Ca<sup>2+</sup>/Calmodulin and Cyclic 3,5' Adenosine Monophosphate Control Movement of Secretory Granules through Protein Phosphorylation/Dephosphorylation in the Pancreatic β-Cell*

MITSURO HISATOMI, HIROYOSHI HIDAKA, AND ICHIRO NIKI

Second Department of Internal Medicine (M.H.), Department of Pharmacology (H.H., I.N.), Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466, Japan

ABSTRACT

We observed the movement of insulin granules in living transformed hamster pancreatic β-cells (HIT T15) with a light microscope, where secretory granules are moving in the cytoplasmic space. Velocity of the typical granule movement was approximately 1.5 μm/sec. A stimulatory concentration of glucose activated the movement of the secretory granules. Forskolin, an activator of adenylyl cyclase, increased the movement, resulting in changes in intracellular localization of the granules. Acetylcholine also activated the granule movement, whereas high K<sup>+</sup> and tolbutamide, which cause Ca<sup>2+</sup> influx through the voltage-dependent Ca<sup>2+</sup> channel, had only little effect. The movement was abolished by BAPTA, the intracellular Ca<sup>2+</sup>-chelator. Activation of protein kinase C by 12-O-tetradecanoyl-phorbol 13-acetate failed to affect this movement. The motile events were inhibited by the calmodulin antagonist, W-7, and dramatically increased by okadaic acid, an inhibitor of protein phosphatases 1 and 2A. These results suggest protein phosphorylation by Ca<sup>2+</sup>/calmodulin- and cAMP-dependent protein kinases play a positive role in the control of the insulin granule movements, which results in potentiation of insulin release from the pancreatic β-cell. (Endocrinology 137: 4644-4649, 1996)

**Materials and Methods**

HIT T15 β-cells (passage numbers 70–85), kindly donated by Prof. S. Seino (Chiba University School of Medicine, Chiba, Japan), were cultured at 37 C in a humidified atmosphere of 95% air/5% CO<sub>2</sub> in Ham’s F-12K medium with kanamycin (300 μg/ml) and 10% FCS. The cells were passaged weekly and harvested by using trypsin/EDTA, and culture medium was replaced every other day. For morphological experiments, HIT cells were seeded onto a glass-bottom tissue culture dish (35-mm diameter, Meridian Instruments Far East, Tokyo, Japan) at a density of 1–5 × 10<sup>5</sup> cells/dish. The culture medium was replaced with HEPES-Krebs solution containing (mM): NaCl 119, glucose 5.5, KCl 5, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 1, NaHCO<sub>3</sub> 25, NaH<sub>2</sub>PO<sub>4</sub> 1, and 10% FCS in Ham’s F-12K medium with kanamycin (300 μg/ml) and 10% FCS. The cells were passaged weekly and harvested by using trypsin/EDTA, and culture medium was replaced every other day. For morphological experiments, HIT cells were seeded onto a glass-bottom tissue culture dish (35-mm diameter, Meridian Instruments Far East, Tokyo, Japan) at a density of 1–5 × 10<sup>5</sup> cells/dish. The culture medium was replaced with HEPES-Krebs solution containing (mM): NaCl 119,
**Results**

**Insulin granules in living HIT cells**

Observation of HIT T15 cells under phase-contrast microscopy revealed that HIT cells were equipped with many small, round granules with $0.75 \pm 0.01 \mu m$ in a diameter ($n = 100$ from 7 experiments). This value is slightly larger than that for insulin granules in islet $\beta$-cells assessed by electron microscopy; $0.3-0.6 \mu m$ (9) or $0.4-0.5 \mu m$ (10) and is comparable to that in living islet $\beta$-cells under the video-enhanced and differential interference contrast microscopy; $0.5-1.2 \mu m$ (5). In this study, identification of insulin granules in living $\beta$-cells was performed by the quinacrine fluorescence study. The dye is accumulated in a low pH condition, such as secretory granules and lysosomes, presumably via protonation (11), and has been demonstrated to accumulate in fixed or living $\beta$-cells (12, 13). As shown in Fig. 1A and B, the intracellular distribution of quinacrine fluorescence was almost coincident with the small granules and lysosomes in the image of the phase-contrast microscopy. Lysosomes were readily distinguished because they are much bigger than secretory granules and lacks in motility. The fluorescent granules demonstrated a heterogeneous distribution in the cytoplasmic area. The magnitude of granule movement varied in individual cells. In some cells, most of the granules were almost still, and in others, those were moving. When we traced the movement for a longer period, we noticed that the granules cannot go into a certain area of the intracellular space. However, the observation did not allow us to identify a fixed route or direction for the granule movement under low temperature (27°C) 16 mm. Okadaic acid (2 μM) 17 μM. Acetylcholine (100 μM) 18 μM. Tolbutamide (100 μM) 19 μM. Glucose (20 mM) 20 mm. W-7 (10 μM, 15 min) 21 min. Forskolin (0.5 μM) 22 μM. dbcAMP (2 mM) 23 mM. TPA (162 mM) 24 μM. Okadaic acid (2 μM) 25 μM. BAPTA/AM (20 μM, 20 min) 26 μM. Glucose (20 mM) 27 μM. Forskolin (0.5 μM) 28 μM. Low temperature (27°C) 29 μM. After preincubation for 2 h in the absence of glucose, HIT T15 cells were incubated under conditions described above. All the experiments were carried out at 37°C unless mentioned. Intracellular movement of the secretory granules in HIT T15 cells were assessed as described in Materials and Methods. For control, the granule movement was measured for 30 sec 3 min before the condition was changed. The movement in the identical square in the microscopic field was reassessed 2-3 min after the challenge unless mentioned. Data are expressed as the mean ± SEM.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Granule movement</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (0.1 mM)</td>
<td>21</td>
<td>3.00 ± 0.48</td>
<td>8.3</td>
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<tr>
<td>Glucose (20 mM)</td>
<td>34</td>
<td>2.53 ± 0.41</td>
<td>45.5</td>
</tr>
<tr>
<td>Tolbutamide (100 μM)</td>
<td>24</td>
<td>3.08 ± 0.46</td>
<td>6.8</td>
</tr>
<tr>
<td>K⁺ (50 mM)</td>
<td>22</td>
<td>2.36 ± 0.37</td>
<td>5.9</td>
</tr>
<tr>
<td>Acetylcholine (100 μM)</td>
<td>32</td>
<td>2.56 ± 0.38</td>
<td>46.5</td>
</tr>
<tr>
<td>Acetylcholine (100 μM) + nifedipine (2 μM)</td>
<td>23</td>
<td>1.22 ± 0.23</td>
<td>82.0</td>
</tr>
<tr>
<td>W-7 (10 μM, 15 min)</td>
<td>25</td>
<td>2.56 ± 0.40</td>
<td>39.1</td>
</tr>
<tr>
<td>W-7 (10 μM, 15 min) + forskolin (0.5 μM)</td>
<td>28</td>
<td>2.25 ± 0.27</td>
<td>1.7</td>
</tr>
<tr>
<td>Forskolin (0.5 μM)</td>
<td>23</td>
<td>3.13 ± 0.64</td>
<td>33.2</td>
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<tr>
<td>dbcAMP (2 mM)</td>
<td>26</td>
<td>3.04 ± 0.55</td>
<td>49.3</td>
</tr>
<tr>
<td>TPA (162 mM)</td>
<td>28</td>
<td>2.32 ± 0.40</td>
<td>-13.8</td>
</tr>
<tr>
<td>Okadaic acid (2 μM)</td>
<td>28</td>
<td>1.29 ± 0.26</td>
<td>185.3</td>
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<tr>
<td>BAPTA/AM (20 μM, 20 min) + glucose (20 mM)</td>
<td>25</td>
<td>2.60 ± 0.88</td>
<td>d</td>
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<tr>
<td>Forskolin (0.5 μM) + glucose (20 mM)</td>
<td>42</td>
<td>2.64 ± 0.32</td>
<td>d</td>
</tr>
<tr>
<td>Low temperature (27°C)</td>
<td>16</td>
<td>3.75 ± 0.44</td>
<td>-40.0</td>
</tr>
</tbody>
</table>

After preincubation for 2 h in the absence of glucose, HIT T15 cells were incubated under conditions described above. All the experiments were carried out at 37°C unless mentioned. Intracellular movement of the secretory granules in HIT T15 cells were assessed as described in Materials and Methods. For control, the granule movement was measured for 30 sec 3 min before the condition was changed. The movement in the identical square in the microscopic field was reassessed 2-3 min after the challenge unless mentioned. Data are expressed as the mean ± SEM.

a $P < 0.05$, b $P < 0.01$ vs. control value assessed by paired $t$ test.

*Movement of the granules was assessed after BAPTA treatment for 20 min.

*The movement was nullified by the BAPTA treatment.

*Glucose or forskolin was further added to the medium after 20 min treatment with BAPTA. The movement was measured 2 min after the addition.
unstimulated conditions. Two distinct types of the movement were observed. The granules were migrating with short amplitude (~1.5 μm), and occasionally we could trace the movement for a longer distance (5–10 μm). The velocity of the latter movement was approximately 1.5 μm per second (129 mm/day), which is equivalent to those in the normal β-cell (4) and for the fast anterograde axonal transport in neural cells; ~100-400 mm/day (14). However, the value in these studies must be underestimated because it is unable to follow the movement along the z-axis. The small movement did not allow us to measure its velocity. We believe that it does not result from Brownian nature as suggested in the islet cell by Somers et al. (15) because the granules became completely still when Ca²⁺ was chelated by BAPTA (see Table 1).

Granule movement with a longer distance was observed only in 30–40% of HIT cells, which may occur because of the heterogeneity of the secretory sensitivity in the individual β-cells.

Motile events of insulin granules in HIT β-cells under glucose stimulation

When assessed by counting numbers of the granules that move into or out of a 3.5 x 3.5 μm square per 30 sec, the granule movement was obviously increased by a stimulatory concentration (20 mM) of glucose (Fig. 2A, B and Table 1). The velocity of the fast movement after glucose stimulation was approximately 1.3–1.5 μm per second (from four independent experiments). It was, therefore, suggested that the number, but not the velocity of the moving granules, was increased by glucose stimulation. We consider that the activation of the movement by glucose may result from a shift of the rapidly moving granules from the other population with small movements. The movement by glucose was nullified when the β-cell was pretreated by the intracellular Ca²⁺ chelator BAPTA/AM at 20 μM for 20 min. A substimulatory concentration (0.1
mM) of glucose did not cause any detectable change in the movement (Table 1).

**Ca^{2+} and the motile events in the pancreatic β-cell**

The insulinotropic sulphonylurea, tolbutamide, did not have a significant effect on the movement. Depolarization by high K+ (50 mM) also failed to change the frequency. Acetylcholine at 100 μM had a significant effect to increase the movement (Fig. 3 and Table 1). The increasing effect of acetylcholine was not influenced or rather increased by simultaneous addition of 2 μM nifedipine, a blocker of the voltage-dependent Ca^{2+} channel (Table 1).

W-7, the calmodulin antagonist (16), inhibited the movement in unstimulated cells at 10 μM (Fig. 4A). W-5, the negative control compound of W-7 (17), did not affect the movement under the parallel condition. When the extracellular solution was depleted of Ca^{2+}, a gradual decrease in the granule movement was observed. The granules were in motion even 20 min after the Ca^{2+} depletion, which is at variance with the early observation using islet β-cells reported by Lacy et al. (4).

**Effects of substances which modulate insulin release on insulin granule movement**

Forskolin (5 μM), an activator of adenylate cyclase and a strong potentiator of insulin release, remarkably increased the frequency of the motile movement (Fig. 5A). The frequency was also increased by the membrane-permeable cAMP analogue, dbcAMP at 2 mM. In a few cases, we found that these granules were directed to a certain part of the β-cell after the addition of forskolin (Fig. 5B, lower right panel), whereas such directed movement was not detected before the addition (Fig. 5B, lower left panel). After the 3-min incubation with 5 μM forskolin, the granules eventually gathered in a right lower portion of the cell (Fig. 5B, upper right panel). Such redistribution of the granules was reproduced by dbcAMP (data not shown). Forskolin, however, did not cause any changes when added after pretreatment with 20 μM BAPTA/AM for 20 min (Table 1). Okadaic acid (2 μM), which inhibits protein phosphatases 1 and 2A, had an enormous increasing effect on the granule movement (Fig. 6 and Table 1). TPA at 162 nM, which increases insulin secretion via activating protein kinase C, failed to affect the granule movement in these experiments (Table 1).

**Discussion**

The present study leads us to suggest that protein phosphorylation and dephosphorylation may control secretory granule movement in the pancreatic β-cell. Glucose, which causes a dramatic change in the pattern of β-cell phosphoproteins (19), evoked a moderate increase in granule movement of the pancreatic β-cell. Because the hexose activates
after 2 h incubation in HEPES/Krebs buffer, forskolin (5 μM) was added to the buffer and the granule movement before and after the addition of forskolin was observed. Frequency after the addition of forskolin was plotted against that before the stimulation. B, Translocation of the insulin granules in a HIT β-cell by forskolin. Images before (left panels) and after (right panels) the addition of forskolin are shown. In the bottom panels, the granules were traced for 5 sec (arrows) by Argus-20. Note that the granules are directed to the right lower portion of the cell after forskolin stimulation.

several different second messenger systems (1), we tried to investigate the second messenger system(s) involved in the control of the granule movement using various pharmacological substances. Treatment with the intracellular Ca²⁺ chelator BAPTA abolished the granule movement, which was not overcome by further addition of glucose. This indicates that the granule movement is dependent on intracellular Ca²⁺. Inhibition by W-7 of the movement suggests the motile events may be controlled by the Ca²⁺-calmodulin system. It is interesting that acetylcholine had a prominent effect on the granule movement, whereas the movement was not much affected by high K⁺ or tolbutamide, both of which cause Ca²⁺ influx through the voltage-dependent Ca²⁺ channel as a result of membrane depolarization. Therefore, Ca²⁺ mobilization seems to be more responsible than Ca²⁺ influx for activation of the motile events. This happens presumably because of the heterogeneous distribution of intracellular Ca²⁺ maintained by buffering action of the cytosol on the free Ca²⁺ concentration (19). Thus, translocated vesicles may be prepared to be released from the β-cell by Ca²⁺ influx. This idea may explain the difference in the magnitude and time course of insulin release by Ca²⁺ influx and Ca²⁺ mobilization: Ca²⁺ influx causes an acute and short-lived release, whereas Ca²⁺ mobilization enhances insulin release without eliciting the secretory events by itself. Moreover, this could be one of the reasons why glucose, which causes both Ca²⁺ influx and Ca²⁺ mobilization, evokes insulin release in a biphasic manner. It is also possible that the change by acetylcholine may result from its effect on the intracellular concentration/distribution of ATP because there is evidence suggesting that intracellular concentrations of ATP and Ca²⁺ are related in this cell line (20).

Forskolin had a strong effect on the secretory granule movement in this study. Activation of the cAMP-dependent system enhances, but does not evoke, insulin release from the islet β-cell (3), although HIT cells are reported to be stimulated by cAMP-relating agents as forskolin in the absence of any secretagogues (7). Directed movement by cAMP (Fig. 5B) generates heterogeneous distribution of the granules, resulting in localization of the granules to a hot region (6) for an advantageous rearrangement for exocytosis. Because forskolin failed to influence the movement when the intracellular Ca²⁺ was chelated using BAPTA/AM, substimulatory concentrations of Ca²⁺ are definitely required for the movement, and cAMP potentiates the event. In the present study, we found limited numbers of the cell showed the typical movement of the granules. It was, however, reported that many particles were moving in monolayer culture of the islet cells (15). This may result from the simultaneous presence of a
cAMP-producing hormone, glucagon secreted from the concomitant pancreatic α-cell in their preparations. That could explain why FACs-purified pancreatic β-cells dramatically restore its secretory response by reaggregating them with purified α-cells (21). Involvement of protein phosphorylation in the control of the granule movement is further supported by the finding that okadaic acid, the inhibitor of protein phosphatases 1 and 2A, also dramatically enhanced the movement, although the inhibitor suppressed insulin release from the pancreatic islets (22). It must be noted that TPA, an activator of protein kinase C, has little effect on the movement, although TPA as well as forskolin enhances insulin output (2). It may be suggested that protein kinase C phosphorylates (a) distinct substrate(s) from those for protein kinase A in the secretory machinery as suggested in synaptic membranes (23). This may explain that simultaneous activation of protein kinase A and C results in a vast synergistic enhancement of insulin release from the pancreatic β-cell (2, 24). Little effect of TPA also reconfirms the idea that the increasing effect of acetylcholine was derived from Ca²⁺ mobilization by IP₃ production rather than activation of protein kinase C by diacylglycerol.

The one or more mechanisms that underlie this facilitation by Ca²⁺/calmodulin are still missing. It has been reported that at least two calmodulin-dependent protein kinases, calmodulin-dependent kinase II and myosin light chain kinase (MLCK), may be involved in the control of insulin secretion (25, 26). Recently, we found that monoclonal antibodies against MLCK attenuated Ca²⁺- or GTP-γS-induced insulin release from permeabilized rat islets (submitted for publication). We suggested that MLCK may act on a proximal step, presumably on the granule translocation, rather than on the final step of the secretory events. Furthermore, it has been reported that phosphorylated MLC is dephosphorylated by type 1 protein phosphatase (27) and okadaic acid inhibits dephosphorylation of this protein (28). Taking the present findings and other reports into our consideration, it could be suggested that MLCK activated by released Ca²⁺ from intracellular Ca²⁺ stores enhances insulin release via activation of the secretory granule movement. It may be also suggested that protein kinase A enhances insulin release partly via potentiating the movement of secretory granules.

Acknowledgments

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References