Regulation of Ca\textsuperscript{2+} Transport by Isolated Organelles of a Rat Insulinoma

STUDIES WITH ENDOPLASMIC RETICULUM AND SECRETORY GRANULES*

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The regulation of extramicrosomal Ca\textsuperscript{2+} concentration maintained by suspensions of rat insulinoma microsomes was studied using Ca\textsuperscript{2+}-selective minielectrodes. The Ca\textsuperscript{2+}-transporting activity was MgATP dependent and correlated with the endoplasmic reticulum marker NADPH-cytochrome c reductase. When incubated in a high KCl medium containing Mg\textsuperscript{2+} and phosphate, the microsomes lowered [Ca\textsuperscript{2+}] within less than 10 min to around 0.2 μM. They had a high Ca\textsuperscript{2+}-seques
tering activity since they were able to take up and retain several small Ca\textsuperscript{2+} additions. No evidence for a Na\textsuperscript{+}/Ca\textsuperscript{2+} countertransport was obtained. The accumulated Ca\textsuperscript{2+} was released by the Ca\textsuperscript{2+} ionophore A23187 or upon transforming ATP into ADP using glucose plus hexokinase. The addition of ADP, at concentrations present in cells, resulted in a dose-dependent and reversible net Ca\textsuperscript{2+} efflux from the microsomes until a higher [Ca\textsuperscript{2+}] steady state was reached. This was specific for ADP since GDP, UDP, CDP, IDP, and the nonhydrolyzable analogue methylene-ADP as well as AMP and cAMP did not reproduce the effect.

Insulin secretory granules were unable to lower medium [Ca\textsuperscript{2+}] or to take up a pulse addition of Ca\textsuperscript{2+}. However, most of the large granular calcium content was released by A23187. The addition of Na\textsuperscript{+} and lowering or increasing medium pH by 0.2 pH unit did not induce Ca\textsuperscript{2+} uptake or efflux from the secretory granules.

The results indicate that insulinoma endoplasmic reticulum but not insulin secretory granules may play a critical role in the regulation of cytosolic Ca\textsuperscript{2+}. A variation in cellular ADP content following secretagogue addition might modulate Ca\textsuperscript{2+} fluxes across the endoplasmic reticulum and contribute in raising cytosolic Ca\textsuperscript{2+}.

The level of cytosolic free Ca\textsuperscript{2+} concentration is thought to be of critical importance in the action of many secretagogues inducing insulin release in the endocrine pancreas (1). Although an increased cytosolic Ca\textsuperscript{2+} concentration may not necessarily be a prerequisite for the action of all secretagogues, a correspondence between the level of cytosolic free Ca\textsuperscript{2+} and the action of some agents inducing hormone release has recently been documented with the fluorescent dye quin 2 in the insulin-secreting RINm5F cell line (2). The action of cytosolic Ca\textsuperscript{2+} in insulin secretion requires a precise regulation by transport systems located not only in the plasma membrane but probably also in membranes of mitochondria, endoplasmic reticulum, and perhaps secretory granules (1, 3). There is now much interest in measuring quantitatively the ability of isolated organelles to maintain Ca\textsuperscript{2+} steady states in the range of intracellular levels of free Ca\textsuperscript{2+}. Thus, as a first step in the understanding of Ca\textsuperscript{2+} handling by intracellular organelles of an insulin-secreting tissue, we have characterized the Ca\textsuperscript{2+} transport by rat insulinoma mitochondria (4). We found that mitochondria were able to buffer the ambient medium free Ca\textsuperscript{2+} around 0.8 μM in a medium simu
lating the composition of the cytosol with respect to the main ions (4). Evidence is now accumulating which indicates that another organelle, the endoplasmic reticulum of noncontractile tissues (5-8), has the ability to take up and release Ca\textsuperscript{2+} as observed in the sarcoplasmic reticulum of muscle tissues (3). We report in the present study that microsomes isolated from a transplanted rat insulinoma have a high, MgATP-dependent, Ca\textsuperscript{2+} transport activity which is associated with the endoplasmic reticulum. They are able to lower medium [Ca\textsuperscript{2+}] to approximately 0.2 μM, a value close to cytosolic Ca\textsuperscript{2+} concentrations. This Ca\textsuperscript{2+} steady state is considerably lower than the one observed with mitochondria under similar experimental conditions. We demonstrate furthermore that ADP at concentrations present in cells is able to release Ca\textsuperscript{2+} from the microsomes.

EXPERIMENTAL PROCEDURES

Insulinoma Fractionation—The tumor and the rat strain (NEDH) used were originally supplied by Dr. W. L. Chick, University of Massachusetts, Worcester, MA. Insulinomas were obtained from subcutaneously transplanted animals as described previously (9, 10). 7-12 tumors were removed from ether-anesthetized rats, dissected, and homogenized as described in a previous report (4) in ice-cold homogenization medium containing 0.25 M sucrose, 0.25 mM EGTA, 0.4 mg/ml of bovine serum albumin, and 5 mM Hepes that was adjusted to pH 7.4 with KOH. The homogenate was centrifuged for 5 min at 1,400 g in a Beckman type JA20 rotor (Beckman Instruments, Palo Alto, CA) which was used for all subsequent centrifugations. The resultant pellet consisting of unbroken cells and nuclei was rehomogenized in 3 ml of the same sucrose medium and centrifuged for 5 min at 1,400 g. The combined supernatants thus obtained were filtered through a nylon mesh and used for both microsomes and secretory granule isolation. For the isolation of microsomes, the post-nuclear supernatant was centrifuged at 9,800 g for 10 min to sediment mitochondria. The resulting supernatant was centrifuged at 24,000 g for 20 min to eliminate some remaining mitochondria and the bulk of secretory granules. Microsomes were then obtained by centrifugation of the post-mitochondrial and post-secretory granule supernatant fraction at 100,000 g for 60

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† The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Ca\(^{2+}\) Transport by Insulinoma Endoplasmic Reticulum

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min in a Beckman ultracentrifuge using an SW 50.1 rotor. The supernatant medium from this final centrifugation was discarded.

The microsomal pellet (around 6 mg of protein) was gently washed three times with a medium containing 0.25 M sucrose and 5 mM Hepes (pH 7.0) (sucrose medium) in order to remove the EGTA present and finally resuspended in 0.1 ml of sucrose medium, kept on ice, and used immediately for Ca\(^{2+}\) transport experiments. In four separate experiments, the microsomal fraction (around 10 mg of protein) was further fractionated on a sucrose density gradient to obtain several fractions enriched either in endoplasmic reticulum or separate experiments, the microsomal fraction (around 10 mg of protein) was used for Ca\(^{2+}\) transport analysis.

For the isolation of insulin secretory granules, the post-nuclear supernatant was centrifuged at 23,700 × g for 15 min. The resultant pellet was resuspended in 1.5 ml of homogenization medium. Three aliquots of 0.5 ml of this crude fraction were gently layered onto three preformed gradients from a solution of 30% Percoll (v/v), 0.27 M sucrose, 0.25 mM EGTA, and 5 mM Hepes at pH 7.0. Gradients were preformed by a brief high-speed centrifugation of 9 ml of the Percoll solution for 5 min at 22,000 × g in 10-ml polycarbonate tubes (4).

After the layering onto the gradients, the tubes underwent low-speed centrifugation for 50 min at 8,700 × g. As the top of the gradient contained the bulk of the mitochondria plus microsomes, while the bottom of the gradient contained insulin secretory granules plus some contaminating dense mitochondria, only the bottom part of the gradient (around 2 ml) was collected from the three gradient tubes. This was diluted with 10 volumes of the 0.25 M sucrose medium and centrifuged at 23,700 × g for 15 min. The secretory granule pellet was subsequently washed twice with the same medium by centrifugation at 23,700 × g for 15 min to remove Percoll, EGTA, and contaminating organelles. The final secretory granule pellet (around 1 mg of protein) was resuspended in 0.1 ml of the sucrose medium, kept on ice, and used immediately for experiments.

Analysis of Fractions—Aliquots of fractions to be assayed for constituents were frozen in liquid nitrogen and stored at −70 °C for no longer than 5 days. Cytochrome c oxidase was assayed according to Ref. 11. NADPH-cytochrome c reductase was assayed as described previously (12). 5′-Nucleotidase was measured by the technique of Avruch and Wallach (13). Immunoreactive insulin was assayed according to a method previously described, using rat insulin as standard (14). Proteins were determined by the Coomassie Blue method using a commercially available kit (Bio-Rad Laboratories, A. G., Glattbrugg, Switzerland).

Measurements of Free Ca\(^{2+}\) Concentration and Incubation of Microsomes and Secretory Granules—The medium free Ca\(^{2+}\) concentration was measured with a Ca\(^{2+}\)-selective electrode. The experimental setup and the manufacture and calibration of the Ca\(^{2+}\)-electrodes have been described in a previous report (4). All the compounds tested, including the Ca\(^{2+}\)-ionophore A23187, did not interfere with the Ca\(^{2+}\)-electrode. The Ca\(^{2+}\)-electrodes were periodically rechecked for sensitivity, and response time and to maintain optimal conditions they were changed after three to four traces. The traces shown in the figures are representative experiments which have been repeated 2–10 times.

Materials—Analytical grade chemicals were obtained from Sigma (Munich, Federal Republic of Germany), Fluka A. G. (Buchs, Switzerland), and Merck A. G. (Darmstadt, F. R. G.). Bovine serum albumin (fraction V) from Sigma was charcoal treated and dialyzed as described previously (15). Percoll was obtained from Pharmacia (Uppsala, Sweden).

RESULTS

Isolation and Characterization of Microsomes and Insulin Secretory Granules—The microsomal fraction from rat insulinoma was enriched about 3-fold in the endoplasmic reticulum marker enzyme (NADPH-cytochrome c reductase) but contained some contamination by the plasma membrane marker (5′-nucleotidase) (Table I). The mitochondrial enzyme marker (cytochrome c oxidase) was virtually absent from the microsomal fraction. Some insulin secretory granules were still present in this fraction as judged by its insulin content. However, some of this insulin represents immunoreactive forms of the hormone normally present in endoplasmic reticulum and Golgi elements.

Electron microscopic observations (not shown) indicated that this fraction was composed primarily of small rough endoplasmic reticulum vesicles with some free ribosomes, smooth vesicles, and a few secretory granules. Mitochondria were virtually absent.

Various attempts to isolate insulinoma secretory granules by differential centrifugation only were found unsatisfactory due to the mitochondria and the microsomes that tend to sediment with insulin granules over a wide range of speeds. A density gradient step was, therefore, found to be necessary to eliminate most contaminating mitochondria and microsomes.

The secretory granule fraction from rat insulinoma was enriched about 11-fold in the granule marker insulin (Table I). The endoplasmic reticulum marker (NADPH-cytochrome c reductase) was markedly reduced in this fraction compared to the homogenate, while there was still some mitochondrial contamination as revealed by the marker cytochrome c oxidase. We refrained from further purification in order not to lose granule preservation by prolonging preparation times. It should be added that when considering the purpose of the present work, namely Ca\(^{2+}\) transport measurements, mitochondria can be easily rendered inoperative simply by carrying out incubations without substrates or by supplementing the medium with antimycin or ruthenium red.

Electron microscopic observations (not shown) confirmed the biochemical measurements, namely a high enrichment in well-preserved secretory granules. Most granules closely resembled those observed in normal B-cells, while some were atypical with a denser halo than usually observed surrounding the central core. This fraction was slightly contaminated by mitochondria and lysosomes. Rough endoplasmic reticulum was virtually absent. Taken together, these data indicate that the organelles were preserved following isolation and displayed an acceptable degree of purity.

Ambient Free [Ca\(^{2+}\)]\(^{2+}\) Maintained by Insulinoma Microsomes—Ca\(^{2+}\) Sequestration by the microsomes started immediately after their addition to the medium and resulted in a decrease in ambient free Ca\(^{2+}\) concentration. When incubated in a high KCl medium containing Mg\(^{2+}\), phosphate, MgATP, and an ATP-regenerating system, the microsomes lowered [Ca\(^{2+}\)] within 6 min to 0.1–0.2 μM (Fig. 1). They were not easily saturated with Ca\(^{2+}\) since they could rapidly take up several sequential pulse additions of Ca\(^{2+}\). Saturation was reached at about 100 nmol of Ca\(^{2+}\)/mg of protein. It should be added that following their isolation, insulinoma microsomes had a calcium content of 28 ± 5 nmol/mg of protein (mean of four separate preparations ± S.E.) as determined by atomic absorption. The Ca\(^{2+}\) accumulated could be rapidly released upon addition of the Ca\(^{2+}\) ionophore A23187 by lowering the ATP present in the medium by the combined
The data are the mean ± SE of three separate preparations. Values represent nanomoles of cytochrome c reduced per min·mg of protein, micromoles of adenosine formed per h·mg of protein, micrograms of immunoreactive insulin/mg of protein, and nanomoles of cytochrome c oxidized per min·mg of protein. Numbers in parentheses are the final recoveries (in per cent) from the homogenate. ND, not determined. The fraction considered as the homogenate is the postnuclear and cellular debris supernatant.

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>NADPH-cytochrome c reductase</th>
<th>S'-Nucleotidase</th>
<th>Insulin</th>
<th>Cytochrome c oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>27.2 ± 0.6</td>
<td>1.79 ± 0.77</td>
<td>21.9 ± 3.4</td>
<td>301 ± 41</td>
</tr>
<tr>
<td>Secretory</td>
<td>70.6 ± 10.9</td>
<td>6.49 ± 0.58</td>
<td>28.7 ± 4.6</td>
<td>3.9 ± 1.2</td>
</tr>
<tr>
<td>granules</td>
<td>7.9 ± 2.0</td>
<td>(21 ± 1)</td>
<td>(7.2 ± 0.6)</td>
<td>(0.67 ± 0.02)</td>
</tr>
<tr>
<td></td>
<td>(0.65 ± 0.15)</td>
<td></td>
<td>(0.7 ± 2)</td>
<td>(1.2 ± 0.4)</td>
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FIG. 1. Extramicrosomal ambient free Ca\(^{2+}\) concentration maintained by insulinoma microsomes. Microsomes were incubated at 30°C and pH 7.0 in 200 μl of a buffer containing 110 mM KCl, 2 mM KH₂PO₄, 25 mM Hepes, 1 mM MgCl₂, 2 mM MgATP, 2 mM creatine phosphate, 50 μM of creatine kinase, 0.2 μM antimycin, 1 μg/ml of oligomycin, and 0.5 mg/ml of bovine serum albumin. Where indicated, CaCl₂ (5 nmol/mg of protein) or the calcium ionophore A23187 (1 μg/ml) were added to the incubation medium. Hk denotes the combined addition of hexokinase (20 units/ml) and glucose (10 mM). Mc represents the addition of microsomes (final concentration, 0.5 mg of protein/ml).

The Ca\(^{2+}\)—sequestering activity was MgATP dependent. Other nucleotides (UTP, GTP, CTP, ITP, or the nonhydrolyzable ATP analogue P₂,β-methylene-ATP) could not sustain Ca\(^{2+}\) transport, even in the presence of Mg\(^{2+}\). Ca\(^{2+}\) uptake was not inhibited by mitochondrial inhibitors such as antimycin (0.2 μM), oligomycin (1 μg/ml), or ruthenium red (2 μM).

Experiments were carried out in order to determine whether insulinoma microsomes were sensitive to Na\(^{+}\) or to small variations in pH. When added at about 0.2 μM Ca\(^{2+}\), NaCl (10–50 mM) did not modify the ambient [Ca\(^{2+}\)] maintained by the microsomes. In another series of experiments, both pH and free Ca\(^{2+}\) were measured simultaneously with minielectrodes (4). When small aliquots of HCl or NaOH were added at about 0.2 μM Ca\(^{2+}\), respectively, lowering or raising medium pH by 0.1 pH unit, the ambient [Ca\(^{2+}\)] maintained by the microsomes was barely affected (a variation of less than 0.02 μM Ca\(^{2+}\)).

**Fig. 2.** Reversible increase in extramicrosomal ambient free [Ca\(^{2+}\)] maintained by insulinoma microsomes upon ADP addition to the medium. Microsomes were incubated as described in the legend to Fig. 1 in a medium containing an ATP-regenerating system only 1 mM creatine phosphate plus creatine kinase. Where indicated, MgATP (1 mM) or CaCl₂ (10 nmol/mg of protein) was added. ADP represents the addition of 2 mM K₂ADP plus 2 mM MgCl₂. CP denotes the addition of 2 mM creatine phosphate. Hk indicates the combined addition of hexokinase (20 units/ml) and glucose (10 mM).
regenerating system. To examine the reversibility of this effect, an amount of creatine phosphate (2 mM) was added in excess of the ADP (1 mM) present. Thus, following the reintroduction of the ATP-regenerating system, medium [Ca]\(^{2+}\) was rapidly lowered to a similar level as before (Fig. 2).

To study the dose dependence of this process, various amounts of ADP were consecutively added to the incubation medium (Fig. 3). As little as 0.5 mM ADP was quite effective in releasing Ca\(^{2+}\) from the microsomes since it increased medium [Ca]\(^{2+}\) from about 0.2 mM to a new steady state of around 0.4 mM. A progressive reduction in this effect was observed with cumulative additions of ADP. In contrast to what is observed with mitochondria (4), the addition of a small amount of EGTA resulted in a lowering of the extramicrosomal [Ca]\(^{2+}\) steady state maintained by the microsomes (Fig. 3).

The net Ca\(^{2+}\) uptake (the balance between Ca\(^{2+}\) influx and efflux) was measured at around 5 mM Ca\(^{2+}\) in the presence of ADP. ADP inhibited markedly and in a dose-dependent manner the net Ca\(^{2+}\) uptake by the microsomes (Fig. 4). This indicates that ADP exerts its effect also at higher Ca\(^{2+}\) concentrations than in the low submicromolar range and suggests that the Ca\(^{2+}\) pump is inhibited in a dose-dependent manner by ADP. We also demonstrated that the capacity of ADP to release Ca\(^{2+}\) from the microsomes was specific; at a concentration of 1 mM, GDP, UDP, GDP, UDP, GDP, and the nonhydrolyzable ADP analogue methylene-ADP as well as AMP or cAMP (0.1 mM) did not release Ca\(^{2+}\) from the microsomes (data not shown).

Since insulinomas were found to possess a high Ca\(^{2+}\)-sequestering activity, it was of interest to know whether under our experimental conditions microsomes isolated from an easily obtainable source and well-studied tissue, i.e. rat liver, would show similar activity.

Liver microsomes were isolated in the same sucrose medium, homogenized with the same Teflon pestle, and centrifuged strictly under identical conditions as those for insulinoma microsomes. It was observed that liver microsomes did show Ca\(^{2+}\)-sequestering activity, as indicated by the lowering of medium [Ca\(^{2+}\)] upon their addition. However, the Ca\(^{2+}\)-sequestering activity of liver microsomes was considerably lower than observed with insulinoma microsomes (Fig. 5).

**Localization of the Microsomal Ca\(^{2+}\) Uptake to the Endoplasmic Reticulum**—The specific activities of either the apparent net Ca\(^{2+}\) uptake or marker enzymes in the four fractions obtained after centrifugation of the suspended microsomes on a sucrose density gradient (see "Experimental Procedures") were determined. The Ca\(^{2+}\) transport activity correlated well with the endoplasmic reticulum marker (Table II). In marked contrast, there was no correlation with the plasma membrane marker. Such experiments demonstrate that the high Ca\(^{2+}\)-transporting activity of the insulinoma microsomes is localized in the endoplasmic reticulum.

**Ambient [Ca\(^{2+}\)] and the Secretory Granules**—Following their isolation, insulin secretory granules had a calcium content of 108 ± 16 mmol/mg of protein (mean of seven experiments ± S.E.) as determined by atomic absorption. Nonetheless, insulin secretory granules were unable, in the presence of MgATP, to lower medium [Ca\(^{2+}\)] or to take up a pulse...
Ca²⁺ Transport by Insulinoma Endoplasmic Reticulum

Specific activities of Ca²⁺ uptake and marker enzymes in four fractions obtained after centrifugation of the resuspended microsomes on a sucrose density gradient

Ca²⁺ uptake in the four fractions was measured from the same amount of material (0.2 mg of protein/ml) under experimental conditions described in the legend to Fig. 1. The apparent net Ca²⁺ uptake was measured during the second minute following the addition of MgATP (1.0 mM) to the medium already containing the fractions. The nanomoles of Ca²⁺ taken up were calculated from the reduction in ambient free Ca²⁺ concentration upon addition of each separate fraction (see also Fig. 1). The data are expressed as percentage of the specific activities in Fraction 1. The specific activities of Ca²⁺ uptake, NADPH-cytochrome c reductase, and 5'-nucleotidase in Fraction 1 were, respectively, 1.78 ± 0.38 (nanomoles of Ca²⁺ taken up per min·mg of protein), 64.8 ± 8 (nanomoles of cytochrome c reduced per min·mg of protein), and 10.4 ± 1.3 (micromoles of adenosine formed per h·mg of protein) (mean ± S.E. of four separate experiments).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Ca²⁺ uptake</td>
<td>100 ± 21</td>
<td>85 ± 20</td>
<td>220 ± 51</td>
<td>271 ± 90</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>106 ± 13</td>
<td>89 ± 9</td>
<td>195 ± 30</td>
<td>206 ± 34</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>100 ± 13</td>
<td>59 ± 11</td>
<td>40 ± 8</td>
<td>28 ± 4</td>
</tr>
</tbody>
</table>

Microsomal Ca²⁺ uptake was strictly MgATP dependent and had characteristics similar to those observed in ⁴⁴Ca²⁺ studies of microsomes in several tissues (6, 7, 22). Insulinoma microsomes lowered within less than 10 min the extramicrosomal ambient free [Ca²⁺] to about 0.1-0.2 μM. The Ca²⁺ accumulated could be rapidly released upon addition of A23187 or by lowering medium ATP by the combined addition of glucose plus hexokinase, indicating that Ca²⁺ is accumulated in an ATP-dependent manner within vesicular elements.

The ability of the microsomes to buffer external [Ca²⁺] appears quite different from that of the mitochondria. Since the rates of Ca²⁺ influx and efflux from the mitochondria are independent of the Ca²⁺ load over quite a wide range, mitochondria can maintain a true Ca²⁺ steady state (4, 23). Whenever small aliquots of Ca²⁺ or EGTA are added, mitochondria restore [Ca²⁺] to the previous level (4, 23). By contrast, the [Ca²⁺] steady state of insulinoma microsomes was decreased by small additions of EGTA. This suggests the absence, under nonstimulatory conditions, of an active and independent Ca²⁺ efflux transporter and that passive Ca²⁺ efflux from the microsomes depends on their Ca²⁺ load. In accordance with such an interpretation, it is known that Ca²⁺ efflux from liver microsomes (22) and sarcoplasmic reticulum vesicles (24, 25) is proportional to the vesicular Ca²⁺ content. Thus, the Ca²⁺-buffering characteristics of insulinoma microsomes are distinct from the mitochondria and are reminiscent of those of Ca²⁺ chelators.

It has been suggested that secretagogue-induced Na⁺ influx through the plasma membrane of chromaffin cells (26) or islet cells (27) could mobilize intracellular sequestered Ca²⁺. We showed previously that Na⁺ increased the Ca²⁺ efflux from insulinoma mitochondria (4). By contrast, Na⁺ is unlikely to exert significant effects on the endoplasmic reticulum or secretory granule Ca²⁺ fluxes since with both organelles there was no change in medium [Ca²⁺] upon Na⁺ addition. cAMP and 3-isobutyl-1-methylxanthine have also been suggested to mobilize intracellular Ca²⁺ stores in pancreatic islets (1). Both compounds were tested on intracellular organelles of rat insulinoma. Neither cAMP nor 3-isobutyl-1-methylxanthine altered Ca²⁺ fluxes across mitochondria (4), endoplasmic reticulum, or secretory granules (data not shown). This suggests either other site(s) of action of these compounds or that they do not directly modulate Ca²⁺ fluxes across intracellular organelles.

One of the most interesting observations in this study is that medium [Ca²⁺] maintained by the microsomes is markedly influenced by the concentration of ADP and that ADP
addition to the medium rapidly releases Ca\(^{2+}\) from the microsomes.

This appears specific for ADP since other nucleotides (GDP, UDP, CDP, IDP, and methylene-ADP) did not induce Ca\(^{2+}\) release. In addition, this effect was reversible (Fig. 2). A precise dose-response curve of ADP-induced Ca\(^{2+}\) efflux could not be done due to the formation of ADP by other ATPases than Ca\(^{2+}\)-ATPase, which are unfortunately inherent in such microsomal preparations (28). We showed, however, marked changes in medium [Ca\(^{2+}\)] with only around 0.5 mM ADP and a dose-dependent inhibition of net Ca\(^{2+}\) influx by ADP. It is known that ADP is capable of inducing Ca\(^{2+}\) release, representing the reversal of ATP-dependent Ca\(^{2+}\) uptake in sarcoplasmic reticulum vesicles (29, 30). It is thus possible that ADP-induced Ca\(^{2+}\) release from insulinoma microsomes is mediated through pump reversal. An alternative is that the pump is less active in its forward direction in the presence of ADP and that Ca\(^{2+}\) escapes the microsomes by other route(s).

These observations should be of interest for studies assessing the contribution of the different intracellular organelles to the regulation of cytosolic Ca\(^{2+}\) (31–33). Indeed, the data reveal that depending on experimental conditions, in particular the presence or the absence of ADP, marked differences in Ca\(^{2+}\) fluxes across the endoplasmic reticulum are observed. By contrast, it is known that ADP only slightly influences mitochondrial Ca\(^{2+}\) transport (34, 35). Thus, caution is needed when interpreting the data of reconstitution experiments. Hence, the Ca\(^{2+}\) content of both endoplasmic reticulum and mitochondria and their relative contribution towards cellular Ca\(^{2+}\) homeostasis might be differently evaluated depending on the level of ADP present.

The exact cytosolic concentration of free ADP is not known, but it has been evaluated from NMR studies (36) to be much lower than the total ADP measured by traditional extraction methods. With these considerations in mind, it has been shown that the free cytosolic ADP concentration is decreased (39). This could be due to the medium and other means. The time course and the magnitude of the Ca\(^{2+}\) changes induced by ADP are compatible with changes in cytosolic Ca\(^{2+}\) during the stimulation of insulin release (2).

Such an action of ADP would be a very simple way to link some metabolic, cationic, and secretory events occurring upon secretagogue addition (39). However, the physiological significance of such an effect remains to be established in the intact cell.

Acknowledgments—We are grateful to Nicole Challet, Janine Bossi, Anne-Sofie Annen, and Charles Jorand for their excellent technical assistance.

Note Added in Proof—In addition to ADP, we have recently shown that inositol 1,4,5-trisphosphate also mobilizes Ca\(^{2+}\) selectively from the microsomal fraction of rat insulinomas (Prentki, M., Bizen, T. J., Janiec, D., Irvine, R. F., Berridge, M. J., and Wellheim, C. B. (1984) Nature 309, 562–564).

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