DETERMINATION OF CYCLOC AMP IN HEAT-SEPARATED
HUMAN EPIDERMAL TISSUE

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Abstract. Heat separation is introduced as a simple, reliable and quick method for obtaining pure epidermis for cyclic AMP analysis. Heating at 60°-65°C for 10 min resulted in a distinct separation of epidermis from dermis. The level of cyclic AMP in epidermal tissue from 9 patients was 3.93±0.31 pmol per mg dry weight. The inter- and intra-assay variations were 10.3% and 8.8%, respectively. The sensitivity of the assay was 0.07 pmol. Recovery analyses revealed that 80.5% of labelled cyclic AMP was refound after extraction and purification procedures. Addition of unlabelled cyclic AMP resulted in a recovery of approximately 100%. The specificity of the assay was high without interference from several nucleotides and the validity was confirmed by zero values obtained after addition of phosphodiesterase or charcoal.

Key words: Epidermal cAMP; Heat separation; Normal human skin

The central biological role for cyclic adenosine 3',5'-monophosphate (cAMP) as intracellular second messenger for various hormonal functions is now well established in different tissues, including the skin.

Methods for exact measurement of tissue levels of cAMP have been available for some years. However, conflicting data have been obtained in human epidermal tissue. Probably this is due mainly to the different methods applied for separation of epidermal and dermal tissue necessitated by the structural complexity of human skin. In previous studies keratoma and microdissection have been used (4, 8, 11).

In the present communication we introduce heat separation as a simple, reliable and quick method for obtaining pure epidermis for cAMP analysis. Thereby we have overcome the potential errors of the keratoma technique and the time-consuming procedures involved in microdissection analysis.

MATERIALS AND METHODS

Reagents
Cyclic AMP assay kits were obtained from the Radiochemical Centre, Amersham (RCA), U.K.; ATP, ADP, AMP, Adenosine and cGMP from Sigma Chemical Company, St. Louis, USA.

Liquid scintillation counting was performed in a Beckman type CS 250 liquid scintillation counter. Scintillation fluid was Instagel 6003059 from Packard Instruments Company, Illinois, USA.

A 1 ml Potter-Elvehjem glass homogenizer was used. The reaction was carried out in 7×11 mm test tubes.

Patients
Biopsy materials from clinically normal skin of 9 male patients suffering from venereal diseases without cutaneous manifestations were examined. Their ages ranged between 18 and 28 years (Table I). The biopsies were taken from the gluteal region in the morning between 10 and 12 a.m. in each case.

Furthermore, surgically removed mammary skin was obtained from one patient with breast cancer without cutaneous involvement.

Sampling
Punch biopsies (4 mm diameter) were obtained from frozen skin areas using ethyl chloride for local freezing anesthesia. The still frozen specimens were immediately placed and kept in liquid nitrogen. The skin was stored for up to one week without any noticeable change in cyclic AMP level.

From the surgically removed skin 4×4 cm skin specimens were used and placed in liquid nitrogen for approximately 30 min after removal. From this material, several 4 mm punch biopsies were used for analysis.

Preparation of specimens
The frozen biopsies were placed in preheated buffer and kept at 60°-65°C in a waterbath for 10 min. After heating, the epidermis could be peeled off easily. Immediately after separation the epidermis was lyophilized to obtain dry weight.

For extraction of cyclic AMP 100 µl 0.1 N HCl was added and the sample homogenized for 3 minutes. The homogenate was transferred to fresh test tubes and combined with 2×100 µl 0.1 N HCl used for washing the
Table 1. Cyclic AMP level in heat-separated normal human epidermal tissue

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Tissue dry weight (mg)</th>
<th>pmol cAMP per mg dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>0.342</td>
<td>4.39</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>0.279</td>
<td>6.09</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>0.299</td>
<td>4.01</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>0.331</td>
<td>3.02</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>0.310</td>
<td>3.23</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>0.236</td>
<td>4.04</td>
</tr>
<tr>
<td>7</td>
<td>26</td>
<td>0.485</td>
<td>3.45</td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>0.295</td>
<td>3.81</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>0.323</td>
<td>3.33</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>3.93</td>
</tr>
<tr>
<td>S.E.M.</td>
<td></td>
<td></td>
<td>0.31</td>
</tr>
</tbody>
</table>

homogenizer. The resulting 300 µl was boiled for 5 minutes, followed by centrifugation at 3,500 g for 10 minutes. For purification the supernatant was lyophilized and redissolved in 300 µl distilled water, followed by addition of 100 µl 0.3 N ZnSO₄ and 100 µl 0.3 N Ba(OH)₂ adjusted to pH 7.5 (5). This mixture was incubated for 10 min in an ice bath and then centrifuged at 3,500 g for 20 min. The supernatant was lyophilized and, after addition of 125 µl assay buffer, 2×50 µl was used for the analysis of cAMP.

Cyclic AMP assay

The RCA assay kit for cyclic AMP analysis is based on a competitive protein binding method (7). The assay was performed at 4°C. 50 µl standard or sample, 50 µl labelled cAMP and 100 µl binding protein were added to test tubes and mixed. Standards and samples were always run in duplicate and a complete standard curve was included in each assay. The test tubes covered with parafilm were then incubated for 10 min in an ice bath and then centrifuged at 3,500 g for 20 min. The supernatant was lyophilized and, after addition of 125 µl assay buffer, 2×50 µl was used for the analysis of cAMP.

RESULT

Heating of the biopsies resulted in a distinct separation of the epidermis from the dermis. Occasionally, a few basal cells adhered to the dermal surface whereas adhesion of dermal components to the epidermis was not seen (Fig. 2).

The mean concentration ± S.E.M. of cyclic AMP in heat-separated epidermal tissue from 9 patients was 3.93±0.31 pmol per mg dry weight (Table 1).

Fig. 1. A typical standard curve. The concentration of cAMP in pmol/tube is indicated as a log-scale on the abscissa. The ordinate shows percentage of bound isotope.
In order to evaluate the amount of cAMP lost during the extraction and purification procedures, labelled cAMP (0.9 pmol) was added to epidermal tissue from 16 biopsies. The recovery (Mean ± S.E.M.) was 80.5 ± 4.2%, range 77.1–86.7.

0.5 and 1.0 pmol of unlabelled cAMP (exogenous recovery) was added to epidermal tissue from seven biopsies, and 105% and 112% were recovered, respectively.

The sensitivity of the binding assay (detection limit) was 0.07 pmol, a value well below the concentrations found in epidermis.

Zero values were obtained when H2O and ZnSO4, Ba(OH)2 or 10 nm ATP and ZnSO4, Ba(OH)2 were mixed in the same ratio as used for purification of tissue extracts.

No interference with the binding capacity was observed when ATP, ADP, AMP or adenosine in concentrations up to 1 mmol were included in the assay. Nor did a ten-fold increase of buffer components influence the assay.

Cyclic GMP interfered with cAMP binding in the assay, but only in concentrations 150 times higher than cAMP concentrations.

The specificity of the method was further validated by addition of phosphodiesterase or charcoal, which resulted in zero values.

DISCUSSION

Slightly different basal levels (about 1 pmol per mg wet weight) of epidermal cyclic AMP have been obtained previously in three different laboratories, probably mainly due to differences in the techniques applied. Our results are placed in the middle of the range for these studies (Table III).

Cyclic AMP is a heat-stable molecule which allows determination of cAMP on heat-separated epidermis. Our results show that enzyme activity is abolished when the tissue is heated to 60°C. No changes in concentration occurred when the tissue was kept at room temperature for up to 30 min after this procedure. However, alterations in the concentration of epidermal cAMP cannot be excluded.

Table II. Cyclic AMP level in heat-separated epidermal tissue. Eight different biopsies from surgically removed mammary skin in one patient

<table>
<thead>
<tr>
<th>Biopsy no.</th>
<th>Tissue dry weight (mg)</th>
<th>pmol cAMP per tube</th>
<th>pmol cAMP per mg dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.439</td>
<td>0.38</td>
<td>2.16</td>
</tr>
<tr>
<td>2</td>
<td>0.426</td>
<td>0.35</td>
<td>2.05</td>
</tr>
<tr>
<td>3</td>
<td>0.825</td>
<td>0.82</td>
<td>2.48</td>
</tr>
<tr>
<td>4</td>
<td>0.390</td>
<td>0.28</td>
<td>1.79</td>
</tr>
<tr>
<td>5</td>
<td>0.482</td>
<td>0.40</td>
<td>2.07</td>
</tr>
<tr>
<td>6</td>
<td>0.437</td>
<td>0.50</td>
<td>2.86</td>
</tr>
<tr>
<td>7</td>
<td>0.442</td>
<td>0.35</td>
<td>1.98</td>
</tr>
<tr>
<td>8</td>
<td>0.266</td>
<td>0.20</td>
<td>1.88</td>
</tr>
</tbody>
</table>

Mean 2.16  
S.D. 0.35
Table III. Cyclic AMP levels in normal human epidermis and unaffected psoriatic epidermis in the literature

<table>
<thead>
<tr>
<th>Reference</th>
<th>Normal epidermis pmol cAMP per mg wet weight ± S.E.M.</th>
<th>Non-involved psoriatic epidermis pmol cAMP per mg wet weight ± S.E.M.</th>
<th>Range</th>
<th>Method of separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voorhees et al. (8)</td>
<td>1.4±0.2</td>
<td>1.4±0.2</td>
<td>0.30–3.30</td>
<td>Keratoma</td>
</tr>
<tr>
<td>Härkönen et al. (4)</td>
<td>0.94±0.17</td>
<td></td>
<td>–</td>
<td>Keratoma</td>
</tr>
<tr>
<td>Yoshikawa et al. (10)</td>
<td>0.91±0.14*</td>
<td></td>
<td>0.46–1.92</td>
<td>Microdissection</td>
</tr>
<tr>
<td>Present data</td>
<td>1.31±0.10*</td>
<td></td>
<td>1.01–2.03</td>
<td>Heat separation</td>
</tr>
</tbody>
</table>

* The data have been converted from the original dry weight basis to a wet weight basis using the ratio of dry weight to wet weight of 1:3 (6).

In the period before the tissue had reached the critical temperature for inactivation of enzyme activity, Halprin and co-workers (10) may have overcome, though not completely ruled out, this potential error. They used microdissected epidermal tissue and heat denaturation of proteins including the enzymes by boiling for 5 min. The error introduced by us may be slightly larger, due to the lower temperature and greater amount of tissue used by us.

The nearly 80% recovery obtained in our experiments is higher than those previously reported (8, 11). Our findings that ATP, ADP, AMP and adenosine did not interfere with the RCA binding protein are in agreement with previous results (7). The concentrations used by us (1 mmol) are well above probable in vivo concentrations (1, 3). Furthermore, it seems unlikely that interference could be due to unidentified substances, since phosphodiesterase and charcoal addition resulted in zero values. Furthermore, external recovery was approximately 100%.

The keratoma technique for epidermal cAMP determination introduced by Voorhees has recently been discussed (2, 9). Several objections have been raised, including the potential error introduced by using local anesthesia, omission of freezing during the sampling procedure, and dermal contamination of the epidermal samples. Halprin et al. (2) have tried to overcome these problems by using microdissection of skin biopsies, thereby eliminating the first two objections. However, free hand microdissection of 20 µm skin slices does not exclude the possibility of contamination with dermal tissue. It would imply that the sections were always vertical and that the level of dermal papillae was uniform throughout the 20 µm thick block.

Heat separation seemed to overcome the objections raised and the potential error due to the observed occasional adhesion of single epidermal cells to the dermis must be negligible.

The range of epidermal cyclic AMP concentrations obtained in our study (1.01–2.03 pmol/mg wet weight) was lower than those previously reported (Table III). This may be due to several factors not previously accounted for. The potential influence of age, sex, regional and diurnal variation was probably eliminated in our study. Only young male patients were examined and biopsies were obtained from the same anatomical region in the morning.

Our cyclic AMP values were related to dry weight, thereby enabling us to compare directly the values obtained with those of other laboratories. cAMP concentration was not put in relation to epidermal DNA and protein, since this might introduce new potential errors (as discussed by Halprin et al. (2)), and thereby hinder comparison.

Thus, in normal human skin, heat separation seems to provide a reliable and quick method for the determination of epidermal cyclic AMP levels, and it may be applied successfully to the biochemical analyses in various human cutaneous disorders.

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REFERENCES


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