The Labile Nature of the Insulin Signal(s) for the Stimulation of DNA Synthesis in Mouse Lens Epithelial and 3T3 Cells*

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A kinetic study was carried out to assess the stability of the intracellular signal(s) generated by insulin in quiescent cells for the stimulation of DNA synthesis. Using murine lens epithelial cells and Swiss 3T3 cells in culture, it was found that insulin stimulated DNA synthesis after a lag of 14.5 h. If, however, 6 h after the addition of insulin to the cells, the insulin-containing media were totally removed, followed by the addition of fresh media (even if insulin was returned to the medium within approximately 10 min), a 14.5-h lag still remained after insulin readdition before DNA synthesis started. In another set of experiments, the insulin was removed after 6 h by diluting its concentration approximately 60,000-fold. In this case, if insulin was at the diluted concentration for approximately 60 min before being added back, a full 14.5 h was necessary for the start of DNA synthesis. The half-time for loss of signal was 2 ± 1 min for total washout and 18.4 ± 0.5 min for the dilution experiment.

These results indicate that the intracellular signal(s) for DNA synthesis produced by the binding of insulin to its cellular receptor are extremely transitory in nature. The signal disappears at approximately the same rate that insulin dissociates from the receptor. Thus, insulin must be constantly binding to the membrane receptor in order to keep the key signal(s) at a high enough level for the cell to progress on to S phase. Early events, such as specific protein synthesis, changes in ion flux, changes in cellular metabolism, and changes in cellular pH, may be essential, but they are not sufficient to cause a cell to progress on to S phase. Addition of sodium vanadate to the cell is found to stabilize the messenger such that there is no loss of signal when insulin is removed. These data are consistent with the tyrosine-phosphorylated insulin receptor or a product of its action being the signal.

Insulin is well known as an anabolic hormone with many effects on metabolism in vivo, especially the events controlling glucose uptake and storage as glycogen. It has also been known for some time that insulin has a growth stimulatory effect in tissue culture (for review see Refs. 1 and 2). Initially, it was felt that the growth-promoting ability of insulin was due to either its stimulation of anabolic processes or its ability to bind weakly to receptors for multiplication stimulating activity (3, 4) or insulin-like growth factor-I (it does not bind to the receptor for insulin-like growth factor-II) (5). However, it has been shown that: 1) insulin will stimulate DNA synthesis in some cells at physiological concentrations (6–8), especially in the presence of other growth factors (9); 2) some cells require both insulin and somatomedins for optimal growth (10); 3) cell variants have been isolated that are insulin-dependent (11); and 4) cell mutants are found which don't have insulin-like growth factor-I receptors, and yet insulin will stimulate DNA synthesis and insulin-like growth factor-II will not if the insulin receptor is blocked (12). Thus, it is apparent that insulin has the potential to stimulate growth by binding to its own high affinity receptor.

The mechanism by which insulin regulates intracellular events is unclear. Recent evidence has pointed to the possible role of the phosphorylation of specific proteins, including the insulin receptor itself, as being possible intermediates (second messengers) in the control process (for review see Refs. 13 and 14).

The present study was carried out with the idea that an analysis of the kinetics of the insulin stimulation of DNA synthesis could help to reveal possible mechanisms of action and rule out others. The results from the lens epithelial cell and 3T3 cell systems are consistent with insulin stimulating DNA synthesis by a mechanism where insulin must bind continuously to its own receptor.

MATERIALS AND METHODS

Nakano mouse lens epithelial cells, explanted as previously described (9), were plated in 24-well plates at a concentration of 1.5 × 10⁵ cells/well, incubated for 48 h in RPMI 1640 with 5% fetal calf serum, washed, and then serum-starved for 48 h to bring them to quiescence. The cells were then washed, and 0.5 ml of media containing 0.5 mg/ml bovine serum albumin (fatty acid-free) was added. To this was added the insulin. For a typical experiment, insulin was added at 3 × 10⁻¹⁰ M final concentration. After a specified time, the insulin was removed by either 1) washing the well twice with media containing bovine serum albumin, or 2) diluting the insulin by adding 1.0 ml of media containing bovine serum albumin to the 0.5 ml in the well, followed by removing 1.0 ml from the well (this process was repeated 10 times). It was determined experimentally that 10 dilutions of this type resulted in an actual dilution of 34,133-fold. Insulin was then added back at the specified time. After the readdition of the insulin, 0.2 μCi of [3H]thymidine was incoated into the well. The total time for the experiment was 23 h. At the end of this time, the cells were washed twice with ice-cold 0.2 M NaCl, incubated at 5°C in 5% trichloroacetic acid for 20 min, washed twice with ice-cold trichloroacetic acid, rinsed with absolute alcohol, digested in 0.5 M NaOH, and an aliquot was removed for counting. The insulin used was Lilly Ultrapure crystalline porcine insulin (kindly supplied by R. Davis, Lilly). There was less than 0.6 zinc atom per molecule of insulin in this material.

The 3T3K cells (kindly supplied by Dr. E. A. Adelberg, Yale University) were plated at 2 × 10⁴ cells/well in 24-well plates in 45% Waymouth's medium (Flow), 45% minimum essential medium (Dulbecco's modification) (Flow), and 10% fetal calf serum. After 12 days of incubation without refeeding, the cells were washed and refed with serum-free medium containing the experimental reagents. These cells were then treated the same as the lens cells above.
For those experiments in which vanadate was used, the vanadate solutions were prepared as previously described using sodium orthovanadate (15). All experiments in this study were done at least twice and all points in each experiment were done in triplicate. The half-times were obtained by linear regression analysis of the data plotted as a first order reaction.

RESULTS

Stimulation of DNA Synthesis by Insulin—In order to determine the range of insulin’s ability to stimulate DNA synthesis with the Nakano len’s cells, a dose-response for insulin was carried out using serum-deprived cells. The response for insulin is shown in Fig. 1. Insulin has an effect at $10^{-9}$ M with a maximum effect at about $10^{-7}$ M. The half-maximal response was at about $2.5 \times 10^{-8}$ M. Thus, these cells are sensitive to insulin’s stimulation of DNA synthesis. The time course of insulin’s stimulation of DNA synthesis is seen in Fig. 2. By monitoring the effect of insulin with 2-h pulses of [3H]thymidine, it was possible to determine the timing of the insulin stimulation. In this case, insulin is added at different times with the [3H]thymidine added just 2 h before the finish of the experiment. Very little stimulation is seen in the first 11-13 h, with a maximum stimulation at about 22-23 h. A similar result was obtained by adding [3H]thymidine for the entire time of the experiment while adding insulin for different time periods (data not shown). This experiment is not capable of showing the maximum time for the insulin stimulation, but the data could be extrapolated to a minimum time for the initiation of DNA synthesis of about 14.5 h. Since there is very little stimulation of the cells before that time, in either experiment, it would appear that the serum deprivation technique results in few cells in the G1 to S phase of the cell cycle.

Insulin Removal—Different methods of removal of insulin from the media bathing the cells were tested in order to determine their effect on the stimulation of DNA synthesis by insulin (Fig. 3). For this experiment, insulin was added for 4 h followed by removal of the insulin for 1 h followed by readdition of insulin for 18 h. Results A, B, C, and D are the controls. Result A has no insulin and result B has insulin for only 4 h. Result C has insulin for only the last 18 h, and result D has insulin for the entire 23 h with no removal. Result B shows that the 4-h treatment with insulin results in very little stimulation above background (result A). Result C shows the amount of stimulation expected for an 18-h treatment with insulin. This would be the minimum amount of stimulation expected for the washout experiments where insulin is added back. This is the case for experiments E-L in which insulin was removed by different means after 4 h, insulin was then added back after 1 h, and the experiment was allowed to go for another 18 h. As can be seen in Fig. 3, the minimum stimulation was approached in results G and L. Result G is where the insulin is removed by washing the cells twice with media containing no insulin. This type of washing exposes the cells to air for a short period of time. This seems to cause a loss of signal by itself. This effect is seen in result F, where the wash solution contains insulin. The fact that F is less than D is likely to be a result of exposure of the cells to the atmosphere. To test for the effect of mechanical manipulation of the cells, the solution was pipetted up and down without exposing the cells to the atmosphere. This can be seen in result E, where the result is essentially the same as where there was no treatment (D). The other wash technique was actually not a wash but a dilution. In these experiments (H-
L), the 0.5 ml of media over the cells was diluted with 1 ml of media without insulin. The 1 ml was then removed, and the process was repeated for a specified number of times. Result H was 2 repeats, result I was 4 repeats, result J was 6 repeats, result K was 8 repeats, and result L was 10 repeats. Result L, which is approximately a 34,133-fold dilution (theoretical is 59,049), approached control levels.

A similar set of experiments were carried out where insulin was added for 4 h, washed out, but was not added back (Fig. 4). Result A is background. Result B is 4 h of insulin treatment followed by total removal of media with 2 washes. Results C through H are 4 h of insulin treatment, followed by dilution of the insulin solution. As can be seen, only the 10X wash (34,133-fold dilution) is close to that achieved with total removal of the media.

Insulin Treatment Followed by Removal—A series of experiments were carried out where insulin was added to the cells for various periods of time, the insulin was removed, and the cells were assayed for DNA synthesis after 23 h. These experiments were carried out using both the total washout and dilution techniques as the methods of removing the insulin. Similar results were obtained in both cases. The results using the dilution method (10X) can be seen in Fig. 5. Treatment with insulin for up to 5 h results in little stimulation of the cells. Also, treatment for as long as 11 h still resulted in less than 50% of the maximum stimulation.

Insulin Removal Time—Experiments were carried out where the time was varied between the removal of insulin and the time when insulin was added back. These results are seen in Figs. 6 and 7. The results in Fig. 6 are for the experiments where total removal of the media was used as the washing technique. This technique, which results in exposure of the cells to the atmosphere, yielded a half-time for the loss of signal in the cells of 2 ± 1 min. The cells were in the presence of insulin for 6 h minimum (the maximum time was 6 h, 57 min), before the insulin was removed. The half-time may actually be less than 2 min because those cells with the shorter washout times would be in the presence of insulin for a longer total time. This was necessary in order to maintain a total treatment time for the cells of 23 h. The results in Fig. 7 are for experiments where the insulin was removed by dilution.

![Fig. 4. Effect of the dilution technique for the removal of insulin (3 x 10^{-7} M) from the NMLE cells, as measured by their incorporation of [^{3}H]thymidine. All experiments were carried out for a total of 23 h and insulin was removed after 4 h for all insulin removal experiments. Insulin was not added back after removal. A, background; B, insulin removed by the washout technique; C, insulin not removed; D, insulin removed by 8-fold dilution; E, insulin removed by 65-fold dilution; F, insulin removed by 524-fold dilution; G, insulin removed by 4,232-fold dilution; H, insulin removed by 34,133-fold dilution.](image)

![Fig. 5. Time of insulin (3 x 10^{-2} M) addition to NMLE cells before removal by the washout technique versus percent maximum response to [^{3}H]thymidine incorporation. All experiments were for a total of 23 h.](image)

![Fig. 6. Time between removal (by the washout technique) and readdition of insulin (3 x 10^{-7} M) to NMLE cells versus [^{3}H]thymidine incorporation. Time of readdition of insulin was 7 h for each point. Insulin was added back for 16 h. Total time for the experiment was 23 h. For one point (C), insulin was removed and not added back.](image)

![Fig. 7. Time between removal (by dilution technique) and readdition of insulin (3 x 10^{-7} M) to NMLE cells versus [^{3}H] thymidine incorporation. The time of readdition of insulin was at 7 h for each point. Insulin was added back for 16 h. Total time for the experiment was 23 h. For one point (■), insulin was removed and not added back.](image)
In this case, the dilution factor was 34,133, and the cells were in the presence of insulin for 6 h minimum (the maximum time was 6 h, 54 min). The half-time for this experiment was 18.4 ± 0.5 min or less. Again, the half-time is probably less than 18.4 min because the cells with the shorter washout times would be in the presence of insulin for a longer total time and thus give a higher result than expected. The total treatment time for all cells was 23 h.

**Insulin Washout with 3T3 Cells**—In order to test another cell type for the persistence of the insulin signal, Swiss 3T3 cells were used. A time course experiment similar to that seen in Fig. 2 was carried out using the 3T3 cells (results not shown). The results were essentially the same as those found for the lens epithelial cells in that maximum stimulation was at about 22-23 h and the minimum time for the initiation of DNA synthesis was approximately 12.5 h. A study was then carried out to determine the rate of signal loss for the 3T3 cells using the total removal washout technique. These results are seen in Fig. 8. For this experiment, insulin was added to the cells for 5 h minimum (the maximum time was 5 h, 57 min). Insulin was then removed and then added back after a specific time. The shortest time possible using the total removal technique is 0.5 min. As seen in Fig. 7, the half-time is 2 ± 1 min. As was the case with the lens cells, the half-time may actually be shorter because the cells with the shorter washout time would be in the presence of insulin for a longer total time.

**Insulin plus Vanadate**—The results for insulin added in the presence of 1 × 10⁻⁵ M sodium orthovanadate can be seen in Fig. 9. Vanadate by itself at this concentration has a small effect on DNA synthesis when added for only the first 4 h of a 23-h experiment or for the whole 23 h. Insulin by itself for 23 h has a significant effect, but if added for only the first 4 h it has no effect (see also Figs. 4 and 5). However, when insulin is added with vanadate for only the first 4 h, a large stimulation is observed. Thus, insulin plus vanadate are synergetic when added for only 4 h while insulin by itself has no effect when added for only that amount of time.

**DISCUSSION**

The results presented here with two different cell types demonstrate that insulin must be continually present in the solution bathing a cell in order to stimulate it to progress from G₁/G₀ to S phase. In addition, it was shown that the effect of insulin and the presumed signal produced in the cell by insulin is rapidly lost upon removal of insulin. The loss of the presumed signal causes the cells to reset their internal clocks, thus requiring the full time to reach S phase upon readdition of insulin. Although insulin can stimulate growth of lens epithelial cells at low concentrations (less than 10⁻⁹ M, Fig. 1), and at even lower concentrations when in the presence of other growth factors (9), it has not been established that it is acting through its own receptor when it stimulates the growth of these cells. However, these results are certainly consistent with that mechanism. If insulin is acting through both its own receptor and the insulin growth factor-I receptor in this study, they must both be labile for a total loss of signal is seen.

Two different methods were used to remove insulin from the cells. One method was to remove the insulin from the cells by dilution. In this case, it was found that a dilution of approximately 34,133-fold was required to achieve a result that was similar to that found for a total restart of the progression of the cell through the cell cycle. Less dilution would result in a larger percentage of the cells maintaining their stimulated status. The amount of stimulation was consistent with the results obtained from the dose-response study of insulin with these cells. The second method was to totally remove all media bathing the cells and replace it twice with fresh media. This method resulted in the cells being exposed to air for a short period of time (approximately 15-20 s). This exposure results in a loss of signal, because when the cells were washed with insulin in the wash media, they still showed a decrease in the amount of DNA synthesized. This may explain the fact that the total removal method resulted in a much shorter half-life (2 ± 1 min) for the loss of the progression signal than the half-life obtained for the dilution technique (18.4 ± 0.5 min), even correcting for the fact that the total removal technique took less time to perform (3 min versus 0.5 min). It is interesting to note that the half-time for deactivation with the dilution technique of insulin removal is close to the value of 10 min obtained for the dissociation rate of insulin from its receptor (16). Thus, it appears that there may be a correlation between insulin receptor occupancy and stimulation of DNA synthesis.

The half-time for loss of signal is also similar to the half-time found for return of internalized insulin receptor to the surface.
after removal of insulin ($t_0 < 20$ min) (17). Thus, the present results would not rule out the second messenger being the internalized receptor.

Cells treated with insulin did not show stimulation of DNA synthesis if the insulin was removed unless the insulin was added for a long time. However, even after 8 h in the presence of insulin, if insulin was removed, over 80% of the cells required the full 14.5 h to reach S phase. It is unlikely that the cells which did progress after insulin removal were the result of incomplete arrest at the $G_1/G_0$ point in the cell cycle, since in the timing studies with continuous insulin treatment few cells were seen to be stimulated to reach S phase before 14.5 h. Thus, the cells are not committed to go on to S phase until insulin has been present for a long time. This would appear to indicate that an additional change must occur in the cells to lock them into this commitment, which did not depend upon the stability of the signal. What is interesting is that it takes so long for this change to occur. If insulin is added for 11 h and then removed, less than one-half of the cells can progress on to S phase. This change in the cell may be what determines the time required to go from $G_1/G_0$ to S. Thus, although many early events (synthesis of specific proteins, changes in ion flux, changes in cellular metabolism, changes in cellular pH, etc.) which may be necessary for progression to occur, they are not sufficient to cause the cell to progress on to S phase. One possibility is that this change might be a modification of the signal.

A possible candidate for the signal is the tyrosine-phosphorylated insulin receptor. This could fit into a scheme where insulin binds to its receptor leading to its phosphorylation at a specific tyrosine residue or the binding could cause the insulin receptor to catalyze the phosphorylation of another receptor. Recent evidence points to autophosphorylation as the mechanism (18); however, it could be a combination of these two events since more than one site is phosphorylated in vivo (14). A phosphatase present in the cell could at the same time catalyze the hydrolysis of the tyrosine phosphate. The main role of the phosphatase may be to remove phosphate from the receptor which might result from a non-insulin-stimulated reaction. The presence of this non-insulin-stimulated tyrosine phosphorylation might explain the ability of phosphatase inhibitors such as vanadate to stimulate DNA synthesis (15). This would be consistent with the finding that vanadate by itself is able to result in tyrosine phosphorylation with the same dose-response curve and time course as seen for its stimulation of DNA synthesis (19). In addition, vanadate treatment in the presence of insulin results in the increased phosphorylation of a protein the same size as the insulin receptor,1 and vanadate plus insulin are synergistic in the stimulation of DNA synthesis (19). Further results that are consistent with this scheme come from the present experiments. Treatment with vanadate plus insulin allowed the insulin signal to persist after removal of insulin. In this case, the vanadate concentration was so small that it gave very little signal. However, after waiting 4 h so that vanadate would have time to enter the cell, if insulin and any external vanadate are removed, the synergistic effect of vanadate plus insulin was unaffected. Thus, vanadate can protect the insulin signal, probably through its ability to inhibit the tyrosine phosphatase-specific phosphatase.

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