Guanosine 3':5'-Cyclic Monophosphate and the Action of Insulin and Acetylcholine

(cyclic AMP/fat cells/liver/receptors/lymphocytes/plant lectin/cell growth)

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ABSTRACT Low concentrations of insulin (120 μunits/ml) and of carbamylcholine (1 μM) increase cyclic GMP content in isolated fat cells by 350%. The maximal amount of cyclic GMP, achieved within 2 min after addition of insulin or carbamylcholine, falls rapidly for insulin and much more slowly for carbamylcholine. 10 pM Acetylcholine can also augment the content of fat-cell cyclic GMP, but by 5 min (37°) the amount falls to that of unstimulated cells. Atropine abolishes the effects of carbamylcholine and acetylcholine but does not modify that of insulin, indicating that the ability of insulin to regulate cyclic GMP levels is not mediated by cholinergic receptors. Insulin and carbamylcholine increase the concentration of cyclic GMP in rat liver slices by 400%; the effects of both agents occur rapidly and are relatively transient. Insulin does not alter cyclic GMP concentrations in purified human peripheral lymphocytes or in rat-spleen lymphocytes, cells which possess few insulin receptors and which are insensitive to the metabolic effects of the hormone. Carbamylcholine, however, causes a substantial increase in the cyclic GMP content of these lymphocytes. The data support the view that close and reciprocal relationships may exist between the concentrations and actions of cyclic AMP and cyclic GMP, as well as between the enzymes responsible for biosynthesis and degradation of these nucleotides.

Many of the biological effects of insulin are fundamentally opposite to the known biological effects of adenosine 3':5'-cyclic monophosphate (cyclic AMP). Insulin can suppress the increased amounts of cyclic AMP in fat (1, 2) and liver (3, 4) cells that occur upon stimulation by certain agents (epinephrine, glucagon, and theophylline). Furthermore, physiological concentrations of insulin can inhibit the stimulated as well as the basal activities of adenylate cyclase in fat-cell ghosts, in crude membrane preparations obtained from homogenates of liver and fat cells (5), and in isolated membranes from Neurospora crassa (6). Treatment of intact tissues with insulin can also cause an increase in the cyclic AMP phosphodiesterase activity of homogenates prepared from these tissues (7). However, it is not clear whether all of the biological effects of insulin are basically a consequence of changes in intracellular amounts of cyclic AMP. Decreased concentrations of cyclic AMP have not been unequivocally demonstrated in cells treated with insulin in the absence of compounds that elevate the intracellular concentrations of this nucleotide. The insulin–receptor interaction may modify very early events in cell membrane that lead to formation of other regulatory chemical substances that can modulate other cellular processes, possibly including the activity of enzymes that synthesize and degrade cyclic AMP.

Recent evidence has implicated guanosine 3':5'-cyclic phosphate (cyclic GMP) as a fundamental chemical mediator of cholinergic receptor activity in various tissues, and in some of these tissues the metabolic effects of this nucleotide appear to be opposite to those of cyclic AMP. Intracellular levels of cyclic GMP rise abruptly upon addition of acetylcholine or cholinergic agonists to preparations of heart (8, 9), brain (9–11), ileum (10), thyroid (12), liver (12), and ductus deferens (13). Very low concentrations of cyclic GMP can mimic the effects of cholinergic drugs on DNA synthesis by hematopoietic cells (14), and acetylcholine inhibits the epinephrine-stimulated rise in cyclic AMP in rat-uterine smooth muscle (15). In heart muscle, acetylcholine greatly reduces the isoproterenol- and glucagon-stimulated increase in cyclic AMP and also reduces slightly the basal levels of this nucleotide (16).

Cholinergic agents augment lymphocyte-mediated cytotoxicity (17). Cyclic GMP has been implicated as a mediator of mitogenic transformation of lymphocytes stimulated by phytohemagglutinin and concanavalin A, since these plant lectins cause a marked, early increase in intracellular amounts of cyclic GMP (18). Cyclic GMP can itself stimulate proliferation of thymic lymphocytes at concentrations as low as 5 μM (19). These effects on lymphocytes appear to be opposite to those that result from agents (e.g., adrenergic drugs) that increase the cellular amounts of cyclic AMP. The view has been emerging (8, 10, 18) that the physiological effects of adrenergic and cholinergic agents may be mediated by the relative intracellular concentrations of cyclic GMP and cyclic AMP.

The present studies demonstrate that insulin, like cholinergic drugs, can cause a prompt and marked increase in the intracellular concentration of cyclic GMP in tissues (fat cells and liver) that are responsive to this hormone but not in tissues (certain lymphocyte populations) that are insensitive to the hormone. The effects of insulin are not apparently mediated by cholinergic receptors since they are not blocked by specific blocking agents. These results may have important implications in understanding the mode of action of insulin, cholinergic hormones, and other processes (e.g., lymphocyte transformation and cell growth) that may be associated with “insulin-like” metabolic effects and diminished activity of adenylate cyclase.

MATERIALS AND METHODS

Isolated fat cells were prepared from epididymal fat pads of 140- to 180-g Sprague–Dawley rats (20). 5 to 9 × 10⁶ cells per ml were incubated at 37° in 2 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.1% bovine-serum albumin and, where indicated, insulin, carbamylcholine, atropine, or acetylcholine. Theophylline was not used unless indicated. Incubation was stopped by addition of 2 ml of ice-cold 10% (w/v)
trichloroacetic acid. The suspension was homogenized at 4° for 50 sec with a Polytron PT-10 homogenizer (Brinkman) at 600 rpm. For preparation of liver slices, male Sprague-Dawley rats (140-180 g) were killed by decapitation, and the livers were quickly perfused by injection of 20 ml of ice-cold Krebs-Ringer bicarbonate buffer (pH 7.4) into the portal vein. The livers were removed, rinsed with cold buffer, and slices (about 0.5-mm thick) were prepared (at 4°) with a Stadie-Riggs manual apparatus. Samples (120-200 mg wet weight) were incubated at 37° in 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.1% albumin and insulin or cholinergic drugs. The incubation was stopped by addition of 2 ml of 10% (w/v) trichloroacetic acid, followed by homogenization (Polytron) for 90 sec. Rat-spleen lymphocytes were prepared (21), and human circulating lymphocytes were prepared from freshly obtained, heparinized blood by passage through nylon filter columns (22). The lymphocytes (1 to 1.5 X 10⁶ cells per ml) were incubated at 37° in 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.1% albumin; incubation was stopped by addition of 2 ml of 10% (w/v) trichloroacetic acid, followed by sonication (8 sec).

**Extraction, Separation, and Assay of Cyclic GMP.** Cyclic GMP was extracted from the homogenates and purified by ion-exchange chromatography (9, 23). It was quantitated with a cyclic GMP-dependent protein kinase isolated from lobster-tail muscle. The tissue homogenate, containing 5% (w/v) trichloroacetic acid, was centrifuged at 27,000 x g for 30 min. 50 μl of 4 N HCl were added to 4 ml of the supernatant, and this solution was extracted three times with 5 ml of ether. 2 ml of the water phase was lyophilized, redissolved in 1 ml of 50 mM sodium acetate buffer (pH 4), and applied to a Dowex AG1-X8 (formate form) column (0.5 X 5 cm) equilibrated with distilled water. The column was washed with 10 ml of water and 10 ml of 2 N formic acid before the cyclic GMP was eluted with 14 ml of 4 N formic acid; recovery of cyclic GMP at this step is 80%, and less than 5% of cyclic AMP applied to such a column is recovered in the fraction containing cyclic GMP. The column eluate was lyophilized and redissolved in 0.5 ml (or smaller volume, if necessary) of 10 mM sodium acetate buffer (pH 4) for determination of cyclic GMP concentration.

Cyclic GMP was assayed by a modification of described methods (9, 23, 24) that used cyclic GMP-binding protein kinases. The incubation mixture contains, in a total volume of 0.1 ml, 500 μg of a cyclic GMP-binding protein purified from lobster-tail muscle through the ammonium sulfate (55%) stage (24), 5 mM sodium acetate buffer (pH 4), 10 pmol of [3H]cyclic GMP (New England Nuclear Corp., specific activity, 3.46 Ci/mmol), and the tissue sample of standard amounts (2-30 pmol) of cyclic GMP. After the mixture was incubated at 4° for 90 min, 10 μl of 1% bovine gammaglobulin was added (as carrier), followed by 0.9 ml of 65% (w/v) ice-cold ammonium sulfate. The samples, which were kept at 4° for 10 min to permit complete protein precipitation, were filtered under reduced pressure through AH or EG (Millipore Corp.) filters and washed with 10 ml of ice-cold 55% (w/v) ammonium sulfate. The filters were placed in counting vials and incubated at 24° for 15 min in 1 ml of 10% (w/v) sodium dodecyl sulfate before radioactive content was determined (45% efficiency), with 10 ml of TLX toluene Fluorolloy (Beckman) and 2 ml of Biosolv Solubilizer BBS-3 (Beckman). All determinations were performed in duplicate.

The use of protein precipitation in the assay of cyclic GMP results in considerable improvement over the methods (9, 23) that depend on adsorption of protein kinases on cellulose filters. A standard curve is depicted in Fig. 1. With 10 pmol of [3H]cyclic GMP, nearly 4000 cpm are bound to the filter and addition of 6 pmol of unlabeled cyclic GMP causes a displacement of 1200 cpm. Under similar conditions, but in the absence of precipitation, less than 500 cpm are adsorbed on the filter and 20 pmol of cyclic GMP cause a total displacement of about 150 cpm (23).

**Table 1. Effect of insulin concentration on the cyclic GMP content of isolated fat cells**

<table>
<thead>
<tr>
<th>Insulin concentration (μU/ml)</th>
<th>Cyclic GMP content (pmol per 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>12</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>120</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>1.2</td>
<td>29 ± 3</td>
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</tbody>
</table>

The conditions were as described in Fig. 1.
RESULTS

Studies with Isolated Fat Cells. The concentration of cyclic GMP in fat cells increases by about 350% within 2 min after exposure of the cells to low concentrations of insulin (Fig. 2). This early effect is followed by a rapid fall in the intracellular content of the nucleotide. By 10 min, the cyclic GMP content has fallen considerably, although its concentration is still nearly 100% higher than that of the cells that were not exposed to insulin. The effects of insulin are apparent at physiological concentrations of the hormone, and a half-maximal effect is observed at about 12 μU/ml (Table 1). Furthermore, a maximal response can be achieved with 120 μU of insulin per ml, since an increase of the hormone concentration to 1.2 mU per ml does not further increase the effect.

The cholinergic drug, carbamylcholine (1 μM), also causes an increase in the amounts of cyclic GMP in fat cells; this increase occurs as rapidly and its magnitude is nearly as great as that observed with insulin (Fig. 2). The effect of carbamylcholine, however, is more sustained than that of insulin since by 10 min the cyclic GMP concentration is only slightly lower than that observed at 2 min.

A rapid and significant elevation of cyclic GMP concentration occurs upon exposure of isolated fat cells to concentrations of acetylcholine as low as 10^-10 and 10^-11 M (Fig. 3). However, the effects of these low concentrations of acetylcholine are transient. By 5 min the amounts of cyclic GMP in acetylcholine-treated cells are indistinguishable from those of untreated cells. The transient nature of the effect of acetylcholine may be a reflection of the high susceptibility of this drug to hydrolytic cleavage (compared to carbamylcholine) by acetylcholinesterase. However, this apparently transient effect may be a true reflection of the physiological mode of action of this hormone. Very high concentrations (10^-4 M) of acetylcholine result in a "lag" or latency phase, which is followed by a profound rise in the level of cyclic GMP (Fig. 3). This initial latency phase may be an expression of an inhibitory effect, since in certain systems biological responses that occur at concentrations of acetylcholine as low as 10^-11 M are not observed if the concentration of the hormone is raised to 10^-4 M and higher (14, 17). The rapid loss of this "inhibitory" effect, observed at 4–5 min, may reflect the rapid hydrolytic destruction of acetylcholine with the consequent achievement of lower, stimulatory concentrations of the drug.

Atropine, which blocks the muscarinic effects of cholinergic agents, completely prevents the effects of carbamylcholine but does not alter the response of the cells to insulin (Fig. 4). Thus, the ability of insulin to enhance the intracellular content of cyclic GMP in isolated fat cells is apparently not mediated by cholinergic receptors. Atropine can itself increase slightly the content of cyclic GMP in fat cells (Fig. 4).

Studies with Liver Slices. Insulin and carbamylcholine cause a rapid increase in the content of cyclic GMP in liver slices (Fig. 5). Both agents elevate the amount of cyclic GMP by about 400%. The effects of insulin and of carbamylcholine are relatively transient, since with both hormones the concentrations of cyclic GMP approach that of untreated liver slices by 10 min. The maximal effects of insulin are clearly
delayed compared to those of carbamylcholine. This apparent “lag” phase in the effect of insulin may be related to barriers in diffusion of this hormone into the interior of the tissue slice.

Studies with Lymphocytes. Although carbamylcholine causes significant elevations in the content of cyclic GMP in human peripheral lymphocytes and rat-spleen lymphocytes, no such effects could be demonstrated with insulin (Table 2). Insulin receptors are not demonstrable in resting human lymphocytes prepared and purified by procedures identical to those used in these studies (22). Furthermore, inhibition of adenylyl cyclase activity by insulin has not been demonstrated in membrane preparations from these cells or from spleen lymphocytes, despite the demonstration of such an inhibition with the hormone somatostatin (25). Studies in this laboratory have failed to demonstrate unequivocal metabolic responses (glucose transport and oxidation, amino-acid transport, or glycogen synthesis) to insulin in these cells. Thus, the inability of insulin to alter the cyclic GMP content of rat-spleen and human circulating lymphocytes correlates well with the inability of this hormone to elicit biological effects in these cells.

DISCUSSION

Cholinergic agents and insulin produce similar effects on the intracellular content of cyclic GMP in sensitive tissues. The effects of these two hormones appear to be mediated by different receptors, since cholinergic (muscarinic) blocking agents do not modify the effects of insulin. Cyclic GMP may act as the principal mediator of certain (or possibly all) of the biological effects of these hormones.

The apparently transient nature of the elevation of cyclic GMP content observed here, and in studies with cholinergic agents in heart (8, 9), brain (9), and lymphocyte (18) preparations, does not negate the possible importance of this nucleotide in regulating intracellular metabolic events. The patterns of change of cyclic GMP induced by insulin and cholinergic agents are similar to the changes in cyclic AMP that follow exposure of cells to adrenergic drugs and peptide hormones whose actions are mediated by cyclic AMP. In the absence of the phosphodiesterase inhibitor, theophylline, epinephrine causes only a relatively small increase in the total intracellular content of cyclic AMP in fat cells, and the effects are as transient (2) as those described for insulin and cholinergic drugs on cyclic GMP. Despite the fact that by 10 min after exposure of fat cells to epinephrine the cellular amounts of cyclic AMP have nearly returned to the baseline values, the biological effects of this hormone continue unabated for a long time provided the hormone is not removed from the medium (2). Relatively small changes in the steady-state concentration of the cyclic nucleotides may result in profound alterations in certain biochemical events; furthermore, compartmentalization and segregation of nucleotide pools may exist within the cell (4).

The possible interrelationships between cyclic GMP and cyclic AMP in regard to the mechanism of action of insulin should be considered, especially in view of the ability of insulin to modify the activity of adenylyl cyclase when it is added directly to cell membranes obtained from cell homogenates (5, 6, 26). If insulin does not simultaneously and independently modify separate membrane functions by interacting with distinctly separate receptors, then the effects of insulin on cyclic AMP and cyclic GMP must occur either simultaneously through a common enzyme system or sequentially in a way that the effects of one or the other nucleotide must be primary. Since the enzyme that is presumably responsible for synthesis of cyclic GMP from GTP is thought to occur in “soluble” form in the cell cytoplasm (27) while the receptors for insulin (28) and adenylyl cyclase are localized in the cell membrane, and since effects of insulin on the membrane-localized adenylyl cyclase are demonstrable in subcellular systems (5, 6, 26), the present weight of evidence is in favor of a hypothesis that suggests that a fall in the concentration of cyclic AMP, even though small, may somehow cause a reciprocal elevation in the cyclic GMP concentration. The subsequent metabolic events could be mediated by consequences of both events.

Alternative possibilities must be considered. A unique insulin–receptor (or cholinergic hormone–receptor) interaction at the cell membrane may transform the substrate specificity of the membrane-localized adenylyl cyclase into a form that now expresses specificity for GTP. The same membrane-localized enzyme system would be responsible for synthesis of both cyclic nucleotides, and the “balance” of ATP- or GTP-utilizing forms of the enzyme would depend on the relative occupancy of receptors that favor the ATP form compared to the GTP form. This hypothesis could explain the simultaneous “inhibition” of adenylyl cyclase activity and elevation of cyclic GMP levels (by enhanced
Cyclic GMP and Insulin Action


