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The Effects of Epidermal Growth Factor on the Cyclic Nucleotide System in Pig Epidermis

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Incubation of pig skin slices with epidermal growth factor (EGF) caused an increase in intracellular cyclic GMP concentration. A significant increase was found after 1 hr of incubation and reached a peak by 6 hr. EGF caused no change in the cyclic AMP level nor did it affect epinephrine-induced cyclic AMP responses.

Epidermal growth factor (EGF) was first isolated from extracts of the male mouse submaxillary gland in 1962 by Cohen [1]. In 1975, human EGF was isolated from the urine of pregnant women [2]. Preliminary data indicate that the EGF concentration in human plasma is 2-4 ng/ml [3]. As far as the mechanism of action of EGF is concerned, 2 models are currently considered. One is that EGF binds to cell surface receptor(s) and then triggers an intracellular cyclic nucleotide second messenger without entering the cell [4]. Another is that EGF is internalized and bound to control site(s) inside the cell and acts without the need for cyclic nucleotides [5]. In favor of the former concept is the finding of a rise in cyclic AMP and adenylate cyclase activity after EGF treatment in corneal epithelium and epidermis of mice [6,7]. However others reported that some effects, especially the mitogenic effects, of EGF on epithelial cells and fibroblasts may be mediated through lowered levels of cyclic AMP [4,8,9]. Reports on the effect of EGF on cyclic GMP levels are essentially limited to a report of a significant increase in cyclic GMP by EGF in rabbit lens epithelial cell cultures [6]. We have undertaken studies on the effect of EGF on cyclic AMP and cyclic GMP systems in pig skin (epidermis).

MATERIALS AND METHODS

Cyclic AMP antiserum, ^{125}I-iodinated 2′-O-succinyl cyclic AMP, cyclic GMP antiserum, ^{125}I-iodinated 2′-O-succinyl cyclic GMP, and epidermal growth factor (source = mouse submaxillary gland) were obtained from Collaborative Research Inc. (Waltham, Mass.). Triethylamine and acetic anhydride were purchased from Eastman Kodak (Rochester, N.Y.). Epinephrine hydrochloride was the product of Park Davis (Bloomfield, N.J.). Hank's balanced salt solution, RPMI 1640 medium, antibiotics and fetal calf serum were purchased from GIBCO (Grand Island, N.Y.). All other chemicals and drugs were of analytical reagent grade.

The skin slices were taken from the back of domestic pigs, weighing 6–8 kg, by a Castroviejo keratome set at 0.3 mm depth. The "skin slices" contain mainly epidermis (70~85%) but also include small amounts of dermis. During this procedure, pigs were anesthetized with Nembutal (dose = 20–30 mg/kg) intraperitoneally. For incubation experiments, skin slices were kept in Hank's balanced salt solution at 4°C. Slices were cut into 5 × 5 mm squares and used within 30 min.

After 15 min preincubation at 37°C, treatment with EGF was started. For simultaneous cyclic AMP and cyclic GMP assays, 4 pieces of skin were used. For cyclic AMP assay alone one piece of skin was used. For short treatments (within 60 min), incubation was carried out with Hank's balanced salt solution in a water bath. For longer treatments, incubations were performed with RPMI 1640 media, containing 2 mM glutamine, 100 units of penicillin per ml, 100 units of streptomycin per ml, and 0.25 mcg of fungizone per ml, and were equilibrated with 5% CO_2 in air in a humidified incubator. After each incubation period, skin squares were immediately frozen between 2 Dry Ice plates, and kept in a −70°C freezer.

The cyclic AMP and cyclic GMP in skin squares were extracted by homogenization of the tissue in 2 ml of 1% perchloric acid at 4°C. Tritium labeled cyclic nucleotides were added during homogenization of the tissues for calculating recoveries, which are generally 70% for cyclic AMP and 90% for cyclic GMP. Perchloric acid was then removed by precipitation with 6 N KOH, and the pH was adjusted to 7.0, and the extracts were applied to Dowex-1 (column = 0.5 × 4.0 cm, chloride form 1 × 8, 400 mesh) which had been prewashed with formic acid. The cyclic AMP and cyclic GMP fractions were separated as described by Murad, Manganiello, and Vaughan [10], with slight modification. Cyclic AMP was eluted with 10 ml of 2 N formic acid and cyclic GMP with 12 ml of 5 N formic acid. When cyclic nucleotides in the media were measured, the media were applied to Dowex-1 columns and both cyclic nucleotides were eluted as described. The eluates were lyophilized in 20 ml serum bottles dissolved in 500 μl of H_2O, and aliquots were assayed. For only cyclic AMP determinations, one skin square was homogenized in 1 ml of 5% trichloroacetic acid (TCA) at 4°C. Aliquots of the supernatant were assayed after having been lyophilized in the presence of 0.1 N HCl. This procedure removes TCA [11], which otherwise interferes with the radioimmunoassay. Both assays for cyclic AMP and cyclic GMP were carried out by the method of radioimmunoassay [12] after one step acetylation [13]. Protein was determined by the method of Lowry et al [14].

RESULTS

The Effects of EGF on the Cyclic GMP Level

Short treatments with EGF revealed that the intracellular cyclic GMP level started to increase at 60 min (Fig 1). The fluctuation during first 20 min is not statistically significant, nor is the apparent difference at 40 min. Longer treatments showed a peak in the cyclic GMP level at 6 hr. Stimulation was still observed after 24 hr of incubation (Table I). We also measured the cyclic GMP level in the media. The level ranged between 2.5 to 9.0 fmoles/mg protein of the tissue slices incubated. No significant difference in the cyclic GMP levels between the control and EGF treated groups was found. As far as the concentration of EGF is concerned, 0.05 μg/ml had some effect, but 0.5 μg/ml and 5.0 μg/ml had a greater effect on the accumulation of intracellular cyclic GMP (Table II).

The Effects of EGF on the Cyclic AMP Level

Our results indicate there are no significant alterations of intracellular cyclic AMP levels after the addition of EGF for up to 24 hr (Table III). Even the largest difference observed at 20 min (0.65 vs. 0.94 pmol/mg protein) is statistically not significant ($p = 0.1$).

The Effect of EGF Treatment on the Epinephrine-cyclic AMP System

Although EGF showed no direct effect on cyclic AMP levels, it was thought possible that an indirect effect on the respon-

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Abbreviations:
EGF: epidermal growth factor
TCA: trichloroacetic acid
TPA: tetradecanoyl-phorbol-acetate

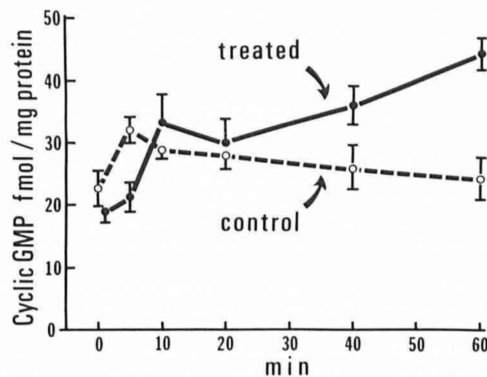

FIG 1. Time course of the changes in intracellular cyclic GMP contents in skin in the absence ("*control*") or the presence ("*treated*") of EGF. The concentration of EGF was 0.5 μg/ml. The data summarize the results (the mean ± SE fmoles/mg protein) of 2 separate experiments. n = Number of samples (n = 4 for controls and 7 for treated except for 0 min control being n = 10). Each sample was assayed in duplicate. The difference in the cyclic GMP level at 60 min of incubation is highly significant ($p < 0.001$) but that at 40 min not highly significant ($0.05 < p < 0.02$).

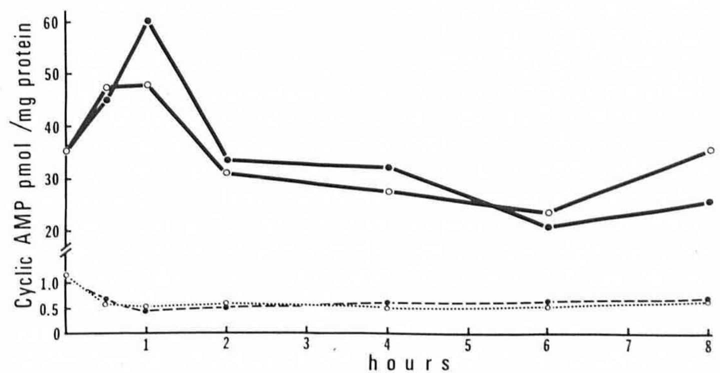

FIG 2. Cyclic AMP accumulation in response to epinephrine following the EGF treatment. Skin squares were treated with 0.5 μg/ml EGF up to 8 hr. At various times during the incubation periods, samples were transferred into the media with 50 μM epinephrine for 5 min. ●——● EGF pretreated; ○——○ control, (no EGF added). *Dotted lines* show base values, i.e. the cyclic AMP levels without epinephrine (●····● EGF pre-treated and ○····○ control groups).

TABLE I. *The effect of EGF on the cyclic GMP content during 24 hours incubation*

	Control	Treated	
0	30.2 ± 0.43		
2 hr	18.2 ± 2.05	26.3 ± 1.65	$0.010 < p < 0.025$
6 hr	19.8 ± 1.56	55.6 ± 2.86	$p < 0.001$
24 hr	12.7 ± 1.24	31.0 ± 6.30	$0.010 < p < 0.025$

EGF concentration was 0.5 μg/ml. Results are expressed as fmol cyclic GMP/mg protein: averages ± SE of 2 independent experiments (n = 4, total number of samples analyzed; each sample was assayed in duplicate).

TABLE II. *The effect of varying amounts of EGF on intracellular cyclic GMP*

	Control	Treated		
		0.05 μg/ml	0.5 μg/ml	5.0 μg/ml
0	13.7 ± 0.88 (n = 11)			
6 hr	15.5 ± 0.93 (n = 11)	18.9 ± .74 (n = 5) $0.025 < p < 0.010$	21.8 ± 1.48 (n = 8) $0.005 < p < 0.001$	26.0 ± 1.02 (n = 5) $p < 0.001$

Results are expressed as fmol cyclic GMP/mg protein.
Skin samples were incubated with EGF for 6 hr. An average ± SE is computed from 2 separate experiments. n = Total number of samples analyzed, each in duplicate.

TABLE III. *The effect of EGF on intracellular cyclic AMP*

	Control	Treated
0	1.26 ± 0.14	—
5 min	0.94 ± 0.08	0.98 ± 0.07
10 min	1.05 ± 0.18	1.35 ± 0.25
20 min	0.65 ± 0.01	0.94 ± 0.16
40 min	0.73 ± 0.03	0.92 ± 0.18
60 min	0.96 ± 0.20	1.04 ± 0.28

Skin samples were treated with 0.5 μg/ml of EGF for various periods. Each value is an average ± SE of 2 separate experiments (n = 6) expressed as pmol cyclic AMP/mg protein. Another set of experiments with longer incubation periods of 0.5, 1, 2, 4, 6, 8 and 24 hr showed no change in the cyclic AMP levels between the control and treated group (data not shown).

siveness of adenyl cyclase might occur. This was tested by "pre-incubation" of skin slices with EGF and then testing the response to a β-adrenergic receptor stimulant. (Fig 2). The "pre-treatment" did not influence the responsiveness of the skin cyclic AMP system to epinephrine. We have also pre-treated the skin squares with hydrocortisone for 24 hr, and the samples were subsequently tested with EGF to see if hydrocortisone could alter the EGF effect on cyclic AMP accumulation. No significant alterations in the cyclic AMP levels were found between EGF-treated groups and control groups for incubation periods up to 24 hr (data not shown).

DISCUSSION

Cyclic nucleotide tissue measurements pose several problems. That due to their extremely minute content appears to be overcome, at least in part, by the use of a sensitive radioimmunoassay. However, one still may note that the baseline cyclic GMP content varies considerably from one experiment to another (e.g. those at 0 time in Tables I, II and Fig 1). This variability may be largely due to the variation in the dermal contamination in the skin slices, since dermis contains much smaller amounts of cyclic nucleotides than epidermis. Also, once skin is removed from an animal, the level of cyclic GMP falls and it is not restored to a normal level during the 15 min pre-incubation period. The extent of this fall may vary for each experiment. However, within each experiment where control and experimental skin squares are from the same skin, the results are reproducible.

It is always difficult to determine a proper dosage for a chemical agent in an *in vitro* system and this is also true for EGF. The *in vivo* level is about 5 ng/ml and this concentration (5~20 ng/ml) has effects in the *in vitro* systems of Rheinwald and Green [15], Baker et al [16] and Shechter, Hernaez, and Cuatrecasas [17]. However, these systems dealt with pure keratinocyte or fibroblast cell cultures. Hassell and Pratt [9] used an EGF concentration of 2000 ng/ml for their organ culture system of unfused palatal shelves and quoted that the level was comparable to that used by Cohen [18] and Hassel [19] in previous organ culture studies. Our floating skin slice is an organ culture system and the use of EGF concentrations of from 50 ng/ml to 500 ng/ml does not seem excessive.

Hollenberg and Cuatrecasas [4] proposed that EGF could alter the level of intracellular cyclic AMP in human fibroblasts and modulate the ability of the cells to initiate polynucleotide synthesis. They stated that the EGF-induced synthesis of RNA and DNA in human fibroblasts was inhibited by theophylline and/or dibutyryl cyclic AMP as well as by cholera toxin, whose actions were believed to be mediated through cyclic AMP. Recently, Frati et al [7] presented evidence that EGF decreased both adenylate cyclase activity and cyclic AMP in mouse epidermis by about 30%, but only after 3–5 days of treatment with

EGF. Our results show that the adenylate cyclase system of pig skin is not affected by EGF.

In mouse fibroblasts in culture [20,21], in lymphocytes [22], and in catecholamine-stimulated rodent salivary glands [23], an increase in cell proliferation is preceded by an immediate increase in cyclic GMP. With respect to epidermal systems, there are only 2 reports on cyclic GMP available: i.e., hyperproliferative psoriatic epidermis had a higher cyclic GMP level than uninvolved and normal epidermis [24] and mouse epidermis after multiple treatments with the tumor promotor phorbol myristate acetate showed increases in cyclic GMP [25]. In our *in vitro* floating system, intracellular cyclic GMP started to increase at one hour after the addition of EGF and reached its maximum at 6 hr. There was no evidence of an immediate response to EGF. This is contrary to the finding of rapid cyclic GMP increases in the rabbit lens epithelial system [6].

EGF and tetradecanoyl-phorbol-acetate (TPA) share several biological effects, such as the induction of plasminogen activator [26], growth stimulation, enhancement of sugar transport, induction of ornithine decarboxylase and prostaglandin synthesis. TPA and other phorbol esters have an ability to diminish the β-adrenergic responsiveness of epidermal cells, though they are not involved in any change of the basal cyclic AMP level [27]. The accumulation of cyclic AMP in response to isoproterenol or the naturally occurring catecholamine, epinephrine, was significantly diminished 9 to 24 hr after topical application of TPA. These reports prompted us to search for the same effect of EGF on epidermal cells. Although the previous results ruled out any direct effect of EGF on adenylate cyclase, it was still possible that EGF might alter the responsiveness of adenylate cyclase to stimulation. Thus we tested the effects of "preincubation" with EGF on the cyclic AMP accumulation in response to epinephrine (Fig 2). For up to 8 hr no significant differences between controls and EGF-treated skin were detected.

Baker et al [16] reported that dexamethasone enhanced the mitogenic response of human foreskin cells to EGF. Paralleling their increased responsiveness to EGF growth stimulation, dexamethasone treated cells exhibited a 50–100% increased ability to bind ^{125}I-EGF during the prolonged exposure to EGF that was required to stimulate cell division. In our system, however, the pre-treatment of skin with hydrocortisone for 24 hours did not change its sensitivity to EGF as tested by cyclic AMP accumulation.

Two different explanations for enhancement of biological effect by EGF are available. Recently, Shechter et al [17] reported that the enhancement of DNA synthesis by EGF in cultured human fibroblasts was demonstrable 24 hr after incubation of cells at 37°C with very low concentrations of the hormone for short periods (30 min) followed by washing of the cells to remove the free hormone in the medium. Also the addition of specific, purified anti-EGF IgG as late as 8 hr after initial hormone exposure completely reversed the biological effects of the hormone. Thus EGF may not have to enter the cell to exert its biological effect. Although several authors [5–9] have reported that EGF acts through the cyclic AMP system in different tissue systems, we could not demonstrate any alterations of the cyclic AMP level in skin following the treatment by EGF. Therefore cyclic AMP is not an intracellular second messenger for the biological effect of EGF.

Is EGF stimulation of epidermal cell proliferation mediated through cyclic GMP? Carpenter, King, and Cohen [28] studied the phosphorylation by EGF in a cell-free membrane preparation from a human epidermoid carcinoma cell line, and reported that maximal stimulation of the membrane phosphorylation occurred in the presence of 3×10^{-8} M EGF, but the presence of cyclic AMP or cyclic GMP ($10^{-4} \sim 10^{-6}$ M) had no discernable effect on the phosphorylation reaction. These results plus our finding that stimulation of cyclic GMP formation in pig skin by EGF does not occur quickly but requires hours leads to the conclusion that this effect on cyclic GMP is a consequence of the action of EGF and probably not related to its action.

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Announcement

A meeting on anthralin will be held at Sophia Antipolis near Nice, France on Oct 17 and 18, 1980. The meeting will focus on current concepts in the treatment of psoriasis with anthralin. Organizers of the meeting are Prof. Malcolm Greaves (London), Prof. Lennart Juhlin (Uppsala), Prof. Hans Schaeffer (Sophia Antipolis).

The main topic, including some clinical experiences, will be covered by guest speakers. Free communications on the theoretical aspects of the topic are invited. For information contact Prof. Hans Schaefer, CIRE, Sofia Antipolis 06560, Zalbonne, France, Telephone 93-74-12-00.