Comparative effects of cholera and *Bordetella pertussis* toxins on cyclic AMP and GTP levels and on lipolysis in rat adipocytes incubated in vitro

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The respective effects of cholera and *Bordetella pertussis* toxins were studied in time and concentration dependent experiments, following glycerol and fatty acid release, GTP and cAMP levels. Cholera toxin, after a lag time of 30 min, stimulated linearly GTP and cAMP accumulation and lipolysis (maximal effect: 2-fold increase at 5 μg/ml). Pertussis toxin presented a biphasic effect both in time and concentration dependent studies. Up to a maximum reached after 2 h with 1.4 units LPF/ml the stimulation affected GTP (3 fold) and cAMP (7 fold) levels, glycerol and fatty acid release (15 fold). Beyond this, an inhibition occurred, yielding a decrease towards basal values of GTP and cAMP content whereas the glycerol and fatty acid release was stopped. These results, which are the first reporting the fluctuation of the GTP content of intact cells challenged with bacterial toxins, show a close relationship between GTP and cyclic AMP levels and lipolytic activity.

Pertussis toxin *Cholera toxin* *Rat fat cell* *GTP* *Cyclic AMP* *Lipolysis*

1. INTRODUCTION

In fat cells, lipolytic activity depends on cyclic-AMP levels [1]. Adenylate cyclase stimulation and inhibition by hormones is mediated by two separate guanine nucleotide-binding regulatory components, N_s [2–4] and N_i [5–7], respectively.

N_s is activated by cholera toxin which ADP ribosylates its α subunit and promotes the dissociation of the heterodimer α-β [4,8,9]. N_i is dissociated by another toxin from *Bordetella pertussis* (IAP) which ADP ribosylates and activates its α subunit [10].

While ADP-ribosylation and N_s inhibits the N_s-associated GTPase activity [11,12], ADP-ribosylation of N_i decreases the rate of formation of GTP bound N_i and prevents its hydrolysis into GTP-bound N_i by the associated GTPase [13–15].

Most work has been done in acellular systems with the concentration of the various components, in particular that of GTP or its stable analogue, fixed by the experimenter [16]. Its seemed of great interest to follow the consequences of toxins treatment of intact cells on their intracellular content of GTP and cAMP and on an integrated final biological effect. The adipocyte fulfills these requirements, indeed it is possible to follow the glycerol and free fatty acid release which is a time-dependent process resulting from the cyclic AMP controlled lipolysis.

Here, we show that the obtained results for each toxin were self-coherent, i.e., that the GTP and cyclic AMP content(s) and the intensity of lipolysis were closely correlated in all the experimental conditions. Nevertheless, these results are difficult to interpret according to the current concepts on the mode of action of toxins. Indeed, pertussis toxin was more efficient than cholera toxin.

2. MATERIALS AND METHODS

DL-Isoproterenol HCl (IPNE) (lot I-5627) and bovine serum albumin fraction V (made fatty acid free as in [17] were from Sigma. Collagenase (163

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U/mg) was purchased from Worthington Biochemicals. Cholera toxin (CT) (23.2 LB/μg) protein) was a gift from Professor Dodin (Institut Pasteur, Paris) and supernatant from cultures of *B. pertussis* strain 8144 (SPT) (2 U LPF/0.1 ml) from Dr Alonso (Institut Pasteur, Paris). (One unit of LPF (lymphocytose-promoting factor) is defined as the amount of material that causes 10^5 lymphocytes/ml above background, 3 days after injection, i.v. in mouse.) Adenylate cyclase activity from SPT was destroyed by heating at 56°C for 30 min. Residual activity was 2.68% of the initial activity. Although other activities may be present in SPT, they do not interfere with the studied parameters as the results are identical with those obtained with pure islet activating protein (IAP). IAP purified approx. 1800 fold from Dr Garcia-Sainz was kindly donated by Dr Lafontan. White fat cells were isolated by collagenase digestion as in [18] from 180 g male Wistar rats.

Drug and toxins (CT, SPT or IAP) were made up freshly in Krebs-Ringer bicarbonate buffer 0.1 M (pH 7.4) with half the recommended Ca^{2+} (1.3 mM) and 4% (w/v) fatty acid-free albumin, except for IAP which was dissolved in 0.1 M phosphate buffer (pH 7.4) plus 2 M urea as indicated by [19] and used at a concentration of 10 μg/ml. Identical vehicle has no effect on lipolysis or on cyclic-AMP production. Free fatty acids were extracted as in [20] and determined as in [21]. Triglycerides were extracted as in [22]. Aliquots of total lipid extracts were saponified in 4% (w/v) in 95% (v/v) ethanol for 30 min at 60°C. Glycerol was assayed as in [23]. Cyclic AMP was measured by the radioimmunological method as in [24], except that bound ligand was separated from the free ligand by polyethylene glycol precipitation. GTP was assayed by the method described in [25] modified by J.L. Aublin (unpublished). Nucleotides triphosphate were subjected to controlled barytic hydrolysis yielding the corresponding 3′,5′-cyclic nucleotide monophosphate in a proportional amount. To test the accuracy of the method, ATP level was determined in rat fat cells stimulated or not by isoproterenol (10^{-6} M) for 30 min, after substraction of control or stimulated cyclic-AMP levels,

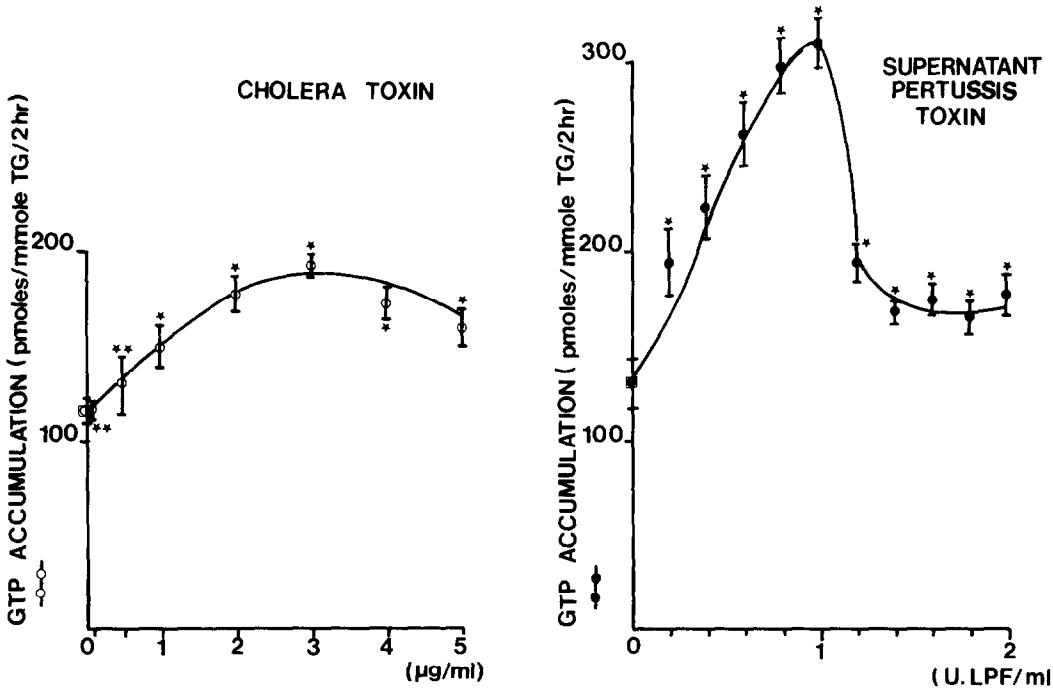

Fig.1. Concentration-dependent effects of cholera toxin (a) and crude pertussis toxin (b) on GTP accumulation in rat fat cells for 2 h. Data are expressed as the mean ± SE of 6 assays. * $p < 0.05$, ** P = NS. Basal values: (a) 115.7 ± 6.2, (b) 130.1 ± 3.7 pmol/mmol triglyceride.

respectively. Obtained values for control (520 pmol/10^5 cells) and for stimulated adipocytes (340 pmol/10^5 cells) are similar to those described in [26]; because of the very low endogenous cyclic-GMP content in rat fat cells, stimulated or not, no correction for GTP level determination was effected. GTP levels were assayed in function of weight of adipose tissue during a 2-h incubation with or without SPT (1.4 U LPF). The following results were obtained: control tissue (no incubation) 224 ± 27 and 470 ± 46 pmol/g and 2 g of tissue, respectively (6 assays); 2-h incubation without SPT: 251 ± 29 and 487 ± 29 pmol/g and 2 g respectively (6 assays); 2-h incubation with SPT: 562 ± 36 and 1042 ± 41 pmol/g and 2 g of tissue, 8 and 5 assays, respectively.

3. RESULTS

A concentration-response curve was established with CT and SPT, directly added into rat fat cells incubation medium. The treatment induced a GTP accumulation in both situations. CT treatment (fig.1a) for 2 h produced a small increase of GTP, levelling off at 168% for 3 μg/ml; higher concentration (5 μg/ml) caused a relative but significant decrease of the effect (110%). In the same conditions, SPT treatment had a very different effect, showing a biphasic curve (fig.1b). The first phase, up to 1 U LPF/ml, presented a concentration-dependent increase (EC_{50} = 0.4 U LPF/ml, maximal effect 240%) followed by a rapid and pronounced decrease for higher concentrations, from 1.4 to 2 U LPF/ml (residual effect vs basal 131%). These results were well correlated with the cyclic-AMP content, and the glycerol and free fatty acid release (fig.2). While for CT all these parameters plateaued, as GTP did, at the same concentration, with SPT, the cyclic-AMP content decreased as observed for GTP but at a higher concentration. Beyond this maximum value lipolysis stopped.

The time-course of the accumulation of GTP

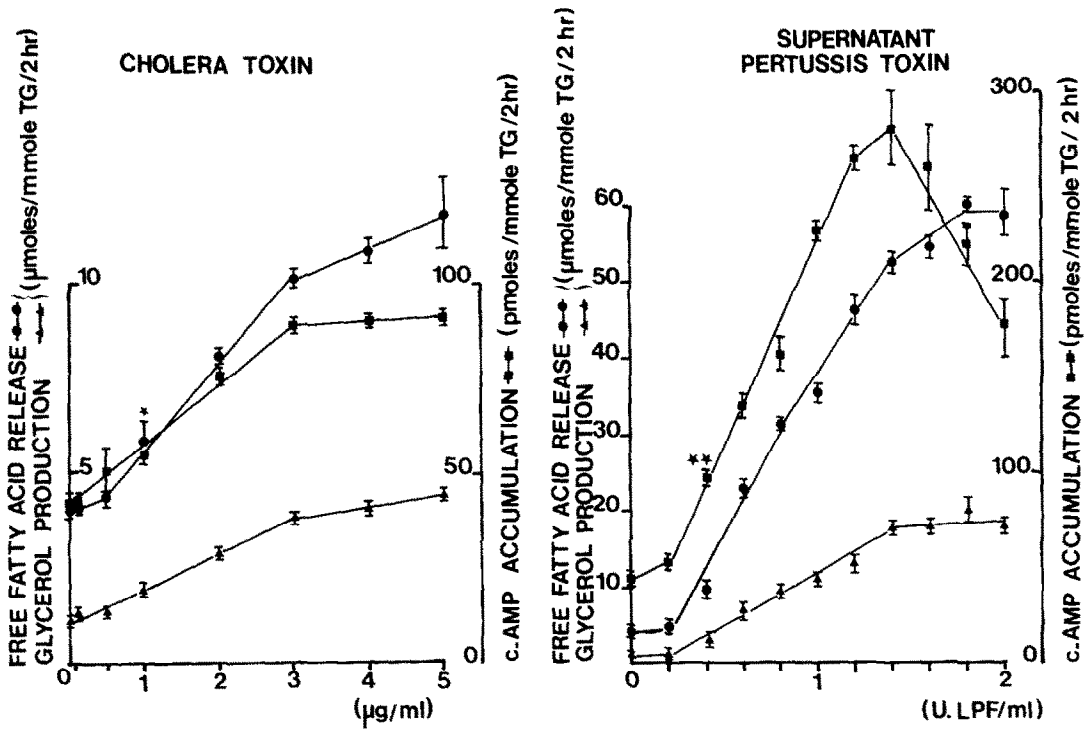

Fig.2. Concentration-dependent effect of cholera toxin (a) and crude pertussis toxin (b) on cyclic AMP accumulation, glycerol and free fatty acid release by rat fat cells in 2 h incubation. Data are expressed as the mean ± SE of 3 (a) and 4 assays (b). * $p < 0.05$ for cholera toxin concentration > 1 μg/ml, ** $p < 0.01$ for pertussis toxin concentration > 0.4 U LFP/ml.

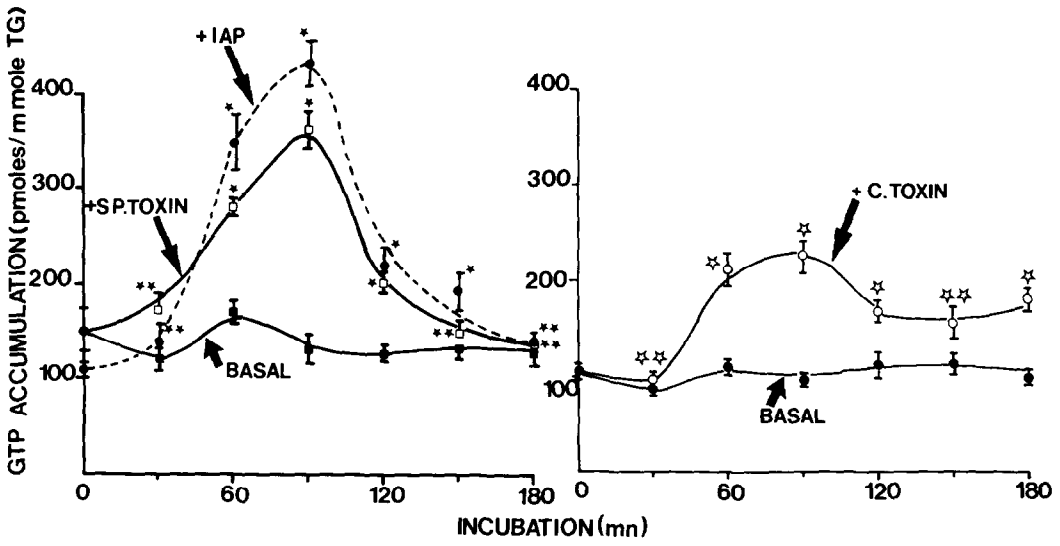

Fig.3. Time course of GTP fluctuations in cells stimulated by cholera toxin (5 μg/ml, ○—○) (a) or by crude (1.4 U LFP/ml, □—□) or purified pertussis toxin (10 μg/ml, ● - - - ●) (b). Results are given as the mean ± SE of 4 (SPT), 6 (IAP) and 4 (CT). * $p < 0.05$, ** P = NS.

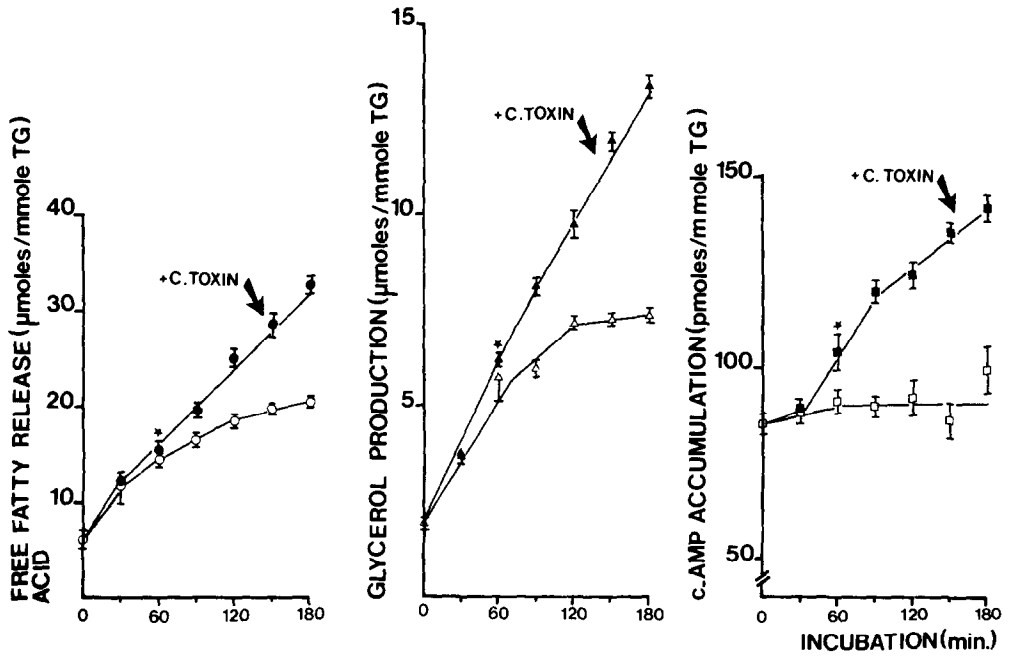

Fig.4. Stimulation by cholera toxin (5 mg/ml) of cyclic AMP accumulation and glycerol and free fatty acid release by rat adipocytes. Data are expressed as the mean ± SE of 3 assays. * $p < 0.05$ vs control for incubation time > 60 min.

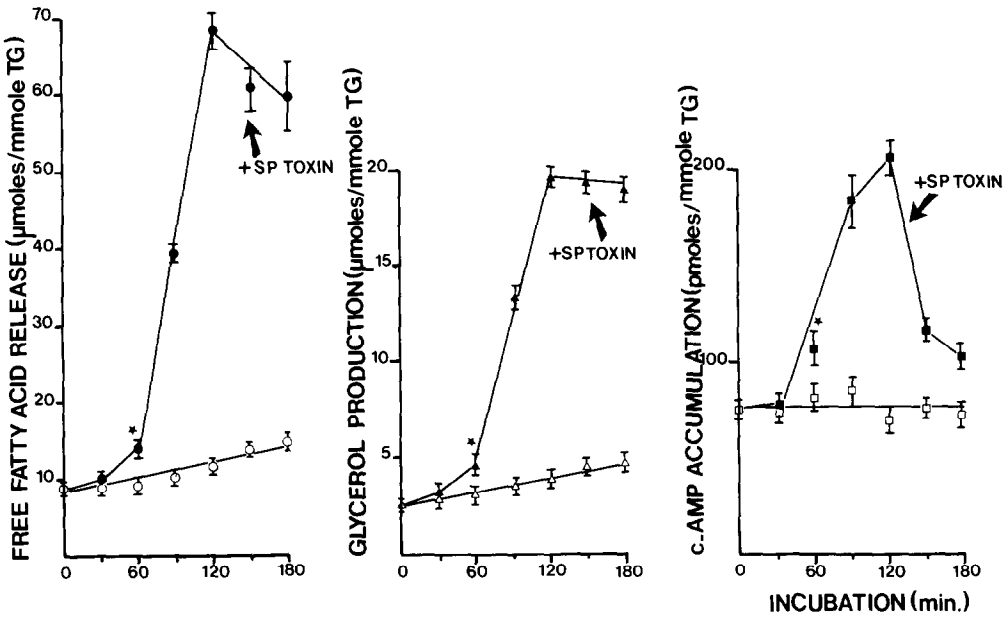

Fig.5. Stimulation by crude pertussis toxin (1.4 U LPF/ml) of cyclic AMP accumulation and glycerol and free fatty acid release by rat adipocytes. Data are expressed as the mean ± SE of 4 assays. * $p < 0.05$ vs control for incubation time > 60 min.

was studied with maximal effective concentrations of CT (5 μg/ml) for 3 h (fig.3). The kinetic study of CT action (fig.3a) showed a time-dependent GTP accumulation with a maximum (230%) obtained at 90 min, followed by a significant decrease to reach a steady-state (160% vs basal) between 2 and 3 h. This effect of CT on GTP accumulation was accompanied by a sustained increase of the cyclic-AMP accumulation and a constant rate of lipolysis up to 3 h. It was not the case for basal lipolysis, though a steady-state of cyclic-AMP accumulation was observed (fig.4).

The kinetic study of the effect of SPT (1.4 U LPF/ml) and of IAP (10 μg/ml) on GTP accumulation showed identical biphasic curves (fig.3b). After a lag time of 30 min, the GTP content increased linearly till 90 min (maximal effect 250 and 400%, respectively), then decreased to finally return to the basal value after 3 h. Cyclic AMP and lipolysis followed this time-dependent increase with a delay. Nevertheless, after a 2-h incubation, cyclic AMP decreased significantly as GTP did, while glycerol and to a lesser extent free fatty acid leveled off (fig.5). The results obtained with SPT were identical to those obtained with pure IAP (not shown).

4. DISCUSSION

The role of GTP in coupling adenylate cyclase was firmly established [27,28], nevertheless, its actual concentration inside the cell is not known. If GTP is equally distributed in the water content of adipocyte, we can estimate it in the micromolar range. This concentration, which lies in the range of those active on membrane preparations, is very low as compared to that in other cell types (millimolar in cultured thyroid cells; unpublished results) but is high enough to inhibit adenylate cyclase [29,30] as well as membrane-bound, low K_m cyclic-AMP phosphodiesterase [31] activities of rat adipocytes. As described for ATP [32], all the cellular GTP content may not participate to the activation of the adenylate cyclase, GTP being sequestered in and provided from different pools within the cell.

Our experimental conditions were chosen to determine the maximal efficient concentration of either toxin and the optimal time of pretreatment, on the cAMP accumulation and the lipolytic rate. It is interesting to compare the fluctuations of the content of GTP with those of cAMP. The maximal rise of GTP given by IAP does not exceed 3 fold;

it is modest as compared to the range of 4 orders of magnitude (10^{-9}–10^{-5} M) efficient on membrane preparations [30]. This limited increase produces a 7-fold elevation of the cAMP content and a lipolysis rate 15 times higher. Our results show a clear temporal relationship between the cellular content of GTP and of cAMP. When the levels of both nucleotides return to basal values, the stimulation of lipolysis stops.

The incubation of the cells in the presence of cholera toxin produces a small but sustained increase of the lipolytic rate, very inferior to that obtained by the stimulation of β receptors [33]. This difference cannot be explained by the negative effect of endogenous adenosine which must be the same in both situations.

Our present results raise more problems than they give answers. The very low concentration of GTP makes it very sensible to the action of toxins, mainly pertussis toxin. It was never reported that either cholera toxin or pertussis toxin stimulated the production of GTP. Then their positive effect on GTP level must be due to an inhibition of their disposal.

The recognized important inhibitory action of endogenous adenosine [34] may be prevented by the treatment with pertussis toxin. The formation of active N_i-GTP is impaired because of the decreased rate of GTP-GDP exchange [35,36], thus, GTP is protected from hydrolysis by the GTPase activity associated to N_i. All available GTP can associate to N_s, promoting its activation, a cyclic-AMP raise and, in turn, the stimulation of lipolysis. The hydrolysis of GTP by the GTPase activity associated to N_s insures a return to basal values which occurs after 180 min. At that time the cyclic-AMP level has returned to basal and lipolysis stopped. Another puzzling observation is the loss of efficiency observed with concentrations of pertussis toxin higher than 1 U LPF/ml (130 vs 240% at the maximal efficient dose). A possible explanation for this paradoxical result is the shift to the left of the time-course curve, when the dose of toxin is higher. The maximum effect being reached earlier, at 120 min, the decreasing phase is already operating.

The cholera toxin effects, less important, are also less transient: the stimulated steady-state reached for GTP at 120 min is sustained for 1 h, allowing a linear accumulation of cyclic-AMP and of lipolysis products. The limited maximal effect of cholera toxin, far below that of isoproterenol and additive to it [33], may be explained by an incomplete ADP-ribosylation of N_s subunits. The remaining native N_s subunits would be susceptible to dissociation under isoproterenol stimulation. This hypothesis would explain the observed additivity [33] between both stimulators. These data obtained with intact cells are difficult to integrate with the results of acellular experiments. Extensive work, evaluating the state of ADP ribosylation of the α subunits of the guanine nucleotide regulatory components of adenylate cyclase, during the stimulation of the cells by the toxins is necessary to understand the causes of our observations.

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