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Cyclic AMP-Dependent Protein Kinase Isozymes of Pig Skin and Human Skin from Normal and Psoriatic Subjects

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Cyclic AMP-dependent protein kinase isozymes of pig and human skin (epidermis) were separated by DEAE-cellulose column chromatography after micromodification for small biopsy samples. Clear-cut separations of type I and type II isozymes, which were of about equal amounts, could be obtained only when the ischemia effect was avoided by in vivo freezing of skin and homogenization for less than 10 s. Intradermal injections of epinephrine caused dose-dependent activation of type I isozyme, but not of type II. Injections of other skin adenylate cyclase stimulators such as histamine, adenosine, and prostaglandin E_2 elevated the local cyclic AMP levels to not more than 5 pmol/mg protein and also stimulated only the type I isozyme.

Incubation of keratome-sliced pig skin under various conditions caused both activation by dissociation and inactivation by reassociation of the subunits, which appeared to be dependent on the cyclic AMP content. Epinephrine added to the incubation medium led to complete activation of both type I and type II isozymes (the intraepidermal cyclic AMP contents ranged from 20–50 pmol/mg protein).

The isozymes of normal skin and involved skin of psoriatics showed identical peaks of type I and type II isozymes of equal amounts. The data indicate that protein kinase in the involved skin is not in an activated (by cyclic AMP) state.

Activation of the cyclic AMP system by hormones or other chemicals is followed by a specific phosphorylation process mediated by cyclic AMP-dependent protein kinase. Since the physiologic effects of an increase in cyclic AMP are solely dependent on this activation process, cyclic AMP-dependent protein kinase activity may be more important than the increase in cyclic AMP level itself. Cyclic AMP-dependent protein kinase has 2 regulatory and 2 catalytic subunits [1]. The inactive holoenzyme dissociates in the presence of cyclic AMP into the active catalytic subunit and the regulatory subunit–cyclic AMP complex [2]. Since Reimann et al [3] first demonstrated two different types of cAMP-dependent protein kinase, these isozymes (types I and II) have been found in most mammalian tissues [2–7]. These two isozymes have the same catalytic subunit, although the regulatory subunits are different [8]. Type I and type II isozymes can be separated by DEAE-cellulose column chromatography [1,4], i.e., a free catalytic subunit can be eluted by 10 mM potassium phosphate buffer, and type I and type II isozymes by 0.1 and 0.2 M of NaCl, respectively. Type I kinase can be rapidly dissociated into the free regulatory and catalytic subunits by salt or substrate. These subunits will slowly reassociate by removing the dissociating agents. In contrast, type II kinase can be only slowly dissociated but will rapidly reassociate [2,9].

It appears that these isozymes have specific functions in some tissues. Byus et al [10] showed that glycogenolysis in rat hepatocytes was regulated by the activation of type I kinase. Costa et al [11] reported that type I kinase activity was high in mitosis while type II kinase activity was associated with the S phase of

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Abbreviations:
cyclic AMP: cyclic adenosine 3′,5′-monophosphate
EGTA: ethyleneglycol-*bis*-(β-aminoethyl ether)-N,N′-tetraacetic acid
IBMX: isobutylmethylxanthine
PGE_2: prostaglandin E_2

cell division. Olson and Russell [12] demonstrated that the activation of type I kinase led to ornithine decarboxylase induction. As far as skin is concerned, Kumar et al [13] have demonstrated the isozymes in monkey and human skin, but the physiologic function of each isozyme is still not clear. In this study, we measured activation of type I and type II isozymes of pig and human skin in relation to the activation of adenylate cyclase. With micromodification of our analytical system, we also measured the isozymes in the involved skin of psoriatics.

MATERIALS AND METHODS

Histone H II A was obtained from Sigma and both DE-52 and phosphocellulose paper (P 81) were products of Whatman. [γ-^{32}P]-ATP was purchased from Amersham.

Skin Treatment

Domestic pigs weighing about 6 to 8 kg were anesthetized with Nembutal i.p. (30 mg/kg). For in vitro studies, skin samples were taken with a keratome set at 0.2 mm in thickness. The skin pieces composed of 80–85% epidermis were incubated in Hanks' medium as described in the following section. For in vivo studies, several concentrations of epinephrine hydrochloride in saline (0.5 ml each) were injected intradermally into the back skin of pigs with a tuberculin syringe. After 10 min, the injected sites were frozen with dry ice and removed with the keratome. Saline-injected skin was removed in an identical manner. Human skin samples, about 5 mm square, were obtained from normal subjects and lesional skin of psoriasis patients by shave biopsy [14] after freezing the skin with liquid nitrogen spray. All samples were kept in a deep freezer until use.

Cyclic AMP Measurement

The cyclic AMP contents in the shave biopsy samples at 5 min after the intradermal injections (0.5 ml each) of epinephrine (0.5 mM), histamine (10 mM), adenosine (10 mM), and PGE_2 (0.5 mM) were measured by a radioimmunoassay method with slight modification [14].

DEAE-Cellulose Column:

DE 52 column procedures were as described by Corbin and Keely [4]. The skin samples kept in a deep freezer were cut into 5-mm squares with scissors as they were frozen, and homogenized for 5 s twice in 1–3 ml of cold 10 mM potassium phosphate buffer, pH 6.8, containing 1 mM EDTA in a glass homogenizer. The homogenate was centrifuged for 30 min at 30,000 × *g*, and the supernatant (generally 3–6 mg protein with specific activity of 150~200 pmol/mg protein/min) was applied to a DEAE-cellulose column (0.6 × 4 cm) equilibrated with the same buffer. After washing the column with 10 ml of the buffer, the isozymes were collected each in 300-μl fractions by a linear NaCl gradient (0–400 mM). The flow rate was 50 μl/min. The variation of the chromatographs was less than 10%.

Cyclic AMP-Dependent Protein Kinase Assay:

The reagent mixture consisted of 100 μg/50 μl of histone H IIA, 10 mM potassium phosphate buffer pH 6.8, 10 mM NaF, 10 mM magnesium acetate, 0.25 mM EGTA, 0.1 mM IBMX, 20 μM ATP (containing 0.5–1 μCi of [γ-^{32}P]-ATP, approximately 8 × 10^5 cpm), 5 μM cyclic AMP (when added), and the isozymes (20 μl) in a total volume of 50 μl. The reaction was started by adding the isozymes from the fractions, and the mixtures were incubated at 30°C for 5 min. After the incubation, 40 μl each of the mixtures were applied on 2 × 2 cm pieces of phosphocellulose paper and washed with tap water for 1 h. These papers were dehydrated with acetone and counted in a liquid scintillation counter. The protein kinase activities were expressed as total ^{32}P incorporated in cpm/5 min (at 30°C)/20 μl each from fractions obtained through the DEAE-cellulose column. Because of rapid decay of ^{32}P, the cpm values were corrected for those on day 1. The initial crude enzyme applied onto the column varied from 3–6 mg protein, hence the protein kinase activities were corrected for 5 mg initial protein.

RESULTS

A typical separation of type I and type II isozymes of pig skin protein kinase is shown in Fig 1*A*. Reproducible separation of the isozymes could be obtained when skin samples were removed after the skin surfaces had been frozen with dry ice and when the samples were homogenized for less than 10 s. When skin samples were left at room temperature for 1 min, the resulting ischemia effect [15] caused a substantial loss of the type I isozyme peak; after 5 min of the ischemia, both type I and type II peaks were lost (Fig 1*B*,*C*). The losses were due to the activation of both isozymes by sudden elevation of cyclic AMP levels after skin removal (ischemia). Fig 1*B* shows that the catalytic unit, free from the regulatory unit, was eluted at the early fractions (c.f., the elution tube #5–#7). Fig 1*B* also shows that the activated catalytic subunit was no longer cyclic AMP-dependent, whereas the type I and type II isozymes were still cyclic AMP-dependent. The losses of the two isozyme

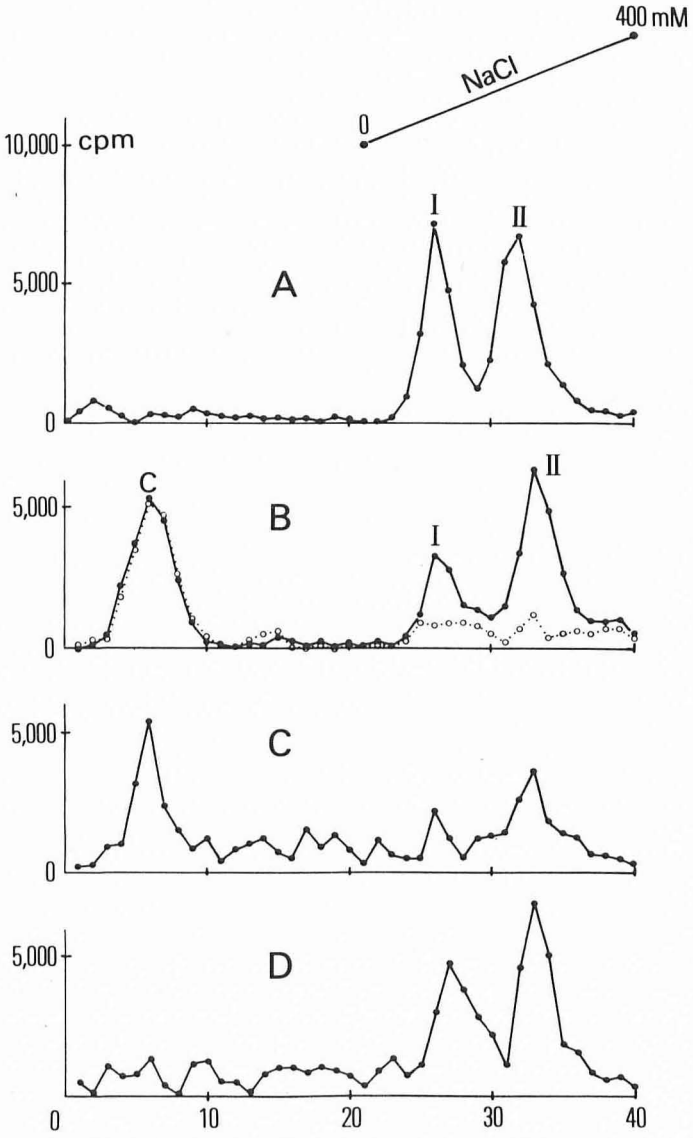

FIG 1. Chromatographic separation of cyclic AMP-dependent protein kinase isozymes of pig skin. *A*, A typical isozyme pattern shows type I and type II peaks. The enzyme activity (cpm) is shown in *ordinate*, the elution tube number is shown in *abscissa*. A linear NaCl gradient to 400 mM started at tube No. 21. *B*, The isozyme pattern after partial ischemia. The skin sample was obtained without in vivo freezing and left in the air for 1 min. Note partial activation of type I isozyme (i.e., partial decrease of peak I) and the appearance of a new peak at tubes No. 5–7. This peak "*C*" is the catalytic subunit. *Dotted line* indicates the enzyme activity in the absence of cyclic AMP in the assay mixture. *C*, The isozyme pattern after 5 min of ischemia. Note marked activation of both type I and type II isozymes. *D*, The effects of preincubation. The skin sample left in the air for 5 min was incubated in Hanks' balanced salt solution with 200 mg/100 cc glucose for 20 min prior to homogenization. Note the recovery of both peaks due to the reassociation of regulatory and catalytic subunits.

peaks could be restored by incubating the keratomed skin samples in Hanks' medium for 15–20 min at 37°C prior to homogenization (Fig 1*D*).

Fig 2 summarizes the results of in vivo activation experiments after epinephrine intradermal injections into pig skin. Skin samples were removed after in vivo freezing by dry ice. The results clearly indicate that type I isozyme was activated in a dose-dependent manner, but type II isozyme was not activated. The cyclic AMP contents at 5 min after the injections were

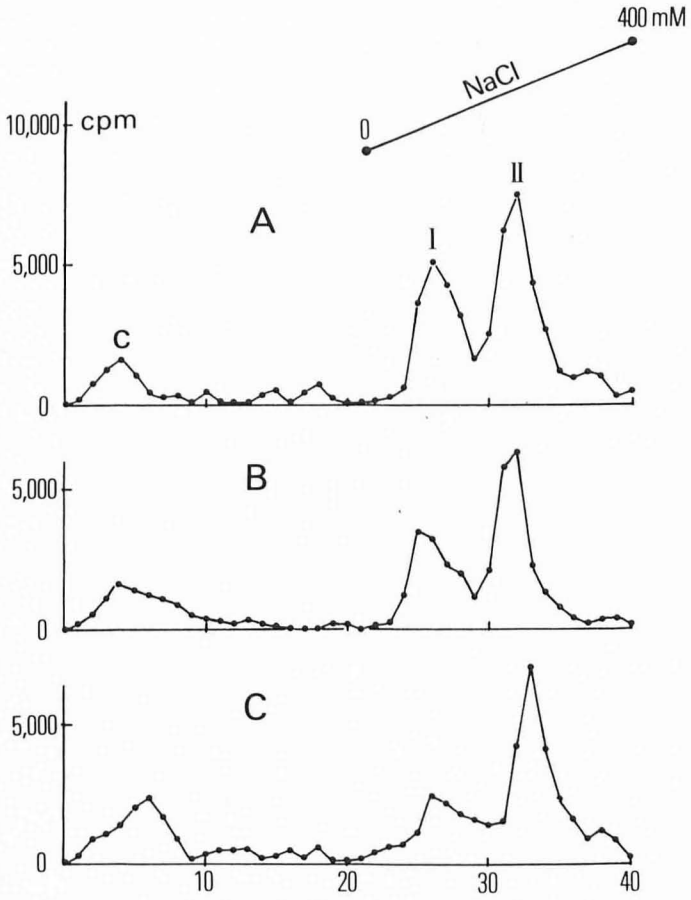

FIG 2. An in vivo activation: effects of intradermal injections of epinephrine on the isozyme patterns. The epinephrine injections were 0.5 ml each of 20 μg/ml (*A*), 100 μg/ml (*B*), and 1000 μg/ml (*C*). For detailed procedures see Materials and Methods.

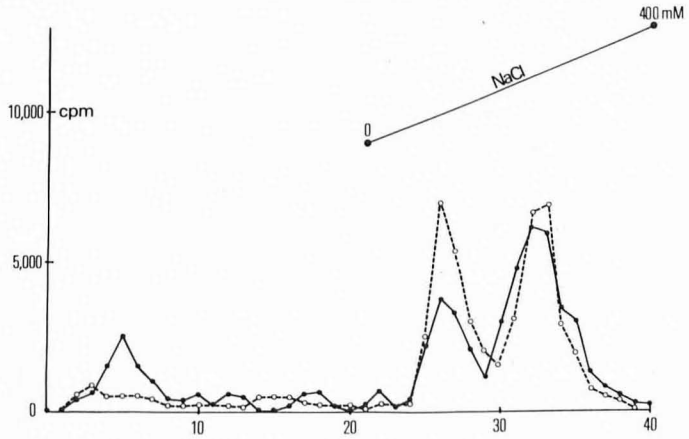

FIG 3. The in vivo activation with PGE_2. PGE_2 (0.5 mM) was injected intradermally. The *dashed line* shows a control pattern. Nearly identical patterns were obtained with the intradermal injections of histamine (10 mM) and adenosine (10 mM).

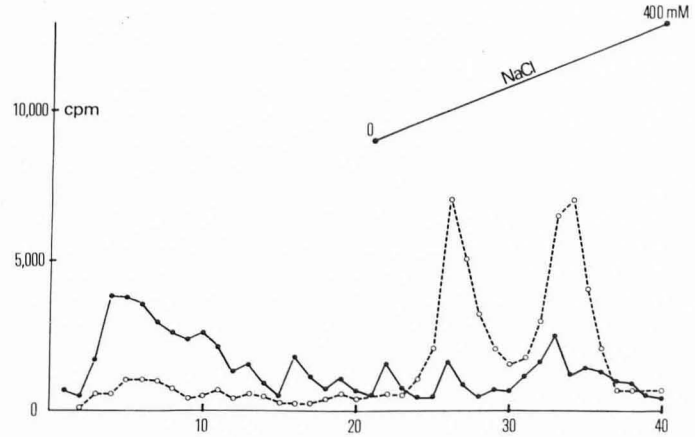

FIG 4. In vitro activation: effects of epinephrine added to the medium. The skin slices were preincubated for 20 min to eliminate the ischemia effect and subsequently transferred to medium containing 5 × 10^{-5} M epinephrine for 5 min. *Dashed line* indicates the control isozyme pattern without added epinephrine.

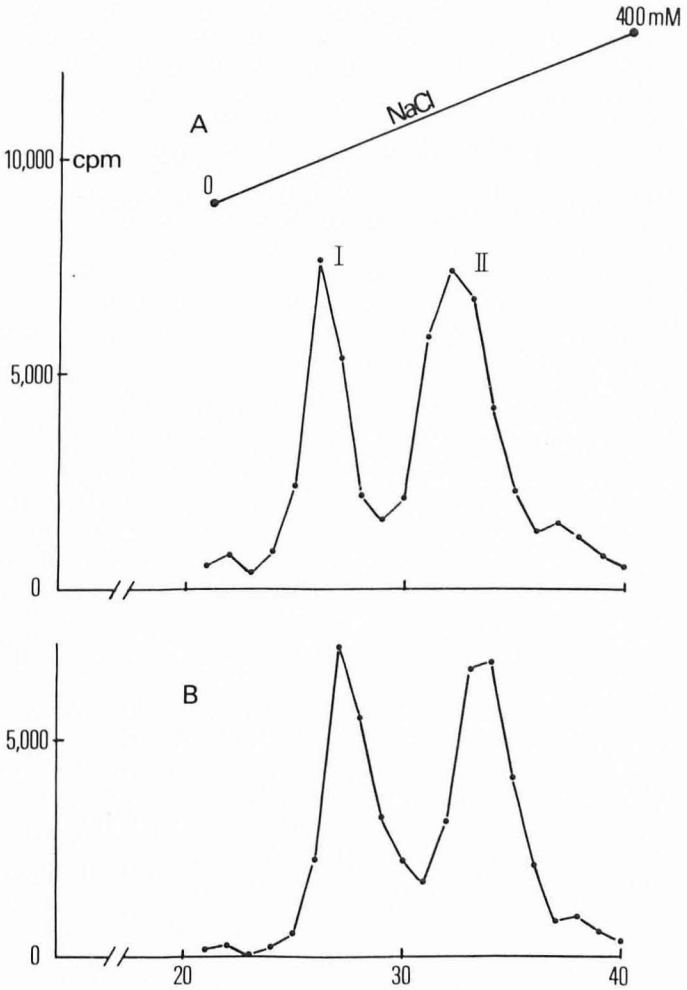

FIG 5. The chromatographic separation of type I and type II isozymes of human skin protein kinase. *A*, Normal skin of normal subject. *B*, The involved skin of psoriatics. The shave biopsies of skin samples (5 × 5 mm) were obtained after freezing the skin surface with liquid nitrogen spray.

about 5 pmol/mg protein as compared with normal value of less than 1 pmol/mg protein.

The effects of intradermal injections of other skin cyclic adenylate cyclase stimulators such as histamine [16], adenosine

[17], and PGE_2 [18] were also examined. Determinations on cyclic AMP contents in triplicate samples showed increased levels up to 5 pmol/mg protein after the injections. Fig 3 shows the effect of PGE_2 injection. Partial activation of only type I isozyme occurred. This is similar to the in vivo injection of epinephrine (Fig 2). The effects of adenosine and histamine were identical to the above and thus the results are not shown here.

The loss of type II isozyme peak was easily observed when the keratomed skin was incubated in vitro with epinephrine. Fig 4 shows one such experiment. Keratomed skin was preincubated in a physiologic medium for 20 min to restore both isozyme peaks and then transferred to the medium containing epinephrine. The complete loss of both peaks was evident.

The isozyme patterns of protein kinase from human skin are shown in Fig 5. Both normal skin from normal subjects and the involved skin of psoriasis patients revealed clear-cut separation of type I and type II isozyme peaks which were of equal amounts.

DISCUSSION

Since Corbin et al [2] reported the occurrence of two protein kinase isozymes that could be distinguished on the basis of DEAE-cellulose elution profiles, similar isozymes have been separated from many tissues and organs including fat, heart, skeletal muscle, liver, kidney, brain, salivary gland, ovary, etc. [2–7]. The separation of 2 isozymes from both pig and human skin as presented in this study is also consistent with data from other tissues. Kumar et al [13] reported the occurrence of 3 peaks of human skin protein kinase, whereas we consistently isolated only 2 peaks. The reason for this discrepancy is unknown. However, reviewing their data, their peaks II and III may be one peak, peak II, since the separation is not complete. Another possibility is that their starting samples might have consisted of whole-thickness skin and subcutaneous tissue, in contrast to ours which were predominantly epidermis. The mixture of epidermis, dermis, and fat might have caused the slight splitting of peak II.

During the early stages of our studies, when we minced and homogenized tissue thoroughly, the chromatographic separations of isozymes were often erratic and unreproducible. Subsequently, we found that the rise in cyclic AMP in skin due to the ischemia effect caused artifactual shifts in the isozyme peaks. The ischemia effect can be prevented by routinely freezing skin in vivo before sampling, and also the samples should be homogenized for not longer than 10 s. In other experiments we found that the in vivo freezing abolished the ischemia effect (unpublished data). The precaution of not over-homogenizing tissue has also been recommended for total skin protein kinase assays [19].

A loss in isozyme peak I (i.e., the dissociation of regulatory and catalytic subunits) appears to correlate with the cyclic AMP contents, since the degree of loss was dependent on the dosages of epinephrine injected intradermally. The intradermal injections of other skin adenylate cyclase stimulators such as histamine, PGE_2, and adenosine also activated the type I subunit, but not the type II. The injections of these stimulators caused increases in intraepidermal cyclic AMP levels up to 5 pmol/mg protein [20]. The higher cyclic AMP level associated with ischemia for 5 min (but not for 1 min) caused activation of both type I and type II subunits. Our previous studies [15] showed that the cyclic AMP levels at 1 min of ischemia ranged from 3–5 pmol/mg dry weight, whereas the level at 5 min reached 10 pmol/mg dry weight.

Preincubation of the keratomed slices in a physiologic medium caused reassociation of the regulatory and catalytic subunits (as indicated by the restoration of two peaks, Fig 1*D*). These data are consistent with the fact that the preincubation caused a decrease in the cyclic AMP level to 2 pmol/mg protein which is close to an unstimulated in vivo level [21]. Further incubation of the skin slice with epinephrine led to complete activation (thus dissociation) of both peak I and peak II which was shown as losses of both peaks (Fig 4). In this in vitro experiment, the intracellular cyclic AMP levels range from 20–50 pmol/mg protein after epinephrine stimulation [21].

It has been shown that adenylate cyclase in the lesional skin of psoriatics responds poorly to epinephrine stimulation as compared with normal skin [22,23]. In search of another abnormality in the cyclic AMP cascade we have looked for an abnormal protein kinase activity ratio but the ratios were nearly identical among normal skin and uninvolved and involved skin of psoriatics [24]. The current protein kinase isozyme study also fails to detect any basic abnormality in the involved skin. Costa et al [11] showed that during the mitotic stage of Chinese hamster ovary cells, the activity of type I isozyme became maximal, but that of type II minimal. The psoriasis lesion where mitosis is markedly increased might have shown a predominant type I isozyme, but we found no change in the isozyme pattern. However, since intraepidermal cyclic AMP levels are about the same in normal skin and in uninvolved and involved epidermis of psoriatics [14,25], we should not expect any change in either protein kinase activity ratio or its isozyme pattern. Our data support the view that the epidermis as an organ has a proper homeostatic control system with regard to protein kinase. In both normal and psoriatic skin including the lesion, protein kinase is in a nonactivated state ready to respond to any increase in cyclic AMP. At any rate, the biochemical abnormality in psoriasis thus far appears to be limited to the β-adrenergic membrane receptor unit of the lesional skin.

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Local Effects of Synthetic Leukotrienes (LTC_4, LTD_4, LTE_4, and LTB_4) in Human Skin

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The local effects of intracutaneous injections into humans of 1–3 nmol of five products of arachidonic acid metabolism, leukotrienes (LT) C_4, D_4, E_4, and B_4 from the 5-lipoxygenase pathways and prostaglandin (PG) D_2 from the cyclooxygenase pathway, were assessed clinically and histologically. In equimolar concentrations, LTC_4, LTD_4, and LTE_4 elicited erythema and wheal formation, in which a wheal with central pallor was present up to 2 hr, and the erythema persisted as long as 6 hr. PGD_2 elicited a wheal that lasted up to 1 hr and erythema that lasted up to 2 hr. The dermal vascular sites affected by LTD_4 and PGD_2 included capillaries, superficial and deep venules, and arterioles. LTB_4 elicited a transient wheal and flare, followed in 3–4 hr by induration that was characterized by a dermal infiltrate comprised predominantly of neutrophils. The combination of LTB_4 and PGD_2 elicited tenderness and increased induration associated with a more intense neutrophil infiltration. Thus, the products of the 5-lipoxygenase pathway of arachidonic acid metabolism in nanomole amounts can induce cutaneous vasodilation with edema formation and a neutrophil infiltrate, and these responses are enhanced by a cyclooxygenase pathway product, PGD_2.

The oxidative products of arachidonic acid appearing with physiologic and pathobiologic perturbations of cellular membrane phospholipids include the prostaglandins, thromboxane, and the leukotrienes. Prostaglandin (PG) D_2, the predominant product of arachidonic acid metabolism in human mast cells in vitro [1,2] and in vivo [3,4], and leukotrienes (LT) C_4, D_4, and E_4 [5–9], recognized as components of slow reacting substance of anaphylaxis (SRS-A), are considered among the mediators of acute allergic processes.

Knowledge of the cutaneous effects of the LT components of SRS-A is limited to observations of alterations in venular permeability in guinea pigs [6,10,11] and rats [11], but not rabbits [11], and the morphologic demonstration of dilated superficial and deep blood vessels in monkey skin [12]. PGD_2, a metabolite of the cyclooxygenase pathway of arachidonic acid, can produce vasodilation in rat skin and elicit a wheal and erythema reaction in human skin [13]. LTB_4, a potent chemoattractant [14] that elicits a neutrophil infiltrate in monkey skin [15], is inactive in the rabbit skin unless combined with a vasodilating prostaglandin [16]. Inasmuch as there is no information on the cutaneous effects in humans of the leukotrienes alone or in combination with PGD_2, the intracutaneous injection of LTC_4, LTD_4, LTE_4, PGD_2, and LTB_4 alone and in some combinations was assessed by clinical and histologic parameters.

MATERIALS AND METHODS

LTC_4, LTD_4, LTE_4, PGD_2, and LTB_4 were prepared as previously described [8,17–20] and stored frozen at a concentration of 25 μg/ml in 0.1 M phosphate buffer (pH 6.8):ethanol (4:1, v/v) under argon until the day of use. Immediately before injection these substances were dried under argon and then redissolved in 0.1 ml of a sterile solution containing 0.15 M NaCl with 2% ethanol. LTC_4 (1.0 nmol/site), LTD_4 (1.0 nmol/site), LTE_4 (1.0 nmol/site), PGD_2 (3.0 nmol/site), LTB_4 (1.6 nmol/site), and vehicle alone or in various combinations were coded and injected intradermally into the ventral aspects of the forearms of 3 adult male volunteers after informed consent was obtained. Clinical

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Abbreviations:
LT: leukotriene
PG: prostaglandin
SRS-A: slow reacting substance of anaphylaxis