Diversity in the Effects of Extracellular ATP and Adenosine on the Cellular Processing and Physiologic Actions of Insulin in Rat Adipocytes*

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ATP or adenosine (1 mM) added to extracellular buffer abolished both chloroquine- and monensin-dependent accumulation of $^{125}$Iodoinsulin in isolated rat adipocytes. The effects of ATP were not secondary to its conversion to adenosine and were mimicked by $\beta,\gamma$-methyleneadenosine 5'-triphosphate. ATP, but not adenosine, partially inhibited the binding of insulin to the cellular receptor. Neither ATP nor adenosine had any significant effect on both internalization of cell-bound insulin and externalization of the internalized hormone. The degradation of cell-bound insulin was reduced to a considerable extent by both 0.1 mM chloroquine and 5 mM ATP, to a lesser degree by 1 mM ATP, and not significantly by 1 or 5 mM adenosine. Physiologically, (a) 1 mM ATP had a strong, while 1 mM adenosine had a mild inhibitory effect on the insulin-stimulated glucose transport without affecting its basal activity, (b) both ATP and adenosine moderately stimulated basal as well as insulin-stimulated glycogen synthase, and (c) ATP, but not adenosine, transiently stimulated basal cAMP phosphodiesterase without affecting the insulin-stimulated enzyme. Phosphodiesterase in cells that had been exposed to ATP for 30 min was refractory to ATP added afresh, but not to insulin. These data suggest that (a) extracellular ATP may block the degradative pathway of insulin processing, (b) adenosine might render the ordinarily irreversible intracellular traffic of insulin reversible or modulate a pathway which is yet to be identified, (c) the previously reported effect of ATP on glycogen synthase may not involve phosphorylation, (d) ATP stimulates cAMP phosphodiesterase by a mechanism which is distinct from that of insulin, and (e) the degradative pathway of insulin processing may not be involved in the physiologic actions of the hormone on glycogen synthase and phosphodiesterase.

The mechanisms of insulin actions at the molecular level are still obscure. However, it is now well established that the cellular insulin receptor is an insulin-sensitive protein kinase (1) while ATP or metabolic energy is involved in some hormonal actions, such as those on glucose transport (2-4) and cAMP phosphodiesterase (phosphodiesterase) (2). Therefore, attempts have been made in our laboratory to reconstitute the physiologic actions of insulin in a cell-free system by incubating ATP with either broken or partially permeated cell preparations. Whereas these attempts are still in progress, it was found during the study that both cellular processing and physiologic actions of insulin are profoundly affected by extracellular ATP in intact, rather than broken, cell preparations. Although the actions of extracellular ATP are by no means physiologic, their effects may not be ignored in the reconstitution studies since, in experiments with broken cell preparations, both outer and inner surfaces of the plasma membrane (hence, those of the insulin receptor and of the glucose transport system) are inevitably exposed to ATP. Therefore, in our present study, we investigated the effects of ATP and adenosine on the processing and physiologic actions of insulin in intact adipocytes; we included adenosine in our study as the compound is readily formed from ATP. Prior to our present study, it has been known that (a) extracellular ATP inhibits both insulin binding to the isolated liver plasma membrane (5) and insulin-stimulated glucose transport in adipocytes (6, 7), while stimulating both basal and insulin-activated glycogen synthase in fat cells (8), (b) in some cell types, extracellular ATP stimulates cellular permeability to metal ions (9, 10) and inhibits cell growth (9) or endocytosis (10), and (c) adenosine is a physiologic regulator of a number of metabolic activities (11-22). Preliminary accounts of our present study have been published in abstract forms (23-25).

**MATERIALS AND METHODS**

UDP [glucose-1-$^{3}$H]glucose and [2,8-$^{3}$H]adenosine 5'-triphosphate were purchased from Du Font-New England Nuclear. UDP-glucose, glucose 6-P, glyogen, ATP, AMP-PCP, and adenosine were obtained from Sigma. The sources of other reagents were listed in our previous publications that dealt with the individual methods described below.

Isolated adipocytes were prepared by the collagenase method (26) from epididymal adipose tissue of Sprague-Dawley rats, weighing approximately 170-220 g. The cells were incubated at 37 °C in KREBS-Henseleit Hepes (30 mM) buffer, pH 7.4, supplemented with 20 mg/ml of crude bovine serum albumin (Fraction V) (27) and 2 mM sodium pyruvate (28). The cellular uptake of [125]Iodoinsulin was determined basically as described previously (29). Briefly, adipocytes were incubated with [125]Iodoinsulin (approximately 0.1 nM or as specified) for the indicated period, washed with ice-cold Buffer A (0.25 M sucrose in 10 mM Tris/HCI, pH 7.5), and homogenized in the same buffer in a Dounce tissue grinder at room temperature. The homogenate was briefly centrifuged, and the fat-free infranatant solution was analyzed for radioactivity and protein. The observed radioactivity was (a) corrected for the so-called nonspecific binding determined in the

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1 The abbreviations used are: phosphodiesterase, cAMP phosphodiesterase; AMP-PCP, $\beta,\gamma$-methyleneadenosine 5'-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CURL, compartment for uncoupling of receptor and ligand.

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The experiment was carried out as in Fig. 1, except that 100 μM monensin (MON) was substituted for chloroquine (n = 6–9).

**RESULTS**

Effects of ATP and Adenosine Added to Extracellular Buffer on the Cellular Processing of Insulin—As is well known and is demonstrated by the control cells in Fig. 1, a considerable amount of [125I]iodoinsulin is accumulated in adipocytes when the cells are exposed to the labeled hormone in the presence of chloroquine (30, 38); chloroquine is an inhibitor of lysosomal function (30, 38), as considered later in more detail (see “Discussion”). However, as depicted in Fig. 1, this chloroquine effect was undetectable when chloroquine-treated cells were exposed to 1 mM ATP for 10 min prior to the incubation with [125I]iodoinsulin. Additionally, it is shown in this figure that extracellular ATP partially inhibited the initial accumulation of [125I]iodoinsulin (see the data at 10 min), in partial agreement with Trischitta et al. (5). As reported in Fig. 2, ATP added to extracellular buffer also abolished the monensin-dependent accumulation of [125I]iodoinsulin in adipocytes. Monensin is an ionophore, which induces an intracellular accumulation of [125I]iodoinsulin by blocking the dissociation of insulin from its receptor (29) (see “Discussion”).

The effects of ATP on the chloroquine- and monensin-dependent accumulation of iodoinsulin were mimicked by AMP-PCP (a nonphosphorylating analogue of ATP) and adenosine (Table I). As may be seen in Fig. 3, the apparent potencies of ATP and adenosine were almost identical; both agents were highly effective at 0.1 and 1.0 mM, but not at 10 mM or less. However, these data do not indicate whether the observed effects of ATP were genuine or secondary to its conversion into adenosine. As recorded in Fig. 4A, extracellular ATP was readily converted into adenosine; note that these experiments were carried out under conditions identical to those employed in Figs. 1–3. However, as reported in Fig. 4B, the accumulation of adenosine (formed from added ATP) was reduced to less than 10% in 40 min when the concentrations of ATP and adipocytes were lowered to 0.1 mM and 10 ng/ml, respectively. By applying these latter conditions to the aforementioned experiments with chloroquine (Fig. 1), we found that ATP itself was apparently effective (Table II). Note that adenosine would not show any positive effect as long as its concentration was 10 μM or less, whereas ATP at 100 μM should give a positive effect if it were indeed effective (see Fig. 3). Although the concentration of endogenous adenosine in this system was unknown, only a weak effect was exhibited by the cell preparation that was supplemented with 10 μM adenosine as a second control (Table II). It was concluded, therefore, that ATP itself was effective in reducing the effect of chloroquine.

We next examined whether ATP and adenosine might inhibit the internalization of insulin into adipocytes. For this study, we utilized our previous observation that most of
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Table 1

Effects of ATP and its analogues on chloroquine- and monensin-dependent accumulation of $[^{125}]$iodoinsulin

The experiments were carried out as described in the legends to Figs. 1 and 2 except that indicated agents (all at 1 mM) were substituted for ATP, and the incubation was carried out for 60 min. CQ, 0.1 mM chloroquine; MON, 0.1 mM monensin. The results are shown in: average ± S.E. (n = 3-6). The data were normalized to 1,000,000 cpm radioactivity/1 ml of the incubation mixture. The experiments with chloroquine and monensin were carried out on different occasions.

| Addition (1 mM) | Basal (A) | +CQ (B) | Difference (B - A) | Basal (C) | +MON (D) | Difference (D - C) |
|----------------|-----------|---------|-------------------|-----------|----------|-------------------|
| None           | 12.2 ± 0.8 | 26.3 ± 1.1 | 14.1              | 7.9 ± 0.8 | 19.3 ± 1.3 | 11.4              |
| ATP            | 10.5 ± 0.5 | 10.8 ± 0.5 | 0.5               | 8.2 ± 0.9 | 8.5 ± 0.8 | 0.3               |
| AMP-PCP        | 10.1 ± 0.7 | 18.2 ± 0.8 | 8.1               | 9.5 ± 0.7 | 12.9 ± 1.2 | 3.4               |
| Adenosine      | 11.2 ± 0.6 | 11.8 ± 0.5 | 0.6               | 8.0 ± 0.7 | 8.1 ± 0.2 | 0.1               |

Table 2

Evidence that ATP itself is effective in suppressing chloroquine-dependent accumulation of $[^{125}]$iodoinsulin

Aliquots of adipocytes (10 mg/ml, 2 ml in total) were mixed with 0 or 1 mM chloroquine plus 0 or 100 μM ATP at -10 min and $[^{125}]$iodoinsulin (approximately 5,000,000 cpm/ml) at 0 min. The incubation was then continued for an additional 30 min, and the samples were analyzed as in Table 1. The level of adenosine formed from ATP in this experiment should be less than 10 μM (see Fig. 4). As a second control, aliquots of cells were incubated as above, except that 10 μM adenosine was substituted for ATP. The results are shown in: average ± S.E. (n = 10).

| Addition | Basal (A) | +CQ (B) | CQ effect (B - A) |
|----------|-----------|---------|------------------|
| None     | 39.2 ± 2.5 | 62.0 ± 2.9 | 22.8             |
| 100 μM ATP | 42.7 ± 1.4 | 48.7 ± 1.1 | 6.0              |
| 10 μM adenosine | 40.0 ± 1.2 | 58.7 ± 1.8 | 18.7             |

Fig. 3. Effects of the concentrations of ATP and adenosine on the uptake of $[^{125}]$iodoinsulin in the presence or absence of chloroquine (CQ). The experiment was carried out as described in the legends to Figs. 1 and 2 except that the concentrations of ATP and adenosine were varied as shown while the time for incubation with the labeled hormone was fixed to 1 h (n = 6).

internalized $[^{125}]$iodoinsulin was associated with slow sedimenting vesicles that were separable from the plasma membrane by linear sucrose density gradient centrifugation (2, 30). As reported in Fig. 5A, adenosine had no significant effects on either the binding of $[^{125}]$iodoinsulin to the plasma membrane (namely on the formation of Peak 1) or the association of the internalized hormone with the slow sedimenting vesicles (i.e. on the formation of Peak 2). In agreement with the view that an ATP-dependent endocytosis was involved in the internalization of insulin, the formation of Peak 2 was blocked when the cells were deprived of intracellular ATP by treatment with 2,4-dinitrophenol, in agreement with our previous data (2). As shown in Fig. 5B, adenosine was also ineffective on the dissociation of $[^{125}]$iodoinsulin from either the plasma membrane (Peak 1) or the intracellular vesicles (Peak 2). Although Fig. 5B does not indicate this, insulin "dissociated" from the intracellular vesicles thought to be eliminated from adipocytes since it was observed earlier that no extra hormone was accumulated in cells that were exposed to $[^{125}]$iodoinsulin for 1 h in the presence of adenosine (Table I and Fig. 3). In agreement with the view that the elimination of insulin from the intracellular vesicles (and from adipocytes) must require ATP (i.e. the ATP-dependent movement of intracellular vesicles, see "Discussion"), the disappearance of Peak 2 was strongly inhibited by treatment of cells with 2,4-dinitrophenol, in confirmation of our previous data (30).

The above approach was not applicable to the experiments with ATP since the subcellular fragments obtained from
ATP-treated cells did not separate well by sucrose density gradient centrifugation (data not shown). Therefore, effects of ATP on the internalization of insulin were studied by measuring the level of \[^{125}\text{I}]iodoinsulin trapped in the whole adipocytes that had been exposed to the labeled hormone, treated with 2,4-dinitrophenol, and then washed with fresh buffer. As shown above in Fig. 5B, internalized insulin would be trapped in cells if the latter were deprived of ATP. The results of this experiment indicated that approximately 30% of the cell-associated hormone was intracellular regardless of whether the cells were exposed to \[^{125}\text{I}]iodoinsulin in the presence or absence of 1 mM adenosine, 1 mM ATP, or 5 mM ATP (Table III). Note that the results of the experiment with or without adenosine were in good agreement with those shown earlier in Fig. 5A. It was concluded, therefore, that ATP, like adenosine, did not inhibit internalization of insulin. The data in Table III further show that, in agreement with those reported in Fig. 1, the uptake of insulin (determined at 10 min of incubation) was partially inhibited by ATP. Indirectly, it was concluded at this point that ATP, like adenosine, may exert little effect on the efflux of internalized insulin from adipocytes since it was observed earlier in Figs. 1 and 2 that no extra insulin was accumulated in cells that had been exposed to \[^{125}\text{I}]iodoinsulin for 1 h in the presence of ATP.

Since ATP and adenosine strongly inhibited the chloroquine-dependent accumulation of insulin (Table I and Figs. 1 and 3), we next examined whether the agents might also block the chloroquine-sensitive (i.e. lysosomal) degradation of the hormone. As may be seen in Table IV, the results of this examination were highly complex. The data show that approximately 20–22% of cell-bound insulin was degraded in 30 min in the absence of any inhibitors (second column). The level of this degradation was reduced approximately to 5–7% in the presence of chloroquine (third column) suggesting that roughly 15% of the cell-bound hormone was degraded by the lysosomal pathway. The effect of chloroquine was mimicked to a large extent (approximately 75%) by 1 mM ATP and completely by 5 mM ATP (upper two data in the fourth column). The inhibitory effects of the two agents did not appear to be additive (fifth column); the apparent differences in the effects of chloroquine alone and chloroquine plus ATP were not statistically significant (p > 0.05). In contrast, neither the total nor the chloroquine-sensitive degradation of cell-bound insulin was affected to any significant extent by 1 or 5 mM adenosine (last two lines in the fourth and fifth columns). Although it was not tested in our present work, it was assumed that the chloroquine-insensitive degradation of cell-bound insulin was mediated by "insulinase" on the cell surface, as we reported previously (30).

**DISCUSSION**

As is well known, the insulin-receptor complex formed on the cell surface (PK1 in Fig. 7 = Peak 1 in Fig. 5) is rapidly internalized by the cell (39, 40). Most of the internalized hormone is associated with the slow sedimenting vesicles that are detectable by sucrose density gradient centrifugation (2, 30; PK2 in Fig. 7 = Peak 2 in Fig. 5). At a steady state of internalization, the ratio of insulin molecules bound to the plasma membrane and the intracellular vesicles is approximately 2:1 (2, 30; see also Fig. 5 and Table II). Of the internalized hormone that is associated with the slow sedimenting vesicles (Peak 2), approximately one-third is simply recycled back into extracellular buffer without degradation (30), presumably via Route A in Fig. 7 (30, 41). The physiological significance of this apparently wasteful energy-dependent recycling of insulin (30) is still obscure. The rest of
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TABLE III

Effects of adenosine and ATP on the total cellular binding and internalization of [125I]iodoinsulin

| Addition | Cell-bound [125I] activity | Level of internalization |
|----------|---------------------------|-------------------------|
|          | Total, before washing (A) | After 2nd incubation | (B) | (C) | (D) = (B - C) | (R) = (D/A) |
|          | cpm/µg protein | +DNP | -DNP | | % | |
| Experiment A | | | | | | |
| None | 4.83 ± 0.07 | 2.52 ± 0.12 | 1.07 ± 0.12 | | 1.45 | 1.37 |
| 1 mM Ado | 4.61 ± 0.17 | 2.24 ± 0.08 | 0.87 ± 0.04 | | 1.51 | 1.37 |
| Experiment B | | | | | | |
| None | 4.70 ± 0.04 | 2.61 ± 0.08 | 1.10 ± 0.05 | | 1.51 | 1.37 |
| 1 mM ATP | 3.09 ± 0.07 | 2.07 ± 0.30 | 1.06 ± 0.06 | | 1.01 | 1.01 |
| 5 mM ATP | 1.72 ± 0.22 | 1.63 ± 0.02 | 1.23 ± 0.04 | | 0.50 | 0.50 |

TABLE IV

Effects of chloroquine, ATP, and adenosine on the degradation of cell-bound insulin

| Addition | Degradation of cell-bound [125I]iodoinsulin in cells treated with | |
|----------|---------------------------------------------------------------|---|
|          | "X" in the 4th and 5th columns | Degradation | CQ | "X" | CQ + "X" |
| 1 mM ATP | 21.2 ± 0.7 | 5.0 ± 0.8 | 5.0 ± 0.8 | 6.7 ± 0.7 | 8.4 ± 1.4 |
| 5 mM ATP | 20.8 ± 0.9 | 5.2 ± 0.3 | 4.9 ± 1.3 | 5.9 ± 1.2 | 7.7 ± 1.4 |
| 1 mM Ado | 21.6 ± 0.6 | 6.9 ± 1.1 | 23.2 ± 1.0 | 30.1 ± 1.7 | 36.4 ± 2.1 |
| 5 mM Ado | 21.5 ± 1.0 | 8.6 ± 0.6 | 21.7 ± 1.4 | 30.3 ± 1.8 | 38.9 ± 2.4 |

the hormone in Peak 2 is dissociated from the receptor (42, 43), degraded in lysosomes (30, 38, 43), and eventually eliminated from the cells, via Route B (42, 43). However, if the lysosomal activity is inhibited by chloroquine, unmodified insulin would be accumulated in cells (30, 38), presumably in a specific type of lysosome (30). The internalized receptor, which has been uncoupled from insulin, is recycled back onto the cell surface without any modification via Route C (42, 43). The uncoupling of insulin from its receptor is facilitated by acidification of specific vesicles which may be functionally identical to CURLs (Compartments for Uncoupling of Receptor and Ligand) that were identified in the liver (43). The acidification of the vesicles, hence the dissociation of insulin from the receptor, is inhibited by monensin (29). As a result, insulin (presumably still bound to the receptor) is accumulated in monensin-treated adipocytes (29).

Based on these lines of background information, our present ATP data on the cellular processing of insulin can be interpreted as indicating that extracellular ATP may partially inhibit the binding of the hormone to the surface receptor and, at the same time, may strongly block the degradative pathway for the processing of insulin at a certain step between Peak 2 (PK2) and CURL in Fig. 7. With these two assumptions, one can readily explain the experimental data which indicate that in the presence of ATP (a) the binding of insulin to the cellular receptor is partially inhibited (Figs. 1 and 2 and Table III), (b) the cell-bound hormone is internalized normally (Table III), (c) the lysosomal degradation of insulin is minimized (Table IV), and (d) no detectable amount of extra insulin is accumulated in cells in the presence of monensin (Fig. 2) or chloroquine (Fig. 1).

The above interpretation is not applicable to the actions of

TABLE V

Effects of 1 mM adenosine and ATP on the physiologic actions of insulin

| Addition (1 mM) | Glucose transport | Glycogen synthase | Phosphodiesterase |
|-----------------|------------------|------------------|------------------|
|                 | Basal | Insulin | Basal | Insulin | Basal | Insulin |
| pmol/min/mg cells | pmol/min/mg | pmol/min/mg | pmol/min/mg |
| None           | 6.1 ± 0.7 | 5.3 ± 0.4 | 10.8 ± 0.2 | 72 ± 6 | 231 ± 5 |
| Ado            | 6.0 ± 0.7 | 7.0 ± 0.3 | 14.2 ± 0.4 | 72 ± 6 | 232 ± 8 |
| ATP            | 6.3 ± 0.5 | 6.6 ± 0.3 | 13.0 ± 0.5 | 133 ± 2 | 228 ± 3 |

* See "Materials and Methods."

a Average ± S.E. (n = 4-6).

b Significantly different from the control (p < 0.01).

c Significantly different from the control (p < 0.05).
adenosine; however. According to our data, adenosine (a) does not inhibit the binding of insulin to the surface receptor (Fig. 3) and (b) abolishes both chloroquine- and monensin-dependent accumulation of insulin (Figs. 1 and 2) without affecting the chloroquine-sensitive (lysosomal) degradation of the hormone (Table IV). The second part of these observations (b above) is difficult to delineate at the present stage of investigation; for lack of any other reasonable explanations, we tentatively suggest that adenosine may render the hormonal traffic irreversible. If the traffic is rendered irreversible, no excess insulin would accumulate in cells when the hormonal processing is blocked at certain steps (Figs. 1 and 2 and Table IV). This hypothetical mechanism may also explain a part of the effects of ATP since the latter at 1 mM does not completely inhibit the lysosomal degradation of insulin (Table IV). Another possibility is that the mechanism of intracellular processing of insulin might be more complex than it is now generally accepted and illustrated in Fig. 7, and different pathways of insulin processing might be affected differently by ATP and adenosine.

Physiologically, adenosine is a natural regulator of various metabolic activities (11–22). The agent is known to interact with the specific cellular receptor (12) and, among other things, inhibits adenylate cyclase (11) by a GTP-dependent process (16). In rat adipocytes, adenosine reduces the cellular level of cAMP (16–19), inhibits lipolysis (11), stimulates basal glucose transport (13), blocks the catecholamine-dependent inhibition of insulin-stimulated glucose transport (20–22), and stimulates phosphodiesterase (14, 15). However, these physiologic effects are typically elicited by the agent at relatively low concentrations, such as 10 nM–1 μM (11–22). This appears to be consistent with the data that the apparent K<sub>a</sub><sup>ad</sup> value for the interaction of adenosine with its cellular receptor is 0.65 μM (12). In contrast, the effects of adenosine considered in our present study are elicited by the agent when its concentration is 0.1–1 mM (Fig. 3). At these high concentrations, the aforementioned adenosine receptor should be saturated with the ligand. It is suggested, therefore, that the mechanism of actions of adenosine observed in our present study is distinct from that involved in the physiologic actions of the agent.

ATP in extracellular buffer is known to affect various metabolic activities when the nucleotide concentration is 1–10 mM (5–10). According to Sung et al. (10), ATP at 5–10 mM makes mouse macrophages leaky to metal ions and strongly inhibits their phagocytic activity. In our present studies, however, neither ATP nor adenosine had any significant effect on the endocytotic internalization of insulin (Fig. 5 and Table III). Extracellular ATP is also known (a) to inhibit insulin-stimulated glucose transport<sup>2</sup> (6, 7) and (b) to stimulate both basal and insulin-activated glycogen synthase (8).

<sup>2</sup>This does not necessarily mean that the basal and insulin-stimulated glucose transport activities have different responses to ATP. Although the expression, "insulin-stimulated," is widely used in literature, the exact terminology should be, "the insulin-dependent stimulation of glucose transport." In fact, ATP was considerably less inhibitory when administered to adipocytes that had already been stimulated with insulin (Y. Shibata and T. Kono, unpublished data).
as confirmed in our present work (Table V). However, in apparent disagreement with our present observation, the previous investigators reported that the effects of ATP on glycogen synthase were not mimicked by nonphosphorylating analogues of the nucleotide (8). Because the effects of ATP on glycogen synthase are mimicked by adenosine (Table V), we suggest that phosphorylation may not be involved in these actions of ATP. However, since ATP and adenosine further activate glycogen synthase that is maximally stimulated by insulin, the mechanism involved in the actions of ATP and adenosine on glycogen synthase may be different from that of insulin. The effect of ATP to stimulate basal phosphodiesterase is not additive to that of insulin; therefore, it is suggested that the two agents may stimulate the same species of phosphodiesterase. However, the mechanisms involved in the actions of ATP and insulin are apparently different since (a) the effect of ATP is transient while that of insulin is not (Fig. 6), and (b) the enzyme in cells exposed to ATP for 30 min is refractory to ATP, but not to insulin (Table VI). This stimulatory effect of ATP on phosphodiesterase must be considered carefully when attempts are made to reconstitute the hormonal effect on this enzyme by incubating broken cell preparations with ATP.

Since extracellular ATP does not inhibit the actions of insulin on glycogen synthase and phosphodiesterase (Table V), it may be suggested that the degradative pathway of insulin processing (namely, the pathway that appears to be largely blocked by extracellular ATP, see above) may not be involved in the generation of the (hypothesized) signal of insulin to these enzymes. This suggestion is consistent with our previous observations that (a) the actions of insulin are not significantly inhibited by either monensin or chloroquine (29,30), (b) insulin that is accumulated in chloroquine-treated adipocites has no physiological effects in situ (although the hormone is fully active if extracted from the cell and tested on fresh adipocytes), and (c) the hormone-receptor complex that is accumulated in monensin-treated adipocytes (presumably in CURLs) retained significant, but only very weak, physiologic activities in situ (29). These considerations imply that the hormonal signal may be generated and transmitted from the insulin-receptor complex associated with either the plasma membrane (Peak 1) or the slow sedimenting intracellular vesicles (Peak 2 in Fig. 5). Although it is generally assumed that the hormonal signal is transmitted from the insulin-receptor complex in the plasma membrane, the internalized hormone-receptor complex associated with the slow sedimenting vesicles could (also) be the signal generator since the formation of Peak 2 is rapid (39), ATP-dependent (2,30), and temperature-sensitive (2). Peak 2 is not an artifact created at the time of cell homogenization; instead, it is formed in intact cells from the hormone that has been bound to the surface receptor (30). In agreement with the hypothesis that internalization of insulin might be involved in its physiologic actions, it was recently reported by Jochen and Berhanu (45) that internalization of insulin and its action on glucose transport are both inhibited by certain peptides, and we previously observed that the above two processes are both inhibited by 1 mM bromophenacyl bromide, 3.3 mM phenylglyoxal, or 1 mM iodoacetamide. It should be noted, however, that there is no experimental evidence to indicate that the agents used in the above two studies might also inhibit the action of insulin at a step other than the internalization of the hormone.

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