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Lipolytic Action of *Buthus occitanus tunetanus* Venom: Involvement of the β Adrenergic Pathway

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Scorpion venoms contain active neurotoxins known to act selectively at the level of voltage sensitive Na^+ and K^+ channels on mammal nervous system. In the present report, we show for the first time that the venom of scorpion *Buthus occitanus tunetanus* (*Bot*) countains compounds able to activate another cell function in non excitable cells. Addition of this venom to the culture media of 3T3-L1 adipocytes or freshly dissociated rat adipocytes rapidly increases lipolysis as estimated by glycerol release ($\sim$3 to 4 fold over basal values) in a dose-dependent manner (EC_{50} $\sim 12 \pm 1.25$ μg/ml; n=3). Bot venom effect was lower and not additive to the effect produced by isoproterenol (IPE) (10 μM), a main lipolytic agent, n=3. In Sephadex G-50 size exclusion chromatography, the lipolytic activity was excluded and not associated to the included neurotoxic fraction. Furthermore, no lipolytic effect could be detected in the Na^+ channel specific toxin II purified from *Androctonus australis hector* (AaHII) or the K^+ voltage-dependent channel toxin from *Androctonus mauritanicus mauritanicus* (KTx). Propranolol (a non selective β adrenoreceptor (βAR) antagonist), alprenolol and pindolol (selective $\beta1/\beta2$ antagonists) totally inhibited in a dose-dependent manner the lipolytic response to Bot venom (IC_{50} $\sim 1\times10^{-7}$, 7.5×10^{-8} and 3×10^{-7}M, respectively), suggesting that venom stimulated lipolysis through the β AR pathway. The pharmacological profiles of molecules acting more selectively on β AR subtypes such as CGP 12177 ($\beta1/\beta2$ antagonist with $\beta3$ agonist properties), CGP 20712A ($\beta1$ antagonist) and ICI 118551 ($\beta2$ antagonist) strongly suggest that lipolytic action of venom mainly involves the $\beta2/\beta1$ AR subtypes.

Scorpions venoms are sources of abundant pharmacologically active compounds (1). They contain a great number of small molecular weight polypeptides (5 to 7 kD) which are highly selective probes of Na^+ and K^+ channels mainly located in nervous, cardiac and skeletic muscles excitable tissues (2,3). Two types of 7kD toxins (α and β toxins) have been characterized according to their specific binding to two distinct receptor sites on the voltage-gated Na^+ channel and their different electrophysiological activities (4). The 5 kD toxins block voltage-gated inactivating channels: K^+ channels (5), high conductance (maxi) Ca^{++} activated K^+ channels (3,6,7), low conductance Ca^{++} activated K^+ channels (8) and voltage-gated non inactivating K^+ channels (9). The multiple clinical manifestations following scorpionic envenimation suggest the existence of highly active components other than these neurotoxins. We analyzed such a possibility on adipose cells which are considered as non excitable cells. In the present report we show that *Buthus occitanus tunetanus (Bot)* venom, known to contain highly active neurotoxins, rapidly increases lipolysis in adipose cells. Our results indicate that the active fraction of this venom is distinct from the K^+ and Na^+ channels specific toxins. Pharmacological investigations carried out with various β adrenergic receptor (β AR) agonists and antagonists strongly suggest that the lipolytic response to Bot venom is mainly mediated through the β adrenergic pathway.

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MATERIALS AND METHODS

Venoms were obtained by electric or manuel excitation of Bot scorpions collected in Beni Khedach area (Tunisia). Venom was used as crude extract or after partial purification on Sephadex G-50 (two columns of 26×100mm) equilibrated with 0.2M ammonium acetate (10). Three main fractions were resolved on the basis of A280 nm : excluded fraction I, included fractions II and III. Toxin II from *Androcotonus australis hector* (AaHII) and Kaliotoxin (KTx) from *Androctonus mauritanicus mauritanicus* were purified as previously described (6,10).

Periepididymal white adipose tissue was obtained from Wistar rats (100 to 150 g body weight) after overnight fasting and dissected into small fragments (20-30 mg). Fat cells were prepared according to Rodbell (11) with minor modifications. Briefly, adipocytes were obtained by collagenase digestion (1 mg/ml) in DMEM, 6 mM glucose, pH 7.4 (Buffer A). After collagenase digestion (37C, shaking 60 cycles/min) the adipocytes were separated from the stroma-vascular fraction by filtration through a silk screen (spectramesh 2104), centrifuged 1 min at 1000 rpm and washed three times in buffer A to eliminate collagenase. Packed cells were brought to a suitable dilution in buffer A and used immediatly for lipolysis measurement according to Langin et al. (12). Glycerol released into the incubation medium was determined by the fluorimetric adaptation of Wieland's method (13).

3T3-L 1 cells cloned from total Swiss embryo (14) were grown and differentiated in DMEM containing 10 % fetal calf serum, 33 μM biotin, 17 μM sodium pantothenate and antibiotics (standard medium) at 37C in an humidified atmosphere of air 5 % CO_2. At confluence the medium was generally supplemented with 170 nM insulin and changed three times a week. Adipose differentiation of 3T3-L1 cells was followed by morphological changes and development of two lipogenic enzyme markers, glycerol-3-phosphate dehydrogenase (GPDH) and malic enzyme (ME), considered as late phenotypes of adipose differentiation (15,16). Freshly dissociated adipocytes were incubated in 1 ml of prewarmed buffer A in polyethylene tubes at 37C. After addition of venom and/or various pharmacological agents to the cell suspension, 0.1 ml aliquots of the infranatant were taken off at the indicated times for enzymatic determination of glycerol release as an index of lipolysis activity. Analysis of the lipolytic response of 3T3-L1 adipocytes was adapted from previous reports (17). After 24h culture in the same medium deprived of insulin, FCS was removed and replaced by DMEM including 1 % dialyzed fat free bovine serum albumin (BSA). Aliquots of medium were removed for glycerol release estimation as above.

DNA was estimated following Labarca and Paigen (18), with calf thymus as the standard and results are expressed as the mean ± SE for more than two assays.

3T3-L1 cells were from the American Type Culture Collection. Tissue culture reagents were from Gibco. Crude collagenase was obtained from Worthinghton, Freehold, N.J. Fat free Bovine serum albumin (fraction V), adenosine deaminase from calf intestine and enzymes for glycerol assay (glycerokinase and glycerol-3-phosphate dehydrogenase) were from Boehringer Mannheim, Germany. (−)-Isoproterenol(+)-bitartrate salts, S-(−)propranolol hydrochloride, (−)-aprenolol-d-tartrate, and pindolol were purchased from Sigma (St Louis, Mo). CGP 12177 and CGP 20712A were generous gift from Ciba Geigy (Basel, Switzerland). ICI 118551 was provided from Tocris Cookson Ltd (Langford Bristol/BS 18 7DY, UK).

RESULTS

Addition of Bot venom (25 μg protein/ml) to the culture media of differentiated 3T3-L1 cells produced a rapid increase of glycerol release over basal levels (88±7.1 versus 37±3.9 nmol glycerol/μg DNA/hr). Effect of Bot venom was lower and not additive to that obtained with 10 μM isoproterenol (IPE) (106±4.8 nmol glycerol/μg DNA/hr), a β adrenoreceptor (βAR) agonist, known as a potent lipolytic agent. Under these experimental conditions, no sign of cell suffering could be observed. Chronic exposure to venom did not provoque any change in morphological differentiation and lipogenic enzyme emergence (GPDH and malic enzyme), neither in DNA or protein content per dish. This indicates that venom does not display any toxic or lytic effect to the cells and does not interfere with the adipose differentiation process.

We conducted the same investigations with freshly dissociated rat adipocytes. Fig. 1A shows a typical time-course study of lipolysis activity. Addition of Bot venom (25 μg protein/ml) to the cells enhanced lipolysis over a low baseline level. This effect was significant after 15-20 min, increased linearly up to 60 min and plateaued after 80-100 min at a level 3-4 fold higher than basal level. Nevertheless, this stimulation was always lower than that observed in the presence of 10 μM IPE with generates a high lipolytic activity in freshly isolated adipocytes (8 to 10 fold over basal level). Stimulating effect of venom was dose-dependent between 2.5 and 25 μg protein/ml ($EC_{50} \sim 12 \pm 1.25$ μg/ml ; n=3). Similar results were obtained when

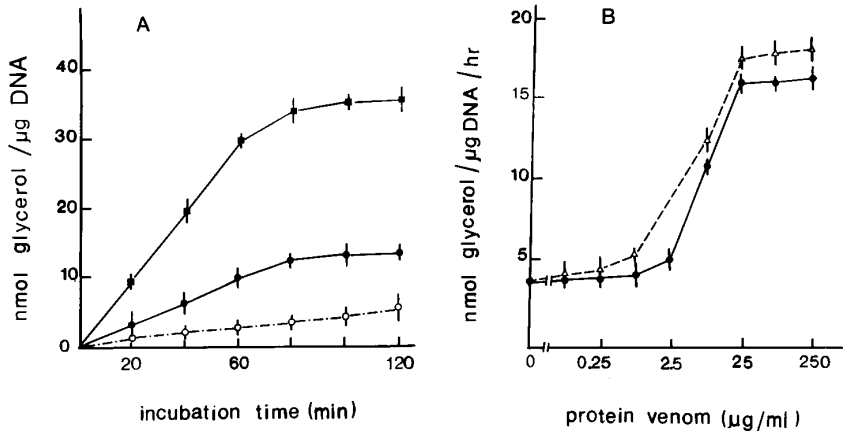

FIG. 1. Effect of Bot venom on lipolytic activity of freshly dissociated rat adipocytes. (A) Time-course study. Freshly isolated adipocytes (~10 μg DNA) were preincubated at 37°C in 1 ml standard medium (DMEM + 3 % fat free BSA). After 15 min (time 0), aliquots (50 μl) were withdrawn and the incubation continued without addition (○), and after supplementation with 25 μg protein/ml Bot venom (●) or 10 μM isoproterenol (■). At the indicated time-periods, aliquots were withdrawn to determine glycerol release as described in Material and Methods. Glycerol released to the medium during the 15 min preincubation period was substracted. Lipolysis activity is expressed as nmol of glycerol released per μg DNA at the indicated time-periods. Each data point represents the mean values of triplicate assays. Panel (B) Dose-response study. Freshly isolated adipocytes (~ 10 μg DNA) were incubated under the above conditions at 37°C during 90 min with increasing concentrations of venom obtained by electric (●) or manual excitation (△). Lipolysis activity was estimated from the amount of glycerol release and expressed as nmol glycerol produced / μg DNA/hr. Data values are the means ± SE of three different cell preparations with assays in duplicate.

using manual Bot venom suggesting that the active coumpounds should be present within the physiological secretion of venom (Fig. 1B). Lipolytic response to Bot venom remained when adipose cells were incubated in the presence of adenosine deaminase added at different concentrations (1-5 μg/ml).

In an attempt to gain further information on the nature of active lipolytic component(s), the venom was size fractionated on Sephadex G-50. Figure 2 shows that the lipolytic activity was found to be associated with excluded material (fraction I). No significant lipolytic activity could be detected in the included toxic fraction (fraction II), in low-absorbing material eluting between fractions I and II, or in the low molecular weight fraction III. Furthermore AaHII and KTx, which act selectively on Na^+ and K^+ channels respectively, did not display any lipolytic effect up to 1×10^{-7}M.

Since catecholamines are known as main lipolytic agents (19,20), the eventuality of interferences of venom with IPE action was investigated. Fig.3 shows the lipolytic effect of increasing concentrations of IPE when added with or without 25 μg protein/ml of Bot venom. An additive effect was observed at low IPE concentrations (below 5×10^{-8}M), which progressively attenuated and disappeared at $\geq 1\times10^{-7}$M, both conditions of stimulation giving the same plateau. This suggests that venom could stimulate lipolysis through the β AR pathway. This hypothesis is strenghtened by the use of various β AR agonists and antagonists. As shown in Fig. 4A, propranolol (a non selective β AR antagonist), alprenolol and pindolol (selective β1/β2 antagonists) totally inhibited in a dose-dependent manner the lipolytic response to Bot venom. The IC_{50} values (concentration of the antagonist required to induce half-maximal inhibition of Bot venom action) are ~ 1×10^{-7}, 7.5×10^{-8} and 3×10^{-7} M, for propranolol, alprenolol and pindolol respectively.

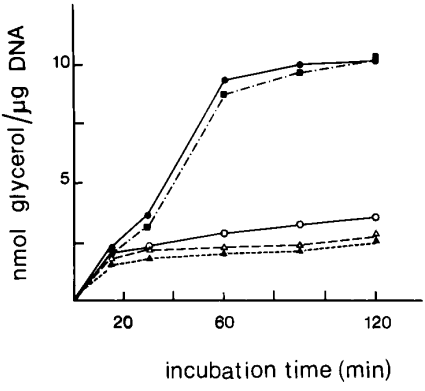

FIG. 2. Comparative time-course studies of lipolytic activity in whole extract of Bot venom and different fractions from Sephadex G-50, size exclusion chromatography. Different fractions were selected on the basis of the A 280 nm elution profile. Adipocytes were incubated in standard conditions and lipolytic activity was determined as described at the indicated period of time. Glycerol release was measured without addition (basal conditions, ○) or after addition of whole extract of Bot venom (25 μg/ml) (●) ; the excluded G-50 Sephadex fraction I (12.5 μg/ml) (■) ; the toxic fraction II (12.5 μg/ml) (△) ; and the low molecular weight fraction III (12.5 μg/ml) (▲). Points are the means of triplicate determinations.

To get a better insight into the nature of βAR subtypes involved in venom action, other pharmacologically active molecules were chosen on the basis of their higher selectivity : CGP 12177 ($\beta1/\beta2$ antagonist with $\beta3$ agonist properties), CGP 20712A (a selective $\beta1$ antagonist), ICI 118551 (a selective $\beta2$ antagonist). Inhibition curves obtained with the different antagonists on venom-induced lipolysis are depicted in Fig. 4. Low concentrations of CGP 12177 (below 1×10^{-7}M) were able to largely counteract the lipolytic response to Bot venom. At higher concentrations ($>1\times10^{-6}$M) this drug revealed its partial $\beta3$ agonist potency (Fig. 4B) as it has been previously described in several reports (12,21,22,23). Increasing concentrations of CGP 20712 A (up to 1×10^{-7}M) exert a partial antagonistic effect ($\sim$40 %) venom action, whereas ICI 118551 acts as a strongest inhibitor, in a dose-dependent manner (IC50 $\sim$ 1×10^{-8}M). Only 10 % residual lipolytic activity was observed at 1×10^{-6}M ICI 118551 suggesting a predominant involvement of $\beta2$ AR in the action of venom (Fig. 4C). Taken together,

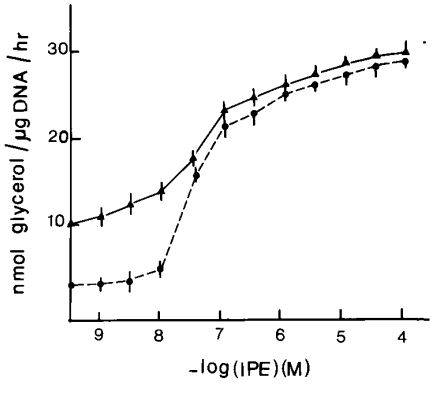

FIG. 3. Effect of Bot venom on lipolysis obtained with increasing concentrations of isoproterenol (IPE) of isolated adipose cells incubated under standard conditions. Incubations were conducted in the absence (●) or with 25 μg/ml of Bot venom (▲). Lipolysis is expressed as nmol glycerol/μg DNA/hr. The data values are mean of duplicate assays from two separate experiments.

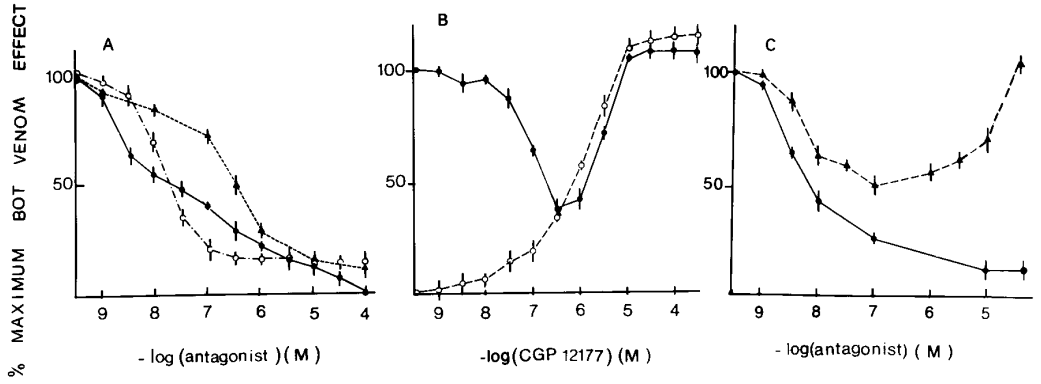

FIG. 4. Effect of different β AR antagonists on lipolysis induced by Bot venom. (A) Comparative antagonistic effect of propranolol (●) , alprenolol (○) and pindolol (▲) on lipolysis induced by Bot venom. Isolated adipocytes were incubated in standard conditions in the presence of Bot venom (25 μg/ml) and increasing concentrations (10^{-9} to 10^{-4}M) of each antagonist. Results are expressed as percent of maximal stimulation of lipolysis by Bot venom in the absence of antagonists. (0 % corresponds to basal values and 100 % to venom stimulated lipolysis). IC_{50} values (concentrations of antagonist inducing 50 % inhibition of lipolysis stimulated by Bot venom) are calculated from the dose-response curve of each antagonist. Values are mean ± SE from three separate experiments. (B) Effect of increasing concentrations of CGP 12177 in the absence (○) or in the presence of 25μg/ml Bot venom (●). Values are expressed as percent of maximal lipolytic effect of venom Values are the means ± SE from two separate cell preparations done in duplicate. (C) Dose-response inhibition curves of inhibition of Bot venom stimulated lipolysis in the presence of increasing concentrations of CGP 20712A (▲) and ICI 118551 (●). Values are the means ± SE of three experiments and expressed as percentage of maximum Bot venom response.

these data (Fig. 4 A,B,C) indicate that the lipolytic action of Bot venom would largely implicate the $\beta 1/\beta 2$ subtypes, with a likely preferential involvement of the $\beta 2$ AR

DISCUSSION

Our results show for the first time that a scorpion venom, besides its known pharmacological specificity against Na^+ and K^+ channels, clearly stimulates lipolysis in adipose cells (3T3-L1 mouse adipocytes and freshly dissociated rat adipocytes). This effect occurs without any toxic effect to the cells or interferences with adipose differentiation process. The lipolytic response increases with protein venom concentration but remains consistently lower than that elicited by IPE, the major lipolytic agent. The lipolytic activity of venom is associated with components size-excluded in Sephadex G-50 chromatography and clearly distinct from the known Na^+, K^+ channels toxins.

Although not detected in the Buthidae family of Scorpion (24), trace amounts of phospholipase A_2 (PLA_2) could be present. Their involvement in the lipolytic action of Bot venom is unprobable for the following reasons: 1/ we never observed any lytic or toxic effect on adipose cells ;2/ no lipolytic activity was detected in *Androctonus australis hector* venom (data not shown) which is known to contain some PLA_2 activity (Rochat et al., personal communication). 3/ PLA_2 detected in venom of *Scorpio maurus palmatus* (Scorpionidae family) is included in Sephadex G-50 size exclusion chromatography (24).

On the basis of pharmacological assays, our results suggest that the lipolytic action of venom is mainly achieved through the βARs. Catecholamine-induced lipolysis in fat cells from various species is modulated through adrenoreceptors (12,25,26). Four subtypes of adrenoreceptor are present on the plasma membrane of rat adipocytes and known to be involved in the lipolytic response ($\beta 1$,$\beta 2$,$\beta 3$ and $\alpha 2$; for review see 27,28). The use βAR antagonists such as propranolol, alprenolol or pindolol totally inhibited in a dose-dependent manner the lipolytic response

to Bot venom. Our results also suggest that the lipolytic response to venom could be largely attributed to the β2AR and to a lesser extend to the β1 subtype. This is evidenced by the finding that the β1 selective antagonist CGP 20712A exhibits a lower potency to counteract venom effect than the β2 selective ICI 118551 which is almost as effective as propranolol in antagonizing the response to Bot. A possible involvement of the β3AR subtype could not be ascertained since the lipolytic response to venom was not totally suppressed under conditions of full β1/β2 blockade (ICI 118551 + CGP20712A) but high concentrations of BRL 37344, a β3 agonist, stimulated lipolysis with the same efficiency whether the venom was present or not. Numerous reports indicate that the β3AR is the predominant βAR subtype in white and brown adipose tissue (21,22,29,30). Similarly, Feve et al. 1991 demonstrated that the β3AR is the predominantly expressed subtype in 3T3-F-442A adipocytes (31). The implication of β3 in venom action is probably limited ; this could explain the lesser lipolytic efficiency of Bot venom compared to that elicited by IPE which activates the three βARs subtypes.

In adipose cells, lipolysis is catalyzed by the hormone sensitive lipase (HSL), catecholamines being the main activators. Classically agonist activation of various fat cell βAR subtype β1,β2 and β3 leads to the generation of cAMP by stimulating plasma membrane adenylate cyclase (29). This pathway is mediated by the guanine nucleotide binding protein Gs. An elevation of intracellular cAMP concentration leads to the activation of cAMP dependent protein kinases which activate HSL through phosphorylation of Ser 563 (32). Although cAMP is found to be increased (2 to 3 fold) in the presence of venom (data not shown), the biochemical linkage between venom effect and HSL activations remain to be explored.

Studies are in progress to purify and identify the active component(s) which, in Bot venom, activate the adipocyte lipolytic response. It is of interest to get further knowledge on such βAR activating factor(s) which might involve not only adipocytes but other cell-tissue types, highly responsive to catecholamines such as cardiac tissue.

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