sorting experiments indicate that a small, but significant, fraction of unlabeled cells remains in S phase during epidermal regeneration, and hence may not be immediately triggered into a more rapid cell cycle progression.

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Regulation of Beta-Adrenergic Adenylate Cyclase Responsiveness of Pig Skin Epidermis by Suboptimal Concentrations of Epinephrine

HAJIME IIZUKA, M.D., KAZUNORI KISHIYAMA, M.D., AND AKIRA OHKAWARA, M.D.

Department of Dermatology, Asahikawa Medical College, Asahikawa, Japan

Although receptor-specific refractoriness has been suggested to be one of the regulatory mechanisms of epidermal adenylate cyclase systems, its physiologic significance has been a subject of controversy because of the requirement of unusually high concentrations of agonists to induce refractoriness. In order to determine whether the epidermal adenylate cyclase system is regulated through a refractoriness mechanism by suboptimal concentrations of receptor agonists, this study was undertaken using pig skin epidermal adenylate cyclase systems.

Pretreatment of pig skin with 0.1–1 μM epinephrine in vitro resulted in the reduction of the maximal epinephrine response (epinephrine-induced cyclic AMP accumulations) to various degrees without alterations in either low or high K_m cyclic AMP phosphodiesterase activities. Repeated pretreatments were shown to be more effective in inducing refractoriness than a single pretreatment. Apparently there was no change in the K_m value for epinephrine, suggesting that the decrease in epinephrine response represents a reduction in the number but not in the affinity of functional beta-adrenergic adenylate cyclase receptor sites. This refractoriness by low concentrations of catecholamine pretreatment was specific to the beta-adrenergic system, since there was no reduction in histamine response after the epinephrine pretreatment.

These results indicate that the epidermal beta-adrenergic adenylate cyclase system is regulated by much lower concentrations of catecholamine than were previously described. It was suggested that physiologic fluctuations of plasma catecholamine levels might have a profound effect on epidermal beta-adrenergic adenylate cyclase responsiveness, resulting in the alteration of the minimal catecholamine level required for the successive

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Reprint requests to: Hajime Iizuka, M.D., Department of Dermatology, Asahikawa Medical College, 3-11 Nishikagura, Asahikawa 078-11, Japan.

activation of cyclic AMP-dependent protein kinase, which is the predominant target of cyclic AMP in epidermis.

Prolonged or repeated treatments with drugs or hormones can result in tolerance to their biologic effects. This phenomenon has been observed in several tissues and has been named desensitization, tachyphylaxis, refractoriness, etc. [1–3]. The epidermal adenylate cyclase system has been known to be regulated by this mechanism [4–6]. Subsequent to exposure of intact epidermal tissue to epinephrine, a reduced capacity to accumulate cyclic AMP is expressed upon addition of epinephrine. This loss of responsiveness is specific to the agonist used, for example, epidermal tissue made unresponsive to epinephrine retained its sensitivity to histamine and vice versa [4]. Although this receptor-specific refractoriness has been assumed to be a physiologically significant regulatory mechanism of the epidermal adenylate cyclase system, previous studies were carried out using unphysiologically high concentrations of agonists [4,5].

Using the pig skin slice system, we have investigated the regulatory mechanism of epidermal adenylate cyclase system in our laboratory [7,8]. During the course of the study, we noticed that responsiveness of an epidermal slice to each agonist varied from tissue to tissue obtained on different occasions. Even the responsiveness of skin from the same pig differed if the skin slice was taken on a different occasion. These findings suggested to us that each receptor adenylate cyclase responsiveness might already be determined in vivo by physiologic fluctuations of each agonist concentration, i.e., with much lower concentrations than were previously described.

The present study was undertaken to determine whether epidermal adenylate cyclase system responsiveness might be influenced by relatively low concentrations of agonists. Among four independent receptor adenylate cyclase systems in epidermis [9], the epinephrine adenylate cyclase system was chosen since it is well known that plasma catecholamine levels fluctuate because of many physical and emotional stimuli, which include exercise, emotional stimuli, cold stress, etc. [10–12].

MATERIALS AND METHODS

Domestic pigs weighing about 10–15 kg were anesthesized with Nembutal (Dainippon, Osaka, Japan) administered i.p. (dose 30 mg/kg) as previously described [5]. Fifteen minutes after the anesthesia, skin slices were taken from the backs of the pigs with a Castroviejo keratome (Storz Instrument Co., St. Louis, Missouri). Keratome shims were selected to give an average thickness of 0.2 mm and the skin slices thus obtained were histologically checked and were confirmed to be predominantly epidermis (around 80%). The skin slice was cut into 5 × 5 mm squares, washed 3 times in RPMI 1640 medium, floated keratin layer up in the same medium, and preincubated for 20–30 min at 37°C to standardize the cyclic AMP level [13]. Skin squares were then treated with various concentrations of epinephrine in the same medium at 37°C in an atmosphere of 5% CO_2 in air for up to 2 h. After an appropriate time, the skin squares were transferred to a new RPMI 1640 medium at 37°C and treated with 50 μM epinephrine or 1 mM histamine for 5 min. These concentrations were chosen to obtain maximal responses for cyclic AMP accumulations [4]. After the incubation, skin squares were quickly frozen between two plates of Dry Ice. The cyclic AMP content in these skin squares was measured by radioimmunoassay using a Yamasa cyclic AMP assay kit (Yamasa Shoyu Co. Tokyo, Japan) after partial purification by the method of Yoshikawa et al [13]. The cyclic AMP phosphodiesterase activities in these skin squares were measured by the method of Scott and Solomon [14] with minor modifications as described by Adachi et al [15]. The substrate cyclic AMP levels were 102 μM for high K_m and 0.75 μM for low K_m enzyme assays. Protein concentration was measured by the method of Lowry et al [16].

Chemicals and drugs were all freshly prepared before each experiment and the pH of the medium was adjusted to 7. The statistical significance was determined by Student's *t*-test.

RPMI 1640 medium was purchased from Gibco (Grand Island, New York). Epinephrine was the product of Daiichi Pharmaceutical Co. (Tokyo, Japan). [^{3}H]Adenosine-3′,5′-cyclic phosphate (sp act 34.5 Ci/mmol) was obtained from New England Nuclear (Boston, Massachusetts). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri).

RESULTS

Fig 1 shows the effect of epinephrine pretreatment on the subsequent epinephrine-induced cyclic AMP accumulations in the skin. When pig skin squares were treated with 50 μM epinephrine (A in Fig 1), there was a marked accumulation of cyclic AMP after 5-min incubation, which was followed by a decrease in cyclic AMP level despite the continuous presence of epinephrine. After 60-min incubation, skin squares pretreated with 50 μM epinephrine did not respond to epinephrine, resulting in minimal increase in cyclic AMP level. On the other hand, skin squares that were not treated with epinephrine responded to 50 μM epinephrine, resulting in marked cyclic AMP accumulations (C in Fig 1). There was no difference in epinephrine-induced cyclic AMP accumulations after 60-min incubation when compared with the initial response of the skin without 60-min incubation (compare A (maximal response) with C in Fig 1).

The effect of suboptimal concentrations of epinephrine pretreatment is shown (B in Fig 1). Pig skin squares were treated with 0.1 μM epinephrine for 60 min and were then incubated with 50 μM epinephrine. The treatment with 0.1 μM epinephrine had a minimal effect on the cyclic AMP levels of the skin. However, after pretreatment with 0.1 μM epinephrine, skin squares responded significantly less than the skin without epinephrine pretreatment ($p < 0.01$) (compare B and C in Fig 1). The response of skin pretreated with 0.1 μM epinephrine was also significantly less than that of skin without 60-min incubation ($p < 0.01$) (compare A and B in Fig 1). There was no difference in the time course of epinephrine response after pretreatment with 0.1 μM epinephrine, and the maximal effect was observed at 5-min incubation time (data not shown).

In Fig 2 concentration effects of epinephrine on the cyclic AMP levels of skin with and without epinephrine pretreatment were compared. Skin squares without epinephrine pretreatment responded markedly and accumulated cyclic AMP by the treatment with various concentrations of epinephrine ranging from 0.1–30 μM. Compatible with the findings in Fig 1, skin squares pretreated with 0.1 μM epinephrine responded to epinephrine significantly less than skin without epinephrine pretreatment. In both cases, however, the maximal accumulation of cyclic AMP was obtained at concentrations of about 3–10 μM and,

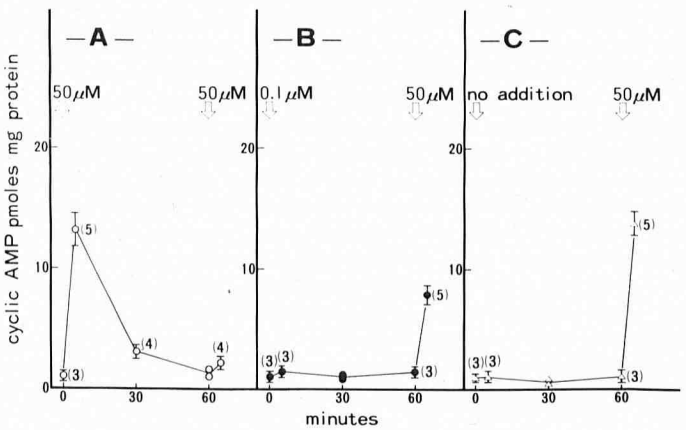

FIG 1. Effects of epinephrine pretreatment on the subsequent epineprine-induced cyclic AMP accumulations. After preincubation (see *Materials and Methods*) pig skin squares were treated with various concentrations of epinephrine ($A = 50$ μM, $B = 0.1$ μM, $C = 0$ μM) and were incubated at 37°C. After 60-min incubation, skin squares were transferred to a new RPMI 1640 medium containing 50 μM epinephrine at 37°C. The cyclic AMP contents of skin squares at each point of time are indicated. The number of each point is shown in parentheses.

despite moderate variations from experiment to experiment, the apparent K_m values for epinephrine were around 1 μM in both skin groups (K_m for control skin, 0.9 ± 0.1 μM; K_m for refractory skin, 1.0 ± 0.2 μM).

Table I shows the effect of repeated pretreatments of epinephrine on the subsequent epinephrine-induced cyclic AMP accumulations. Pig skin squares treated with various concentrations of epinephrine repeatedly for 3 times responded less than the control skin in a concentration-dependent manner to subsequent epinephrine activations. Thus pretreatment with higher concentrations of epinephrine resulted in more marked refractoriness of skin. Furthermore, it was shown that repeated pretreatments were much more effective in inducing refractoriness; for example, pretreatment with 0.1 μM epinephrine (for 3 times) resulted in essentially the complete loss of epinephrine responsiveness, which was not observed by a single pretreatment with the same (0.1 μM) concentration of epinephrine. After epinephrine pretreatment, there was no difference in histamine-induced cyclic AMP accumulations (Table I).

Table II shows the comparison of cyclic AMP phosphodiesterase activities of skin treated with and without 0.1 μM epinephrine. There was no difference in either low or high K_m cyclic AMP phosphodiesterase activities between the two skin groups.

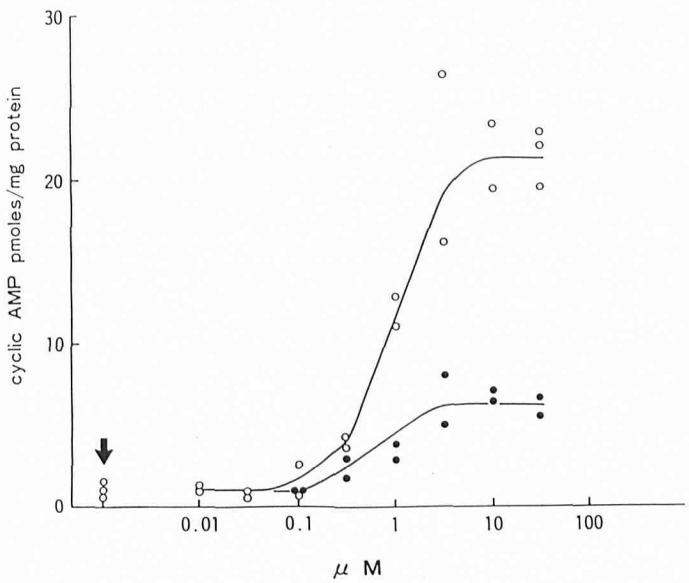

FIG 2. Concentration effect of epinephrine after epinephrine pretreatment. After preincubation, skin squares were treated with and without 0.1 μM epinephrine for 2 h at 37°C. Skin squares were transferred to new RPMI 1640 media at 37°C and incubated with various concentrations of epinephrine plus 5 mM theophylline. Incubations were done for 5 min at 37°C and cyclic AMP contents in these skin squares were measured as described in *Materials and Methods*. Results represent three independent experimental series. O-O-Control; ●-●-0.1 μM epinephrine pretreated skin. *Arrow* indicates 0 value.

DISCUSSION

Physiologic concentrations of plasma catecholamines (norepinephrine and epinephrine) are 2–4 nM, which may be increased to 5–15 nM during stress and exercise [10,11]. Although epidermal beta-adrenergic adenylate cyclase is activated by these agonists, resulting in the spikelike accumulation of cyclic AMP [17], the concentration required for the detection of activated adenylate cyclase was reported to be much higher than these physiologic concentrations. For example, consistent with the results by Yoshikawa et al [17], we could detect only a small increase in cyclic AMP level by 0.1 μM epinephrine treatment (Fig 2), which was about 7 times higher than the physiologic range of plasma catecholamine level. Furthermore, although receptor-specific refractoriness of epidermal adenylate cyclase has been established by Adachi et al [4], the concentration used for the induction of refractoriness was also much higher than the physiologic range of agonist concentrations. Thus there have been no data available suggesting that physiologic concentrations of catecholamine have an effect on the beta-adrenergic adenylate cyclase system in epidermis.

In this study, using receptor-specific refractoriness phenomena, we could clearly demonstrate that epidermal beta-adrenergic adenylate cyclase responsiveness is regulated by relatively low (near physiologic) concentrations of the beta-adrenergic agonist, epinephrine. Although 0.1 μM epinephrine had minimal effect on the cyclic AMP levels of the skin by itself, after the pretreatment of the skin with this concentration of epinephrine there was a significant reduction in beta-adrenergic responsiveness (B in Fig 1 and Fig 2). Repeated pretreatments were more effective in inducing refractoriness than a single pretreatment

TABLE II. *Cyclic AMP phosphodiesterase activities before and after epinephrine pretreatment*

	Cyclic AMP phosphodiesterase activity (pmol/min/mg protein)	
	low K_m	high K_m
Control	8.6 ± 0.7	129.7 ± 10.5
Epinephrine pretreatment	10.4 ± 1.0	142.7 ± 16.5

After preincubation, pig skin squares were treated with and without 0.1 μM epinephrine for 60 min at 37°C. Skin homogenates were prepared and phosphodiesterase activities were measured at 0.75 μM and 102 μM for low and high K_m enzyme respectively. Data are expressed as pmol/min/mg protein (n = 4).

TABLE I. *Effect of repeated pretreatment of epinephrine*

	Control	Epinephrine pretreatment				
		0.01 μM (3 times)	0.1 μM (3 times)	1 μM (3 times)	0.1 μM (once)	50 μM (once)
No addition	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Epinephrine	13.6 ± 2.6	11.1 ± 0.4	3.1 ± 0.2[a]	1.2 ± 0.1[a]	7.7 ± 0.5[b]	2.1 ± 0.1[a]
Histamine	107.5 ± 9.4	N.D.[c]	152.3 ± 20.9	N.D.	N.D.	117.0 ± 16.6

After preincubation, pig skin squares were treated with various (0, 0.01 μM, 0.1 μM, 50 μM) concentrations of epinephrine at 37°C. Skin squares treated with 0.01 μM, 0.1 μM, and 1 μM epinephrine for 3 times were transferred twice at 40-min and 80-min incubation time to new RPMI 1640 media containing 0.01 μM, 0.1 μM, and 1 μM epinephrine, respectively. Control skin squares and skin squares treated with 1 μM and 50 μM epinephrine once were transferred at 40-min and 80-min incubation time to a new RPMI 1640 medium without the addition of epinephrine. After 120-min incubation, these 6 groups of skin squares were transferred to new RPMI 1640 media at 37°C and were then treated either with 50 μM epinephrine or 1 mM histamine. Incubations for cyclic AMP accumulations were done for 5 min at 37°C without the addition of phosphodiesterase inhibitors. Data are expressed as cyclic AMP pmol/mg protein (n = 3).

[a] $p < 0.01$ compared with control.

[b] $p < 0.01$ compared with skin treated 3 times (0.1 μM).

[c] N.D. = not determined.

(Table I). As was reported by Adachi et al [4], the refractoriness was receptor-specific (Table I) and no change in phosphodiesterase activities was observed by suboptimal concentrations of agonist treatment (Table II). Furthermore, the apparent K_m for epinephrine for beta-adrenergic adenylate cyclase activation seemed to be unchanged after the partial desensitization process, suggesting that the decrease in epinephrine response represents a reduction in the number but not the affinity of the functional beta-adrenergic adenylate cyclase system in epidermis.

There are numerous reports concerning the mechanism of desensitization of the adenylate cyclase system, which is a membrane-bound enzyme complex composed of at least 3 distinct subunits: receptor subunit, catalytic subunit, and guanine nucleotide binding site [18]. The activation of the enzyme (catalytic subunit) requires the functional coupling of these subunits, and it is assumed that during the desensitization process, the uncoupling of the receptor and other subunits and/or the reduction in the absolute number of receptor subunits occur [2,3,19,20]. Since we did not directly measure beta-adrenergic binding sites before and after the desensitization process, no data are available as to which is the predominant mechanism of epidermal adenylate cyclase refractoriness at present.

Despite the finding which suggests that activation and desensitization of adenylate cyclase might be a separable process in certain experimental conditions [2,21], it is generally accepted that receptor-specific desensitization is associated with activation of the adenylate cyclase system physiologically. It is well known that only agonists, but not antagonists, which do not lead to coupling and activation of adenylate cyclase, produce desensitization [2,22]. Thus it is possible that epidermal beta-adrenergic adenylate cyclase was activated during suboptimal concentrations of epinephrine pretreatment, resulting in the increased intracellular level of cyclic AMP, although the increase might be too small to be detected. Recently Yoshikawa et al [23] reported that cyclic AMP-dependent protein kinase in epidermis was fully activated by relatively low concentrations of epinephrine, suggesting that protein kinase activation takes place in response to a relatively small increase in the cyclic AMP level. Taken together, it is likely that the epidermal adenylate cyclase-cyclic AMP-protein kinase system does function at near physiologic range of catecholamine concentrations.

In conclusion, our data clearly indicate that the epidermal beta-adrenergic adenylate cyclase system is regulated through a refractoriness mechanism by relatively low concentrations of epinephrine. These results are consistent with those of other cell systems [24,25], where the physiologic concentrations of plasma catecholamine regulate the responsiveness of the beta-adrenergic adenylate cyclase system. The resultant fluctuations of the maximal capacity to accumulate cyclic AMP without an alteration in K_m value for epinephrine, would result in the inversely related fluctuations of the minimal catecholamine level required for the successive activation of epidermal protein kinase. In other words, partially refractory skin would require higher concentrations of catecholamine for the activation of protein kinase. Thus the epidermal adenylate cyclase-cyclic AMP-protein kinase system seems to be under dynamic regulation through an adenylate cyclase refractoriness mechanism, which may be significantly involved in the regulatory mechanisms of cyclic AMP-mediated processes in epidermis.

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