Inhibition by cholera toxin of the antilipolytic action of prostanoids, N^6-(phenylisopropyl) adenosine and insulin

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Received 15 April 1982

1. INTRODUCTION

In rat adipose tissue, the lipolytic activity is negatively controlled by various structurally unrelated biological compounds[1]. Some of them are endogenous substances released by the cells, such as adenosine [2], prostaglandins E_2 and $F_2\alpha$ (PGE_2, $PGF_2\alpha$) [3] and prostacyclin (PGI_2) [4, 5]; others, as insulin, belong to the endocrine pancreatic system. The physiological significance of adenosine, an end degradation product of cyclic AMP is well established [6–8] whereas the efficiency of the negative feedback of endogenous prostaglandins is not convincingly demonstrated [9, 10] and has been questioned [11].

The sites of action of insulin are double and express themselves in the increase of phosphodiesterase activity [12–14] and in the inhibition of adenylate cyclase activity [15–17]. The mechanism of the former effect of insulin is unknown but that of the latter implicates the participation of endogenous prostanoids [18]. Actually, insulin increases the sensitivity of adenylate cyclase to the inhibitory action of prostaglandins.

The aim of our work was to find the site of action of the mentioned antilipolytic substances. Fat cells or adipose tissue were exposed to the action of cholera toxin which activates irreversibly the adenylate cyclase system [19] via an inhibition of GTPase activity [20]. Here, we show that this treatment suppresses the inhibitory action of N^6-(phenylisopropyl) adenosine (PIA) (a non-metabolizable analog of adenosine), of PGE_1 and PGI_2 and of insulin on free fatty acids release and cyclic AMP production. These results suggest that these effectors share a common pathway at the guanosine triphosphatase (GTPase) level.

2. MATERIALS AND METHODS

Crystalline bovine insulin B-grade (25.5 IU/ mg) and collagenase (200 U/mg, Worthington Biochemical Corp.) were purchased from Calbiochem. L-Adrenaline bitartrate (lot 4-4375) and D,L-isoproterenol–HCl (lot 1-5627) were obtained from Sigma as was Bovine serum albumin fraction V, which was purified from fatty acids according to [21]. 1-Methyl-3-isobutyl xanthine (IBMX) (lot 1353) was obtained from Regis and 3-(3, 4 dimethoxybenzyl-2-imidazolidinone (RO 7-2956) was kindly donated by Hoffman-La Roche (Basel). N^6-(phenylisopropyl) adenosine (PIA) was obtained from Boerhinger. Prostaglandins E_1, $F_1\alpha$ and prostacyclin were a gift from the Upjohn Co. (Kalamazoo MI) and cholera toxin (23.2 LB/μg protein was a gift from Professor Dodin, Institut Pasteur, Paris).

Male Wistar rats (180 g) were obtained from Animalabo (Paris), kept at room temperature and given free access to food and water.

Rats were decapitated and the epididymal fat pads were removed as quickly as possible, rinsed in the ice-cold saline (NACl 0.9%, w/v). The fat pads were cut in small fragments (80–100 mg), and distributed among vials containing 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. White fat cells were isolated by collagenase digestion as in [22].

Hormones and drugs were made up freshly in

Published by Elsevier Biomedical Press

00145793/82/0000–0000/$2.75 © 1982 Federation of European Biochemical Societies

buffer to the required final concentrations. Prostacyclin was dissolved in 0.05 M Tris buffer (pH 9) and immediately added to the incubate. IBMX and RO 7-2956 were dissolved in dimethyl sulfoxide which has no effect on lipolysis by itself at the concentration used. Tissues or fat cells were preincubated for 2 h at 37°C with or without cholera toxin and then placed in fresh medium containing the appropriate hormone(s) and drug(s) for different intervals: 1 h for the studies of lipolysis (free fatty acids release) or 6 min for cyclic AMP production.

Free fatty acids were extracted as in [23] and determined as in [24].

Glycerol was measured as in [25]. Triglycerides were extracted as in [26]. Portions of total lipid extracts were saponified in KOH 4% (w/v) in 95% (v/v) ethanol for 30 min at 60°C. Glycerol was measured as above.

Cyclic AMP was assayed by the radioimmunological method in [27] except that bound ligand was separated from the ligand by polyethylene glycol precipitation (P. Mayeux, unpublished).

The statistical differences were calculated with Student's-t-test. The mean values are expressed with standard error of the mean (SEM).

3. RESULTS

3.1. *Conditions of cholera toxin treatment*

The maximal effective concentration of cholera toxin was of 5 μg/ml for 3 h incubation. With this concentration, after a lag period of 30 min, the rate of cyclic AMP accumulation, free fatty acid release and glycerol production was constant with an average doubling time of 1 h (not shown).

In the subsequent experiments, tissue or cells were preincubated with cholera toxin at 5 μg/ml for 2 h when indicated, then incubated in the presence of the various effectors and cholera toxin as in section 2.

3.2. *Effect of cholera toxin treatment on PIA antilipolytic action*

In these experiments, phosphodiesterase activity was inhibited with RO 7-2956 (10^{-3} M) to allow a constant rate of cyclic AMP accumulation and to

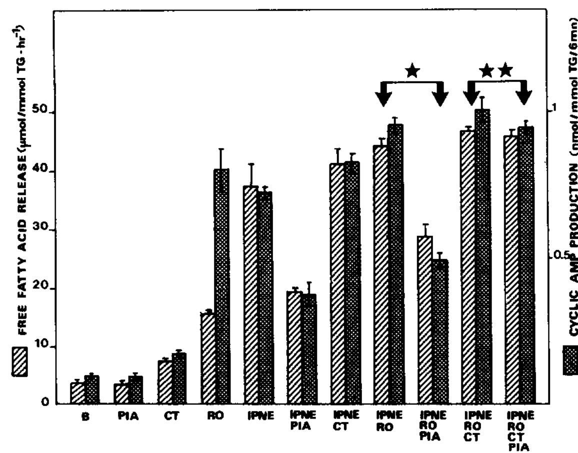

Fig.1. Effect of N^6-(phenylisopropyl) adenosine on isoproterenol and RO 7-2956 stimulated lipolysis in the presence and the absence of cholera toxin. Relationship between cyclic AMP and free fatty acid release. Results are given as means ± SEM calculated from 6 prep. fat cells. Significance of effect of drug: $*P < 0.001$; $**P$ = NS. *Abbreviations*: B, control values; PIA, N^6-(phenylisopropyl) adenosine (10^{-6} M); CT, cholera toxin (5 μg/ml); RO, RO 7-2956 (10^{-3} M); IPNE, isoproterenol (10^{-6} M).

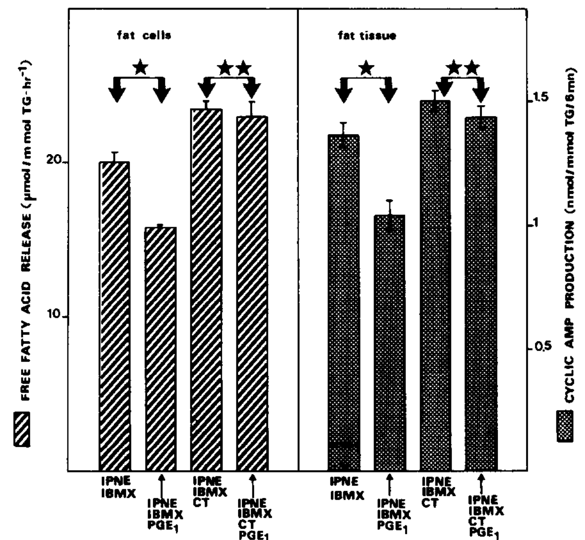

Fig.2. Effect of prostaglandin E_1 (10^{-6} M) on cyclic AMP (fat tissue) and free fatty acid release (fat cells) on isoproterenol (10^{-6} M) and 3-isobutyl-1-methylxanthine (10^{-3} M) in the presence and the absence of cholera toxin (5 μg/ml). Results are given as the mean ± SEM of 6 assays respectively. Significance of effect of hormone: $*P < 0.05$; $**P$ = NS.

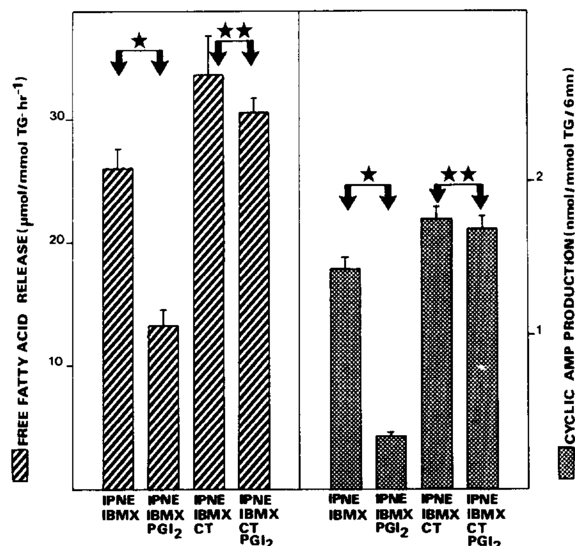

Fig.3. Effect of prostacyclin I_2 (10^{-7} M) on cyclic AMP and free fatty acid release on isoproterenol (10^{-6} M) and 3-isobutyl-1-methylxanthine (10^{-3} M) in the presence and absence of cholera toxin (5 μg/ml) in rat adipocytes. Results are given as the mean ± SEM of 6 assays, respectively. Significance of effects of hormone: $*P < 0.001$; $**P$ = NS.

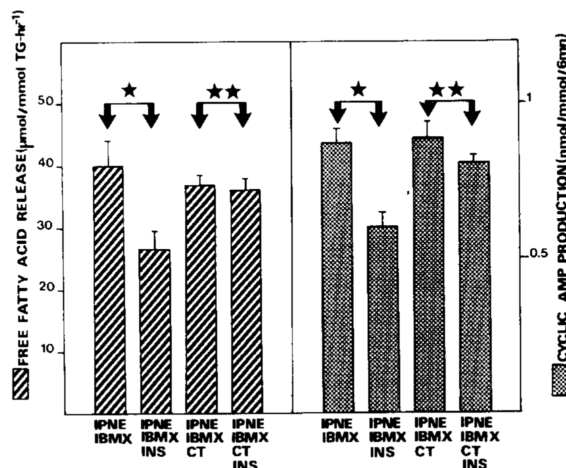

Fig.4. Effect of insulin (100 μU/ml) on cyclic AMP and free fatty acid release on isoproterenol (10^{-6} M) and 3-isobutyl-1-methylxanthine (10^{-3} M) in the presence and absence of cholera toxin (5 μg/ml) in rat adipocytes. Results are given as the mean ± SEM of 6 assays, respectively. Significance of effects of hormone: $*P < 0.05$; $**P$ = NS.

prevent interference at the adenosine receptor level with IBMX. Adenylate cyclase was stimulated by isoproterenol (10^{-6} M).

Results in fig.1 show the complete suppression of the inhibitory action of PIA (10^{-6} M) on cyclic AMP accumulation and on the release of free fatty acids.

3.3. *Effect of cholera toxin treatment on the anti-lipolytic activity of prostanoids*

PGE_1 (10^{-6} M) fig.2 and PGI_2 (10^{-7} M) fig.3 inhibited cyclic AMP accumulation and lipolysis stimulated by isoproterenol (10^{-6} M) with IC_{50} values of 3×10^{-7} M and 3×10^{-8} M, respectively (not shown).

When tissues or cells were preincubated and then incubated with cholera toxin, inhibition by prostanoids no longer occurred. In these experiments PDE activity was inhibited with IBMX (10^{-3} M). Neither $PGF_{2}\alpha$ nor 6-keto prostaglandin $F_{1}\alpha$ were active (not shown).

3.4. *Effect of cholera toxin treatment on the anti-lipolytic activity of insulin*

Incubations were performed in the presence of adrenaline (5×10^{-6} M) and of IBMX (10^{-3} M). The inhibition of phosphodiesterase activity allows a constant rate of accumulation of cyclic AMP and prevents the anti-lipolytic action of insulin at the PDE level. Under these conditions, the inhibitory action of insulin at the adenylate cyclase level disappeared when the tissues have been initially exposed to cholera toxin (fig.4). Neither the cyclic AMP content nor the free fatty acid release were depressed under these conditions.

4. DISCUSSION

Prostanoids (PGE_1 and PGI_2) as well as PIA and insulin at the concentration used showed antilipolytic activities either at the adenylate cyclase or at the phosphodiesterase levels or both in rat fat adipose tissue. Our present aim was to study the effect of these structurally different substances on the stimulated adenylate cyclase activity in the presence

of inhibitors of the PDE activity and in the presence or in the absence of cholera toxin. In rat adipose tissue or fat cells, there is evidence that cholera toxin accelerates lipolysis and cyclic AMP production [28–31] by maintaining the adenylate cyclase activity in a constant activated state [20] through the inhibition of the GTPase activity.

Our results showed that the treatment of fat cells or tissue with cholera toxin prior to stimulation with β-adrenergic effectors, suppresses the antilipolytic action of PGE_1, or PGI_2 and of PIA as described by [32] and of insulin. The inhibitory effects of these substances at the level of cyclic AMP is also suppressed. A similar behavior of cholera toxin against the 4 effectors was now observed, suggesting that they share the same mode and probably the same site of action. This confirms the results [33] for PGE and PIA but is new for insulin and furthermore brings additional support for the evidence of a site of action located at the adenylate cyclase level. Several hypotheses may explain the mode of action of cholera toxin. Since cholera toxin inhibits the GTPase activity of the adenylate cyclase system [31] PGE_1, PGI_2, PIA and insulin may be antilipolytic by increasing the GTPase activity; the enhanced hydrolysis of GTP in turn, would inactivate the catalytic subunit of the cyclase and then reduce the rate of synthesis of cyclic AMP and the efficiency of lipolysis. This hypothesis is reinforced since adenosine has been described as mediating the stimulation of GTP hydrolysis in adipocyte membranes [34]. We cannot discard an alternative explanation [35] suggesting that adenosine and PGE increase the displacement rate of GDP from the regulatory subunit of adenylate cyclase. The mechanism(s) by which these antilipolytic compounds achieve their effects is far from clear and further experiments are necessary to elucidate this problem.

ACKNOWLEDGEMENTS

We are grateful to Mrs O. Legué for her skillful technical assistance. We want to thank Dr. J. Pike, Dodin and H. Cailla, warmly for their respective gifts of prostaglandins, cholera toxin and cyclic AMP antibodies.

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