. Results are compared to basal cortisol production and to the response to 100 mIU ACTH/ml. Zero time indicated in the figure refers to initiation of combined treatment (ACTH + theophylline) following pre-incubation (4 min) and compared to appropriately timed controls. Vertical bar indicates SEM except when smaller than the symbol.

significantly lower than the aldosterone output of the control groups (Table 1). Pre-treatment with ACTH followed by ACTH + theophylline significantly elevated aldosterone output above that of control groups at 2 min (Table 1). These aldosterone levels (2 min) were not significantly different from that of adrenals treated with ACTH alone (Table 1). However, later temporally (4–32 min),

Table 2.

*In vitro* temporal effect of aminoglutethimide (10 µg/ml) upon basal and ACTH (100 mIU/ml) stimulated cAMP levels (pm/mg protein; X ± SEM) in human adrenocortical dice.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>ACTH</th>
<th>Aminogluthetimide</th>
<th>ACTH pre-treatment followed by ACTH + aminogluthetimide</th>
<th>Aminogluthetimide pre-treatment followed by ACTH + aminogluthetimide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.5 ± 0.4</td>
<td>12.9 ± 0.7</td>
<td>5.1 ± 2.0</td>
<td>15.8 ± 4.9</td>
<td>6.5 ± 2.3</td>
</tr>
<tr>
<td>2</td>
<td>5.4 ± 1.0</td>
<td>15.6 ± 0.8</td>
<td>4.0 ± 2.3</td>
<td>18.1 ± 3.3</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>4.7 ± 1.9</td>
<td>22 ± 3.2</td>
<td>4.3 ± 1.2</td>
<td>23 ± 2.6</td>
<td>10 ± 1.2</td>
</tr>
<tr>
<td>8</td>
<td>5.3 ± 2.3</td>
<td>22 ± 2.5</td>
<td>3.7 ± 1.0</td>
<td>28 ± 0.8</td>
<td>12.5 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>5.6 ± 1.9</td>
<td>33 ± 9.9</td>
<td>4.0 ± 1.7</td>
<td>39 ± 5.5</td>
<td>31 ± 6.3</td>
</tr>
<tr>
<td>32</td>
<td>5.1 ± 2.7</td>
<td>32 ± 1.3</td>
<td>3.6 ± 0.6</td>
<td>33 ± 8.0</td>
<td>34 ± 3.2</td>
</tr>
</tbody>
</table>
aldosterone output by the ACTH + theophylline treated group remained at control levels and was significantly lower than that of the ACTH treated group (Table 1). Pre-treatment with theophylline followed by transfer to theophylline + ACTH resulted in a significantly lower aldosterone output than that of control (2–32 min) and ACTH (1–32 min) treated adrenals but not different from that of theophylline treated adrenals (Table 1).

Aminoglutethimide did not significantly alter basal human adrenal cAMP levels at the time intervals examined (Table 2). In addition, pre-treatment with ACTH followed by ACTH + aminoglutethimide resulted in a cAMP response similar to that evoked by ACTH alone (Table 2). However, pre-treatment with aminoglutethimide followed by ACTH + aminoglutethimide delayed the cAMP response to ACTH. Nevertheless, cAMP levels during the 16–32 min interval were not significantly different from those of ACTH treated adrenal dice (Table 2).

Aminoglutethimide significantly depressed basal cortisol output (16 and 32 min) compared to that of the control groups (Table 3). ACTH pre-treatment followed by ACTH + aminoglutethimide produced significantly lower cortisol levels than that produced by ACTH alone, but significantly (16 and 32 min) higher than basal control levels (Table 3). Alternatively, pre-treatment with aminoglutethimide followed by ACTH + aminoglutethimide inhibited cortisol output below that of ACTH treated or control adrenal dice (Table 3). These aminoglutethimide pre-treatment results were not significantly different from the cortisol depression produced by aminoglutethimide alone (Table 3).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>ACTH</th>
<th>Aminoglutethimide</th>
<th>ACTH pre-treatment followed by ACTH + aminoglutethimide</th>
<th>Aminoglutethimide pre-treatment followed by ACTH + aminoglutethimide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80 ± 8</td>
<td>98 ± 10</td>
<td>84 ± 16</td>
<td>100 ± 12</td>
<td>43 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>88 ± 14</td>
<td>159 ± 14</td>
<td>84 ± 19</td>
<td>126 ± 8</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>4</td>
<td>103 ± 18</td>
<td>250 ± 13</td>
<td>105 ± 18</td>
<td>121 ± 24</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>8</td>
<td>140 ± 31</td>
<td>300 ± 44</td>
<td>148 ± 10</td>
<td>147 ± 27</td>
<td>82 ± 14</td>
</tr>
<tr>
<td>16</td>
<td>184 ± 12</td>
<td>502 ± 31</td>
<td>156 ± 2</td>
<td>275 ± 30</td>
<td>132 ± 31</td>
</tr>
<tr>
<td>32</td>
<td>206 ± 19</td>
<td>707 ± 6</td>
<td>157 ± 7</td>
<td>314 ± 12</td>
<td>148 ± 35</td>
</tr>
</tbody>
</table>

Table 3: In vitro temporal effect of aminoglutethimide (10 μg/ml) upon basal and ACTH (100 mIU/ml) stimulated cortisol output (ng/mg protein; X ± SEM) by human adrenocortical dice.
Table 4. 

In vitro temporal effect of aminogluthetimide (10 µg/ml) upon basal and ACTH (100 mIU/ml) stimulated aldosterone output (ng/mg protein; X ± sem) by human adrenocortical dice.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>ACTH</th>
<th>Aminogluthetimide</th>
<th>ACTH pre-treatment followed by ACTH + aminogluthetimide</th>
<th>Aminogluthetimide pre-treatment followed by ACTH + aminogluthetimide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7 ± 0.60</td>
<td>2.8 ± 0.45</td>
<td>1.2 ± 0.32</td>
<td>1.8 ± 0.10</td>
<td>1.3 ± 0.14</td>
</tr>
<tr>
<td>2</td>
<td>2.4 ± 0.25</td>
<td>3.6 ± 0.35</td>
<td>2.0 ± 0.43</td>
<td>2.3 ± 0.30</td>
<td>1.6 ± 0.25</td>
</tr>
<tr>
<td>4</td>
<td>4.2 ± 0.50</td>
<td>5.0 ± 0.50</td>
<td>2.2 ± 0.12</td>
<td>2.8 ± 0.20</td>
<td>2.0 ± 0.32</td>
</tr>
<tr>
<td>8</td>
<td>4.6 ± 0.78</td>
<td>5.8 ± 0.50</td>
<td>2.6 ± 0.21</td>
<td>3.9 ± 0.16</td>
<td>2.7 ± 0.23</td>
</tr>
<tr>
<td>16</td>
<td>4.8 ± 0.47</td>
<td>6.3 ± 0.37</td>
<td>3.6 ± 0.50</td>
<td>5.1 ± 0.08</td>
<td>3.8 ± 0.10</td>
</tr>
<tr>
<td>32</td>
<td>5.3 ± 0.28</td>
<td>8.3 ± 1.00</td>
<td>3.9 ± 0.10</td>
<td>5.5 ± 0.22</td>
<td>4.5 ± 0.30</td>
</tr>
</tbody>
</table>

Aminogluthetimide also significantly depressed basal aldosterone output (4–32 min) below that of the controls (Table 4). Pre-treatment with ACTH followed by ACTH + aminogluthetimide resulted in aldosterone output below that of ACTH treated adrenals but not significantly different from that of the control groups (Table 4). However, pre-treatment with aminogluthetimide followed by ACTH + aminogluthetimide resulted in aldosterone output significantly (2–32 min) lower than both ACTH and control aldosterone outputs (Table 4).

DISCUSSION

Human adrenocortical tissue in vitro shows a low basal rate of cortisol and aldosterone release concomitant with low and consistent cAMP levels throughout the time period interval studied.

The effective intracellular concentration of a cyclic nucleotide is controlled, in part, by the activity of phosphodiesterase (Cheung 1967). Theophylline, an inhibitor of phosphodiesterase activity, is reported to elevate basal adrenal cAMP and steroidogenesis, in addition to acting synergistically in ACTH and cAMP stimulated steroidogenesis (Marton et al. 1971; Sayers et al. 1971; Mackie & Schalster 1973). Nevertheless, some discrepancies exist. Halkerston et al. (1966) reported theophylline (10 mM) to have two antagonistic adrenal actions, a small potentiation of ACTH simulated steroidogenesis, and inhibi-
tion of such in addition to inhibition of protein synthesis. Further, theophylline (Bieck et al. 1969) and caffeine (Kitabchi et al. 1971) have antagonistic effects upon ACTH and cAMP stimulated steroidogenesis. Theophylline also slightly increases adrenal side chain cleavage activity concomitant with decreased adrenal free and esterified cholesterol levels (Leier & Jungmann 1971). In the present study, theophylline induces a prolonged cAMP increase with subsequent increase of cortisol output by the human adrenal. Nevertheless, theophylline depresses basal aldosterone output. When administered shortly after the maximal stimulating dose (100 mIU) of ACTH (Honn & Chavin 1977d) theophylline depresses the subsequent cortisol response to ACTH. These findings agree with those of Halkerston et al. (1966). Further, when theophylline is administered to human adrenals prior to ACTH stimulation, the cortisol output remains at the basal level. ACTH stimulated mineralocorticoid output is also sensitive to theophylline inhibition.

Whether the above findings result from an inhibition of protein synthesis or other action(s) of theophylline is uncertain. However, recent evidence (Horrobin et al. 1977) demonstrates that the methylxanthines, in particular theophylline, are potent antagonists of E and F series prostaglandins. These results are intriguing considering the demonstrated role of prostaglandins in both cortisol and aldosterone output by the human adrenal (Honn & Chavin 1976a,b, 1977a,b,c). In this regard, indomethacin, a potent prostaglandin synthetase inhibitor, and 7-oxa-13-prostynoic acid, a prostaglandin antagonist, have been demonstrated not to alter basal adrenal cortisol output (Honn & Chavin 1977b). However, both substances depress basal adrenal aldosterone output (Honn & Chavin 1976a). Interestingly, theophylline does not alter basal cortisol output, but basal aldosterone output is depressed. Therefore, basal aldosterone secretion may require a maintenance level of prostaglandins (Honn & Chavin 1976a) and such prostaglandin levels may be depressed by theophylline. Prostaglandin antagonism may also explain the theophylline depression of steroid output by human adrenal dice in response to ACTH as indomethacin and 7-oxa-13-prostynoic acid depress both ACTH stimulated cortisol and aldosterone output by the human adrenal (Honn & Chavin 1976a,b, 1977b).

Aminogluthetimide specifically blocks adrenal steroidogenesis both in vivo (Fishman et al. 1967) and in vitro (Kowal 1969; Falke et al. 1975). The site of aminogluthetimide action is the intramitochondrial conversion of cholesterol to pregnenolone (Kahnt & Neher 1966; Uzgiris et al. 1977). However, aminogluthetimide produces several effects upon the adrenal which are unrelated to its primary effect upon the cholesterol to pregnenolone transformation. Kowal (1969) observed that high doses (100 μg/ml) of aminogluthetimide competitively inhibit 11β-hydroxylase activity. Recently, Saez et al. (1977) demonstrated in vivo that aminogluthetimide increases rat adrenal DNA synthesis, an effect which is additive with exogenously administered ACTH. In the
present report, aminogluthetimide treatment, prior to a maximal stimulating ACTH dose, delays the onset of the cAMP response. The mechanism underlying this phenomenon is unclear.

In vitro, aminogluthetimide depresses basal cortisol and aldosterone production by the human adrenal. Qualitatively these results agree with those of other in vitro studies (Kowal 1969). However, the degree of inhibition of basal steroid output produced by aminogluthetimide in the human adrenal is less than that observed in the murine adrenocortical system despite the equivalency of doses employed. It is unlikely that this difference is due to slow diffusion into adrenal dice as aminogluthetimide effects complete inhibition of side-chain cleavage activity in tissue slices within 3 min (Uzgiris et al. 1977). Therefore, the possibility of a non-cholesterol steroidogenic pathway (Tait & Mills 1974) in the human adrenal cannot be overlooked in this regard. Further, the differential sensitivity of various sterols to inhibition by aminogluthetimide has been observed (Falke et al. 1975). Addition of aminogluthetimide to human adrenal incubates immediately prior to ACTH stimulation completely abolishes the subsequent cortisol and aldosterone output in response to ACTH. Aminogluthetimide exposure immediately after ACTH stimulation significantly depresses the extent of cortisol elevation in response to ACTH whereas the aldosterone response remains at basal levels. Thus, in vitro human adrenal aldosterone output is more sensitive to aminogluthetimide inhibition than cortisol output. These in vitro findings agree with the in vivo observations of Fishman et al. (1967) wherein the most consistent effect of aminogluthetimide upon normal human subjects was a decrease in the aldosterone secretion rate.

In addition, the present findings suggest that ACTH simulated steroidogenesis utilizes the cholesterol to pregnenolone pathway exclusively whereas basal steroid secretion may occur via one or more alternative routes. The identity of possible alternative routes and the factor(s) responsible for the steady-state control of steroid output, however, remain speculative.

REFERENCES


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