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Adenylate and Guanylate Cyclase Activities in Isolated Guinea Pig Epidermal Cells at Various Stages of Differentiation

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The enzymatic properties of adenylate and guanylate cyclases were examined in sonicates of trypsinized guinea pig epidermal cells as enzyme source. Adenylate cyclase was found to be membrane-bound, while guanylate cyclase activity was detected in both membrane and cytosolic fractions. The maximal activities of the enzymes were obtained in the presence of Mn^{++} in the pH range 7.8–8.8. The apparent K_m values of adenylate cyclase for Mn^{++}- and Mg^{++}-ATP were 20.5 and 38.6 μM, respectively, while the value of guanylate cyclase for Mn^{++}-GTP was 500 μM. Examinations of cells separated by velocity sedimentation at unit gravity revealed that the basal activity of adenylate and guanylate cyclases was maximal in the germinative cells, falling gradually to the low level as cells differentiated. We assume that in the epidermis, the control and coordination of proliferation require higher concentrations of adenylate and guanylate cyclases as compared with events occurring during terminal differentiation. (Received April 8, 1987.)

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There have been contradictory arguments concerning the effects of cyclic nucleotides on epidermal cells. Reagents that increase the concentration of intracellular cyclic AMP (cAMP) enhance the growth colony formation of cultured keratinocytes (1, 2), while in vitro growth of skin sheets is inhibited by dibutyryl cAMP (3), and cAMP plus theophylline (4). One of the difficulties in interpreting these data stems from the fact that one is dealing with heterogenous epidermal cell populations. The responsiveness of the cyclases to external and internal stimuli and the biological effects initiated by the changes in intracellular cyclic nucleotide levels are varied among epidermal cells at particular proliferative and differentiation points.

Isolated epidermal cells are fractionated successfully into germinative and differentiated populations by various procedures (5–7). In the present study, we characterized the enzymatic properties of adenylate and guanylate cyclases in guinea pig epidermal populations separated by velocity sedimentation at unit gravity.

MATERIALS AND METHODS

Materials

[2-^{3}H]ATP and [8-^{3}H]GTP were purchased from the Radiochemical Centre, Amersham, England; ATP, GTP, cAMP, cyclic GMP (cGMP), creatinine phosphate from Sigma Chemical Co., St. Louis, Mo.; creatine kinase from Boehringer, Mannheim, West Germany; neutral aluminium oxide from Woelm,

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Abbreviations: ATP, adenosine-5-triphosphate; cAMP, cyclic AMP; cGMP, cyclic GMP; EDTA, ethylendiaminetetra-acetic acid; GTP, guanosine-5-triphosphate; IBMX, isobutyl-1-methylxanthine.

West Germany; AG 1×2 resin from BioRad, Richmond, Va.; 3-isobutyl-1-methylxanthine (IBMX) from Aldrich Chemical Co., Milwaukee, Wisc.

Isolation of epidermal cells

The thin skin fragments of outbred female guinea pigs (Hartley strain) were trypsinized to obtain isolated epidermal cells, as previously described (7). The yield was $3–5\times10^7$ cells/animal and the viability was more than 90% as determined by dye exclusion method.

Cell separation by velocity sedimentation at unit gravity

The cells were separated in a chamber (∅ 10 cm; 1000 ml capacity) according to the previous method (7). Among the 25 fractions (40 ml each), the cells in fractions 4 to 6, 8 to 10 and 12 to 14 were combined and designated as lower, middle, and upper epidermal cell fractions, respectively.

Preparation of epidermal homogenates

The dissociated cells were suspended in 0.25 M sucrose containing 2 mM dithiothreitol and 1 mM EDTA at a concentration of 4×10^7 cells/ml. The cell suspension was sonicated in a Sonifier Cell Disruptor (Heat System Ultrasonic, Plainview, N.Y.; Model W185) using a standard microtip at 25 W for 10 s twice with a 30-s interval in an ice bath, and was used as enzyme preparation. In some experiments, the cells were homogenized in a glass homogenizer, or subjected to alternate snap-freezing to −70°C and thawing at 37°C, that was repeated four times.

Assay of adenylate and guanylate cyclase activities (8)

The standard assay mixture for adenylate cyclase contained 0.25 mM [^{3}H]ATP (specific activity, 20 Ci/mol), 1 mM cAMP, 15 mM creatine phosphate, 40 µg creatine kinase, 1 mM $MnCl_2$ (or 1 mM $MgCl_2$), 25 mM Tris-HCl buffer (pH 8.0), 1.5 mM IBMX, and an enzyme preparation in a total volume of 200 µl. For guanylate cyclase assay the same conditions were used except that 0.25 mM [^{3}H]GTP (spec. act. 20 Ci/mol) and 1 mM cGMP were substituted for [^{3}H]ATP and cAMP. The reaction mixture was incubated for 20 min at 37°C. The reaction was stopped by heating for 2 min in a boiling-water bath, following addition of 40 µl of 1 N HCl. The radioactive cAMP or cGMP was isolated by serial passages through neutral aluminium oxide and AG 1×2 resin column.

Protein concentration

The method of Lowry et al. (9) was employed with the use of bovine serum albumin as a standard.

Statistical analyses

Calculations of arithmetic means, standard deviations (SD) and *p*-values were performed by Student's *t*-test.

RESULTS

Designation of fractionated epidermal cells

Our previous study (7) demonstrated that the lower epidermal cell fraction consisted mainly of basal and lower spinous cells devoid of membrane-coating granules and the upper epidermal cell fraction was occupied by upper spinous cells with membrane-coating granules and a few granular cells. The middle epidermal cell fraction was a mixture of basal, and lower and upper spinous cells. Most of the granular cells were distributed in fractions 15 to 25, which were not included in the present study. The viability of cells in these three fractions was always more than 95%.

Basic conditions for formation of cAMP and cGMP in epidermal cell homogenates

We compared activities of adenylate and guanylate cyclases in epidermal preparations obtained by glass homogenization, freezing–thawing and sonication. Adenylate cyclase activities in three different preparations were comparable, while guanylate cyclase showed the maximum activity in the sonicates. In the following experiments, sonicated cell preparations were used as a source of the enzymes.

When [^{3}H]ATP or [^{3}H]GTP was incubated with the sonicates under standard assay condi-

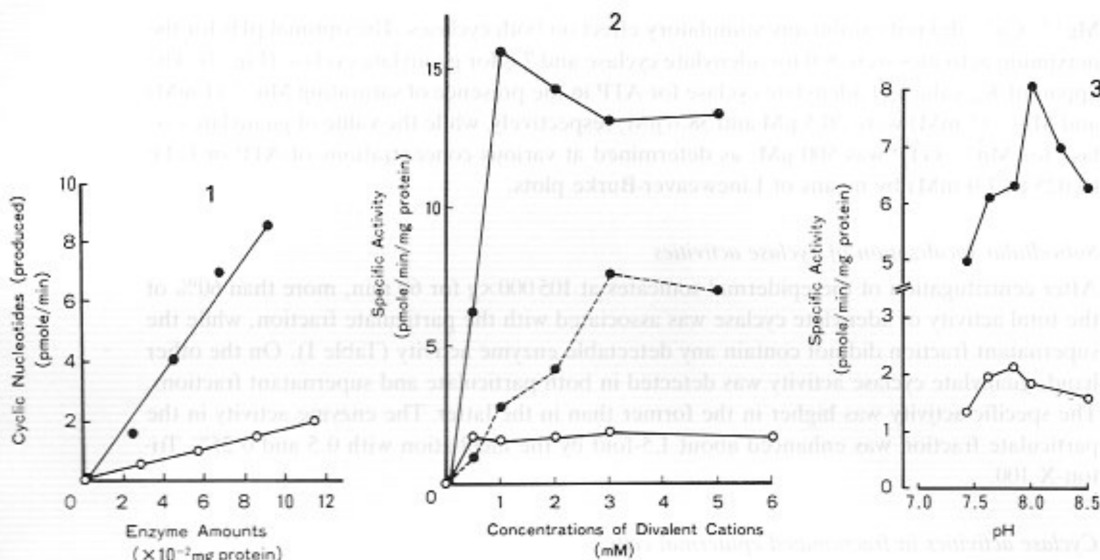

Fig. 1. Formation of cAMP (●) and cGMP (○) by guinea pig epidermal cell sonicates under various enzyme concentrations. The protein concentration of 10^6 epidermal cells were 138.7 µg.

Fig. 2. Formation of cAMP (●) and cGMP (○) in guinea pig epidermal cell sonicates as a function of Mn^{++} (—) and Mg^{++} (- - -) concentrations. The standard assay conditions were employed, except that the different concentrations of either Mn^{++} or Mg^{++} were added.

Fig. 3. Formation of cAMP (●) and cGMP (○) in guinea pig epidermal cell sonicates under various pH conditions.

tions with the use of Mn^{++} as a metal co-factor, radioactive cAMP or cGMP was produced linearly with time for at least 20 min. The formation of cyclic nucleotides was proportional to the quantity of the enzyme preparations (Fig. 1). Maximum activities of adenylate cyclase were obtained at concentrations of Mn^{++} above 1 mM and of Mg^{++} above 3 mM, respectively (Fig. 2). Under standard conditions (1 mM divalent metal ion) the effectiveness of Mn^{++} exceeded that of Mg^{++} by about 5-fold. On the other hand, guanylate cyclase had a requirement for Mn^{++} in concentrations above 0.5 mM which could not be replaced by

Table I. *Subcellular distribution of adenylate and guanylate cyclase activities in epidermal cells*

Enzyme preparations[a]	Specific activities[b] (pmole/min/mg protein)	
	Adenylate cyclase	Guanylate cyclase
Whole sonicates	5.64	1.92
Particulate	3.68	2.41
Supernatant	ND[c]	1.38

[a] Whole sonicate denotes sonicated, whole epidermal cells; particulate and supernatant were obtained after centrifugation of the sonicate at 105 000 *g* for 60 min at 4°C. Protein concentrations of whole sonicate, particulate and supernatant were 103, 73 and 38 µg/10^6 cells.

[b] Means of duplicate assays.

[c] ND, not detected.

Mg^{++}. Ca^{++} did not exhibit any stimulatory effect on both cyclases. The optimal pHs for the maximum activities were 8.0 for adenylate cyclase and 7.8 for guanylate cyclase (Fig. 3). The apparent K_m values of adenylate cyclase for ATP in the presence of saturating Mn^{++} (1 mM) and Mg^{++} (5 mM) were 20.5 μM and 38.6 μM, respectively, while the value of guanylate cyclase for Mn^{++}-GTP was 500 μM, as determined at various concentrations of ATP or GTP (0.025 to 1.0 mM) by means of Lineweaver-Burke plots.

Subcellular localization of cyclase activities

After centrifugation of the epidermal sonicates at 105000×*g* for 60 min, more than 60% of the total activity of adenylate cyclase was associated with the particulate fraction, while the supernatant fraction did not contain any detectable enzyme activity (Table I). On the other hand, guanylate cyclase activity was detected in both particulate and supernatant fractions. The specific activity was higher in the former than in the latter. The enzyme activity in the particulate fraction was enhanced about 1.5-fold by the incubation with 0.5 and 0.25% Triton-X 100.

Cyclase activities in fractionated epidermal cells

Table II shows the activities of cyclases in the epidermal cells that were separated by velocity sedimentation into lower, middle and upper fractions. The lower epidermal cells exhibited the maximum activities of both adenylate and guanylate cyclases, while about 20% of the activities in the whole epidermal preparations was detected in the upper epidermal cells. These results suggested that the intensity of the cyclase activities was reduced as cells differentiated.

DISCUSSION

The major problem inherent to studies of isolated epidermal cells is the possibility that the enzymatic treatment for cell dissociation alters the properties of cell membrane to affect the reactivity of the cyclases to various stimuli. It has, however, been demonstrated that the trypsinized epidermal cells still retain adenylate and guanylate cyclase systems that are sensitive to hormones (10, 11). The present study also showed that the basal activities of both adenylate and guanylate cyclases in the whole-cell sonicate seemed to be comparable to those re-

Table II. *Adenylate and guanylate cyclase activities in separated epidermal cells*

Epidermal cell preparation[a]	Specific activities[b] (pmole/min/mg protein)		
	Adenylate cyclase		Guanylate cyclase
	Mn^{++}	Mg^{++}	Mn^{++}
Whole	10.44	5.72	2.23
Lower	9.34	4.72	2.06
Middle	3.69	2.08	1.03
Upper	2.26	1.19	0.47

[a] The whole cells were subjected to velocity sedimentation at unit gravity to be separated into lower, middle and upper epidermal cells. The proportions of lower, middle and upper cells among the whole cells were 56, 23 and 7%, the remainder being cell aggregates and horny cells. The protein concentrations of whole, lower, middle and upper epidermal cells were 99.6, 69.8, 124.6 and 134.6 μg/10^6 cells.
[b] Means of triplicate assays. The SD were always less than 10% of the means. Mn^{++} and Mg^{++} indicate the divalent cation added to the assay mixtures.

ported in skin sheets (12–15). These findings indicated that impairment of the enzyme activities by trypsinization was minimal in our experimental system.

The adenylate and guanylate cyclases in the guinea pig epidermal cells shared certain characteristics common to the enzyme activities of other tissues, including human and mouse epidermis (12–17). The adenylate cyclase activity was associated entirely with the particulate fraction as in other organs, while both particulate and supernatant fractions contained the guanylate cyclase activity. The association of enzyme activity with the former fraction was further confirmed by the stimulation of the enzyme by Triton-X incubation. According to Marks (15), guanylate cyclase in the mouse skin localizes exclusively in the supernatant of skin homogenates. One possibility is that the guinea pig epidermal cell possesses two different guanylate cyclases, one membrane-bound and the other soluble. Alternatively, a substantial amount of the membrane-bound enzymes might be solubilized during sonication and fractionation procedures.

The activities of both adenylate and guanylate cyclases were maximal in the germinative cells, falling gradually to the low level in accordance with the differentiation. This finding agrees with the previous biochemical and immunofluorescence studies (18, 19) demonstrating higher levels of cAMP and cGMP in the basal layer and, thus, renders the increased production of cAMP by trypsin treatment (20, 21) unlikely. We assume that in the epidermis the control and coordination of proliferation require higher activities of adenylate and guanylate cyclases, as compared with events occurring during the keratinization process. In fact, the long-term cell culture study shows that the high cyclic nucleotide content is a feature of cycling (possibly S phase) keratinocytes, while the onset of differentiation coincides with a small but definite increase in both cAMP and cGMP (22).

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