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INHIBITION OF CALCIUM AND CALMODULIN-DEPENDENT PHOSPHODIESTERASE ACTIVITY IN RATS BY CAPSAICIN

Bharathi P. Salimath* and M.N. Satyanarayana**

*Department of Infestation Control and Protectants

**Department of Biochemistry,
Central Food Technological Research Institute,
MYSORE - 570 013, Karnataka, INDIA

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SUMMARY: Capsaicin, reported to elevate hormone sensitive lipase (HSL), is also found to inhibit the Ca++ and calmodulin-dependent cAMP phosphidiesterase (PDE) activity in adipose tissue of rats, fed high fat diet. The dependence of the enzyme activity on Ca++ and calmodulin in vitro, in control rats, is shown by its substantial lowering in the presence of EGTA and inhibition by trifluoperazine (TFP) (IC50 between 10-20 μM). This enzyme activity is also inhibited by both red pepper extract (80% inhibition with 50 μl) and capsaicin (IC50 between 0.3-1 μM) in a dose dependent manner. Capsaicin has been found to inhibit Ca++-dependent PDE activity by 60% in the test rats. Enzyme inhibition in vivo, due to capsaicin, was overcome by addition of calmodulin to the assay system. Inclusion of fluphenazine or capsaicin in assay inhibited not only the calmodulin-restored enzyme activity from test rats but also that of control rats. These results suggest a possible mechanism for the stimulation of lipolytic activity by capsaicin in vivo.

The intracellular concentration of 3'-5' cAMP is modulated by calmodulin, an endogenous Ca++ -binding protein, by stimulating membrane-bound adenylate cyclase activity and soluble PDE activity (1,2). It is well documented that cAMP mediates the lipolytic effect of many hormones in adipose tissue, by regulating the HSL activity through a cAMP-dependent reversible phosphorylation of the enzyme (3,4).

Recently in this Institute, a synthetic capsaicin analogue (N-vanillyl nonanamide, 0.2 mg%) in 30% mixed fat fed to rats was found to lower liver and serum triglycerides (TG) (5). In these

* To whom correspondence should be addressed.

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rats an elevation of adipose tissue HSL and serum free fatty acids (FFA) was observed, clearly indicating a stimulation of lipolytic activity. Capsaicin also brought about other effects such as the stimulation of TG secretion from liver to serum (6), lowering of adipose tissue lipoprotein lipase (LPL) (5), and the elevation of skeletal muscle LPL (5), pointing to its significant role in preventing TG accumulation in liver, serum and adipose tissue. The earlier observations about red pepper (5 g%) or an equivalent concentration of synthetic capsaicin (15 mg%) lowering even carcass lipids in 10% fat fed rats (7) shows its dose dependent effect. Kawada et. al. (8) from Japan have recently reported a dose dependent lowering of adipose tissue weight and serum TG by natural capsaicin (trans-8-methyl N-vanillyl 6-nonenamide, 7, 14, 21 mg%). They also observed a stimulation of energy metabolism and catecholamine secretion from adrenal medulla in rats (8-10).

There are no reports so far linking the lipolytic activity of capsaicin in adipose tissue with cAMP levels. In studies reported here, capsaicin has been shown to inhibit the Ca++ and calmodulin-dependent PDE in the adipose tissue of rats both <u>in vitro</u> and <u>in vivo</u> conditions.

MATERIALS AND METHODS

Materials: The sources of chemicals etc., were as follows: Sodium azide, dithiothreitol (DTT), 2-amino-2-(hydroxy methyl)-1,3-propane-diol (Tris), 3'5 cAMP, ethylene glycol-bis-(3-aminoethyl ether) -N,N,N'-tetra acetic acid (EGTA), bovine serum albumin (BSA) and alkaline phosphatase (Sigma, USA); TFP hydrochloride (May and Baker, India); Calmodulin from bovine brain and fluphenazine were kind gift from Dr. D. Marmé, Freiburg, W. Germany; Synthetic capsaicin analogue (referred to as capsaicin, Fluka-AG, Switzerland); Red pepper (local market).

<u>Adipose tissue from control and test rats</u>: The perirenal adipose tissue was obtained from rats fed control and test diets for 8 weeks. The diets consisted of 30% mixed fat (1:1 peanut oil and hydrogenated fat, a local brand) adequate in other respects without and with 0.2 mg% capsaicin. The maintenance of rats and other details were as reported earlier (5). The tissue from control rats was used for <u>in vitro</u> studies.

Preparation of a soluble fraction from adipose tissue: The tissue was homogenised using a glass teflon homogeniser (1 g fresh weight/2 ml) in 10 mM Tris-HCl buffer pH 7.8 containing 10% sucrose, 1 mM DTT and 0.02% sodium azide. After centrifugation at 20,000 x g for 30 min, the fat free infranatant was immediately used for the assay of PDE. This extract usually contained 2-4 mg protein/ml, assayed (11) using BSA as a standard. All preparative procedures were carried out at about 4 deg.C.

Assay of cAMP PDE activity: A two stage assay for PDE enzyme activity was done as follows: The reaction mixture (0.5 ml) contained homogenization buffer with 4 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM cAMP, with or without 10 mM EGTA, with or without various concentrations of capsaicin (1 nM-100 µM). The reaction was started by adding the soluble fraction (100 µg protein). After incubation for 30 min at 37 deg C., the reaction was stopped by boiling for 5 min in a water bath. After cooling on ice, 5 units of alkaline phosphatase were added. The reaction mixture was incubated at 37 deg. C for 10 min and terminated by adding 0.5 ml of ice cold 10% (w/v) trichloroacetic acid. After centrifugation at 20,000 x g for 10 min, 100 µl of the supernatant was assayed for inorganic phosphate (12). For the *in vitro* effect, capsaicin was dissolved in ethanol and subsequent dilutions were made with 10 mM Tris-HCl, pH 7.8. The result expressed as Ca++ -dependent-PDE is, the difference between total activity obtained without EGTA and the activity in presence of EGTA (Ca++ -independent activity).

Ethanol extraction of red pepper: One gram of red pepper powder was ground into a paste with 5 ml ethanol in a pestle and mortar. The extract was filtered through cotton wool. Aliquots of the fresh extract were used in the assay system.

RESULTS

Effect of EGTA and TFP on PDE activity: The effect of EGTA and TFP on PDE activity are shown in Fig.1. Inclusion of 10 mM EGTA in the assay mixture caused 50-60% inhibition of the enzyme activity. TFP showed a dose-dependent inhibition of PDE activity. Half-maximal inhibition of the enzyme activity was obtained at concentrations between 10-20 µM of TFP in the incubation medium. However, the Ca++ -independent activity of the enzyme was not influenced by TFP.

Effect of red pepper extract on the Ca++ -dependent PDE activity: Red pepper has been shown to have a significant influence on lipid metabolism (5,13-16). The influence of red pepper extract on adipose tissue PDE activity, checked without and with the addition of EGTA (Fig.2), showed a dose-dependent inhibition of the Ca++ -dependent PDE activity. The enzyme activity was inhibited to the

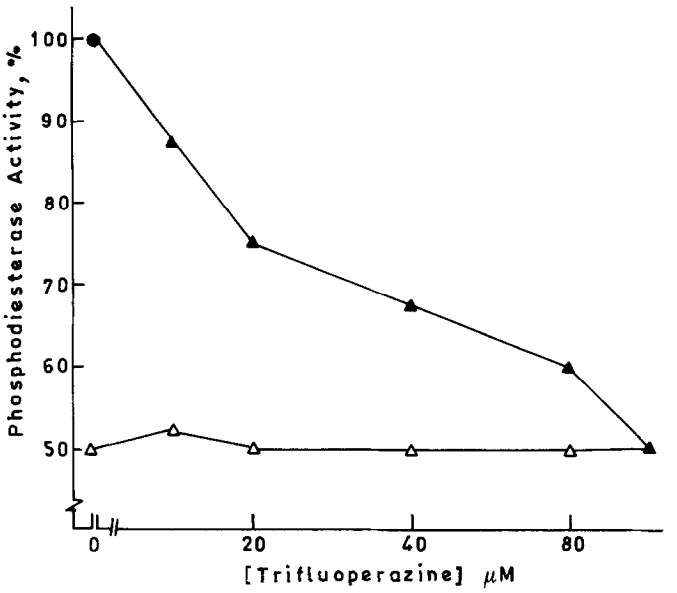

Figure 1. Inhibition by trifluoperazine of adipose tissue PDE activity. 100% refers to the activity without trifluoperazine (○). With trifluoperazine (▲), with EGTA (△), with EGTA + Trifluoperazine (△). Each value represents mean ± S.E. of four values.

extent of 80% by 50 μl of the extract, equivalent in concentration to 60 nM capsaicin in the incubation mixture.

Effect of capsaicin on Ca++ -dependent PDE activity: As the effects of red pepper on lipid metabolism are attributable to its

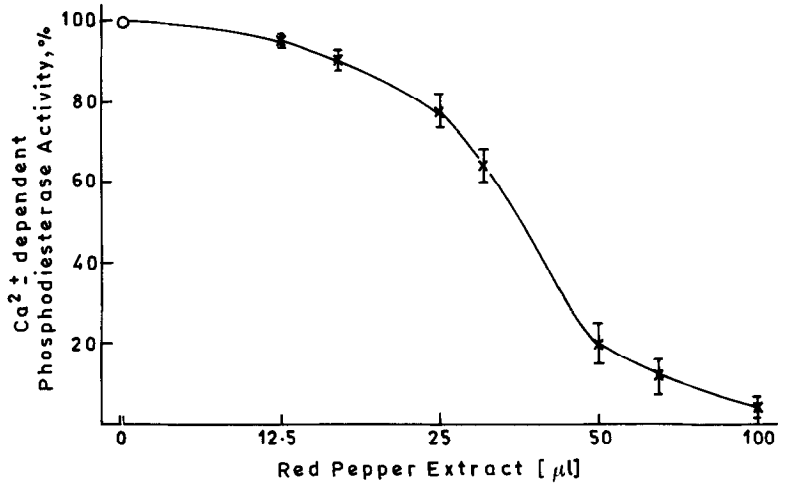

Figure 2. Inhibition of Ca++ -dependent PDE by red pepper extract. Activity, without (○) or with (X) red pepper extract. The blank values with alcohol is substracted. Each point represents mean ± S.E. of three values.

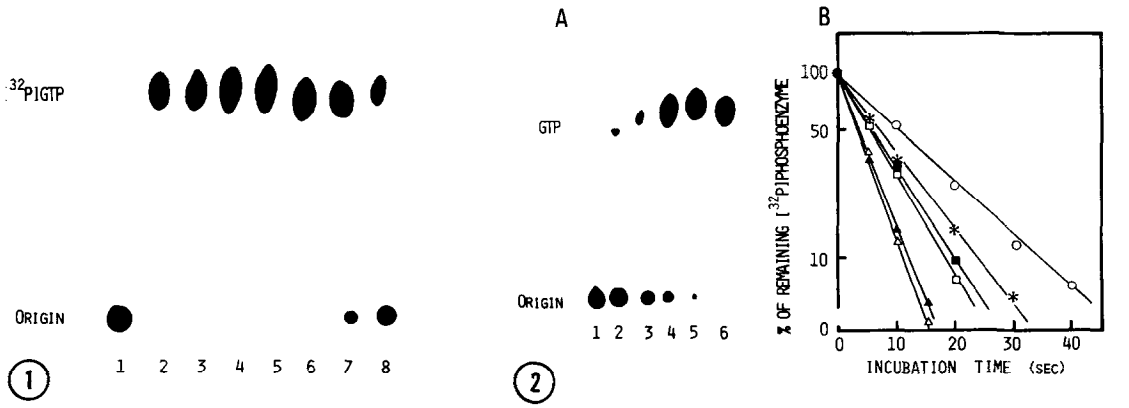

Fig. 1. [^{32}P]GTP formation from the [^{32}P]phosphoenzyme and GDP-bound guanine nucleotide binding proteins. The reaction mixtures (50 µl) contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 0.1 mM EDTA, 1 mM Mg^{2+}, 5 µM ADP, various GDP-bound guanine nucleotide binding proteins (protein, 0.5 µg; GDP, approximately 1 mol/molecule protein) and [^{32}P]phosphoenzyme (0.1 µg of protein, approximately 10^4 cpm), were incubated separately for 3 min in an ice bath. After addition of 0.15 M EDTA (final concentration 25 mM), the [^{32}P]GTP formed was detected by PEI thin-layer chromatography, followed by autoradiography, as described in previous our reports (6,7). Lane 1, no incubation of the [^{32}P]phosphoenzyme; lane 2, incubation of the [^{32}P]phosphoenzyme with GDP-bound Gt; lane 3, with GDP-bound Gi; lane 4, with GDP-bound Go; lane 5, with GDP-bound EF-α_1; lane 6, with GDP-bound r-*ras* p21; lane 7, with GDP-bound r-*ras* p21 and Y13-259 (2 µg); and lane 8, with GDP-bound r-*ras* p21 and Y13-259 (10 µg).

Fig. 2. Kinetics of [^{32}P]GTP formation from the [^{32}P]phosphoenzyme of NDP-kinase and GDP or GDP-bound guanine nucleotide binding proteins. The [^{32}P]phosphoenzyme (0.1 µg of protein, approximately 10^4 cpm) was added to the reaction mixtures (50 µl), which contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM Ca^{2+}, 0.1 M KCl and 10 µM GDP. After the mixtures were incubated for the indicated periods in an ice bath, the enzyme reaction was arrested by the addition of 0.15 M EDTA (final concentration 25 mM). Aliquots of 5 µl were spotted separately on a PEI-cellulose F thin-layer plate. The [^{32}P]GTP formed was detected by autoradiography after development in 0.75 M potassium phosphate at room temperature. [A] No incubation of [^{32}P]phosphoenzyme (time 0, lane 1); incubation of the [^{32}P]phosphoenzyme (0.1 µg) with 10 µM GDP in an ice bath for 10 sec (lane 2), 20 sec (lane 3), 30 sec (lane 4), 40 sec (lane 5) and 50 sec (lane 6), respectively. Figure B (right) represents the percentage of the [^{32}P]phosphoenzyme remaining after incubation with GDP (o) or GDP-bound various guanine nucleotide binding proteins [guanine nucleotide binding protein from NDP-kinase F-I (G_1, △), r-*ras* p21 (▲), EF-α_1 (x), Gt (□) and Go (■)] for the indicated incubation times. 100% radioactivity in 50 µl of the control reaction mixture (time 0, ●, total 50 µl) was approximately 1 x 10^4 cpm.

To determine the phosphate-transfer from the high-energy phosphates on the phosphoenzyme of NDP-kinase to GDP on various guanine nucleotide binding proteins [EF-α_1, r-*ras* p21, Gt, Gi and Go], analytical experiments were carried out. The results are shown in Fig. 1, (i) [^{32}P]GTP was formed within 40 sec at 0°C after addition of the [^{32}P]phosphoenzyme of NDP-kinase from HeLa S3 cells to the reaction mixtures, which contained a different guanine

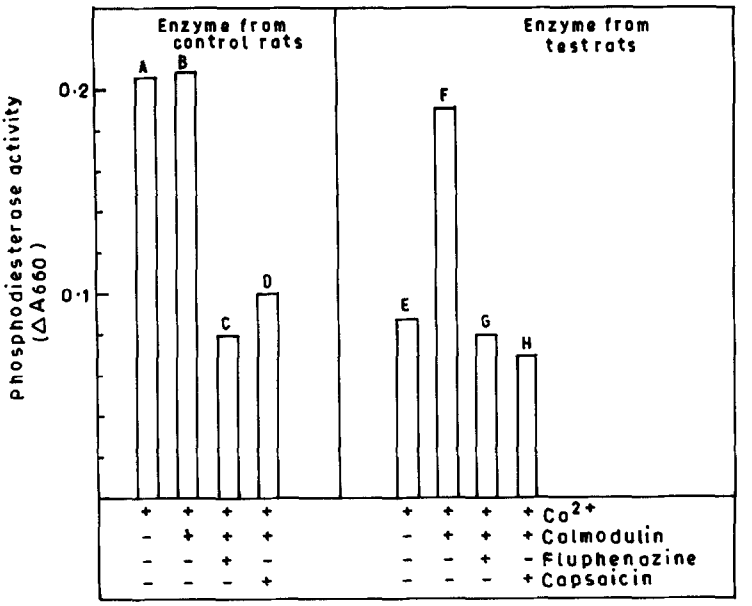

Figure 4. In vivo inhibition by capsaicin of Ca++ -dependent PDE activity in adipose tissue from rats. Reversal of enzyme inhibition by calmodulin.The values are expressed as mean ± S.E. (n = 3).

activity (40%) from test rats (E) was restored significantly (93%) when calmodulin was included in the assay (F). Inclusion of fluphenazine (50 μM), a potent calmodulin antagonist in the assay, inhibited the Ca++ and calmodulin-dependent PDE activity from both control and test rats (C and G, respectively). A similar inhibition was obtained upon addition of capsaicin (10 μM) either to the enzyme obtained from control rats (D) or to that obtained from test rats (H).

DISCUSSION

The present studies demonstrate that capsaicin, which is known to influence lipid metabolism (5-8) inhibits the Ca++ and calmodulin dependent PDE activity in the adipose tissue of rats as tested in both in vitro and in vivo conditions. More than 50% of the cAMP-PDE activity in the adipose tissue from control rats is dependent on Ca++ and calmodulin. A substantial decrease in PDE activity seen in the presence of EGTA indicates the Ca++ -dependence of the enzyme activity and also suggests a role for

calmodulin-dependent regulation of PDE. Specific calmodulin antagonists like TFP can be used to inhibit calmodulin-dependent processes (12). As endogenous calmodulin was not removed from the soluble fraction, we could show that addition of various concentrations of TFP, inhibits the calmodulin-dependent regulation of PDE activity with an IC50 between 10-20 μM. This is comparable to the value found for inhibition of the brain calmodulin-depedent cAMP PDE by TFP (18, 19). The Ca++ -independent form of PDE was not affected by the drug showing that the inhibition by the drug is a Ca++ -dependent process as has been suggested by Levin and Weiss (11). We have clearly demonstrated that both red pepper and capsaicin inhibit the Ca++ and calmodulin-dependent PDE activity. Thus capsaicin appears to be a more potent inhibitor of Ca++ and calmodulin-dependent PDE activity (IC50 between 0.3-1 μM) than other known inhibitors (IC50 between 10-20 μM) (18). It is well established that beta-adrenergic agonists (3,4) bring about elevation of intracellular cAMP, which in turn activates adipose tissue HSL, leading to lipolysis. The same effect may be brought about by the inhibition of cAMP-dependent PDE activity. Interstingly, capsaicin inhibited Ca++-dependent PDE activity by 60% in the test rats. Our results show that addition of calmodulin to the assay system relieved the inhibition of the enzyme, induced by capsaicin *in vivo*. However, when capsaicin was again added to the assay system containing either the restored enzyme from test or enzyme from control rats, it did inhibit the Ca++ and calmodulin-dependent PDE activity. These results suggest that, capsaicin by directly acting on calmodulin, inhibits the Ca++ and calmodulin-dependent PDE activity in the test rats. Capsaicin, by inhibiting the Ca++ and calmodulin-dependent PDE activity in adipose tissue and stimulating the secretion of epinephrine from adrenal medulla (10), which activates adenylate cyclase, may

increase cAMP concentration in adipose tissue, leading to increased lipolytic activity. The spice red pepper, at levels consumed in the diet could be an effective stimulus, evoking a number of cellular responses (5,6,9,10,20), one of the several being lipolysis. Further studies are necessary to understand the molecular mechanism of capsaicin action in lipolysis.

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