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# COMPARTMENTATION OF CALCIUM IN DIGITONIN-DISRUPTED GUINEA PIG PANCREATIC ACINAR CELLS

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**The treatment of guinea pig pancreatic acinar cells with digitonin leads to disruption of the plasma membrane, as judged by the liberation of cytosolic enzymes, without significant alteration of the mitochondrial membrane. The transport of calcium by the particulate residue was studied, and two different pools could be distinguished. One was supported by ATP or ADP, succinate providing the respiratory substrate, and was sensitive to the inhibitors, Ruthenium red and azide. The other pool needed the presence of ATP, ADP being ineffective, and also was unaffected by Ruthenium red or by azide, but was stimulated several-fold by oxalate. The Ruthenium red-sensitive calcium pool has characteristics resembling those of the transport of calcium by a mitochondrial fraction prepared from digitonin-treated acinar cells. In contrast, the Ruthenium red-insensitive calcium transport has characteristics resembling those of a microsomal fraction obtained from guinea pig pancreas. When the transport of calcium in digitonized cells was assayed at a calcium concentration range of $10^{-8}$–$10^{-4}$ M, preferential Ruthenium red-insensitive calcium transport could be observed at submicromolar calcium concentrations.**

## Introduction

It is generally accepted that an increase in free cytosolic calcium serves as a trigger to initiate the secretory process in a wide variety of cells [1]. Transport of calcium across the plasma membrane may play an essential part in the maintenance of the intracellular free calcium concentration within the range $10^{-8}$–$10^{-7}$ M (for a review, see Ref. 2). In different tissues, including guinea pig pancreatic acinar cells [3], mitochondria and microsomes are capable of an active accumulation of calcium, which could serve as intracellular calcium stores. In addition, it has been suggested that there is an ATP-dependent $Ca^{2+}$-storage system in rat pancreatic acinar cells, most likely located in the rough endoplasmic reticulum [4].

One method to render cells permeable is treatment with digitonin or saponin, which allows the separation of mitochondria and other particulate components from the soluble cytoplasm [5–7], and this has been applied to the study of the regulation of free $Ca^{2+}$ in liver [8,9] and pancreas [4,10] tissues.

It is the purpose of the present work to study the transport of calcium in digitonin-disrupted guinea pig pancreatic acinar cells and to compare it with calcium transport in isolated subcellular fractions, e.g., mitochondria and microsomes, from the same tissue. The data to be presented show that when digitonin was washed out, from previously digitonin-disrupted cells, these cells remain permeable to small molecules and that subcellular

Abbreviations: EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

components of calcium transport were stable for at least 40 min. One of the subcellular calcium pools has properties resembling those of calcium transport by isolated mitochondria. The other pool, insensitive to azide and Ruthenium red, has properties resembling those of the calcium transport by a microsomal fraction enriched in endoplasmic reticulum membranes.

## Materials and Methods

*Preparation of digitonin-disrupted pancreatic acinar cells.* Guinea pig pancreatic acinar cells were prepared as described in Ref. 3. Prior to digitonin treatment, cells were incubated for 30 min in a medium consisting of 120 mM NaCl, 1.2 mM $MgCl_2$, 1.4 mM $KH_2PO_4$, 0.6 mM $CaCl_2$, 15 mM Hepes, 14 mM glucose, 0.1 mg/ml soybean trypsin inhibitor and 10 mg/ml bovine serum albumin. Final pH was adjusted to 7.4. Cells were continuously gassed with 95% $O_2$/5% $CO_2$. Cells were sedimented by centrifugation at $1500 \times g$ for 1 min and resuspended in 10 ml of the incubation medium devoid of calcium and supplemented with 2 mM EGTA. After 5 min, digitonin was added to give a final concentration of 0.3 mg/ml and 15s later the mixture was centrifuged for 1 min at $1500 \times g$. The cell pellet was resuspended and washed twice with 5 ml of 300 mM saccharose/10 mM Tris, pH 7. Final resuspension of digitonin-disrupted cells was made in a medium containing 280 mM saccharose, 5 mM pyruvate, 5 mM succinate and 4 mM Hepes, pH 7, at a protein concentration of 4–5 mg/ml.

The effect of digitonin on plasma membrane disruption was tested by comparing the total enzyme activity in the cells with that released into the medium. The percent of total enzyme released after digitonin treatment was $93 \pm 2.1$ (S.E.) for amylase, $101 \pm 3.1$ (S.E.) for lactate dehydrogenase, and $9.7 \pm 0.5$ (S.E.) for glutamate dehydrogenase. Enzymatic activities were assayed as described in Ref. 3. Protein was determined by a biuret method [11], using bovine serum albumin as standard. Corrections for turbidity were made after adding 0.5 M KCN.

*Measurement of $^{45}Ca$ uptake.* For the measurement of $^{45}Ca$ uptake, disrupted cells corresponding to 2–2.4 mg were incubated at 37°C in 2.3 ml of a medium consisting of 40 mM KCl/3.7 mM $MgCl_2$/1.2 mM $KH_2PO_4$/0.08 mM EGTA/170 mM saccharose/3 mM succinate/10 mM Hepes, pH 7. As indicated in individual experiments, the medium was supplemented with $^{45}CaCl_2$ to give the desired free calcium concentration. Free ionic calcium concentrations were calculated according to the method of Portzehl et al. [12]. At intervals, as indicated, duplicate 0.2-ml aliquots of the cell suspension were transferred to Eppendorf tubes containing 1 ml 280 mM saccharose and centrifuged for 1 min at approximately $10\,000 \times g$. The supernatant was discarded and the pellet was dissolved in 0.1 ml 1 N NaOH for counting in a $\beta$-spectrometer.

Uptake of radioactive calcium was also measured by membrane filtration. At specified time intervals, 75-$\mu$l aliquots were removed and filtered under vacuum conditions through type HA Millipore filters of 0.45 $\mu$m pore size, previously soaked with 250 mM saccharose. The filters were washed once with 5 ml 250 mM saccharose/5 mM Hepes, pH 7, dried, and then dissolved in the scintillation cocktail.

*Isolation of mitochondria.* A crude mitochondrial fraction was obtained by homogenizing digitonin-disrupted guinea pig pancreatic acinar cells in 4 ml of a medium consisting of 250 mM saccharose/8 mM succinate/10 mM Hepes, pH 7. The homogenization was carried out in a glass-teflon Potter-Elvejhem homogenizer by three strokes at 900 rpm. The homogenate was centrifuged at $1000 \times g$ for 10 min, The resulting pellet was discarded and mitochondria were spun down by centrifugation at $10\,000 \times g$ for 10 min. The pellet was resuspended in 2 ml of the homogenization medium at a protein concentration of 2–2.5 mg/ml.

The uptake of radioactive calcium by the mitochondrial fraction was assayed in a similar way, as described above for digitonin-disrupted cells. Mitochondria were incubated in 0.2 ml of the incubation medium at a protein concentration of 1–1.2 mg/ml. Reaction was started by adding the nucleotides ATP or ADP after 5 min preincubation. Incubation took place for 5 min. Duplicate aliquots were filtered and washed as described above.

*Preparation of crude microsomes and measurement of microsomal calcium uptake.* Microsomal

membranes were obtained by differential centrifugation of a guinea pig pancreas homogenate as described in Ref. 3. As judged by the enzymatic activities of $Mg^{2+}$-activated ($Na^+ + K^+$)-ATPase, glutamate dehydrogenase and NADPH-cytochrome *c* reductase, this fraction was enriched with endoplasmic reticulum membranes (unpublished data) at least four times as compared to the homogenate. Calcium uptake was assayed by the membrane filtration procedure. The main incubation was carried out at 37°C in 0.2 ml, containing 0.2–0.3 mg of microsomal protein, of a medium consisting of 75 mM KCl/6 mM $MgCl_2$/0.2 mM EGTA/0.18 mM $^{45}CaCl_2$/50 mM Tris-HCl, pH 7,5. This medium was supplemented as described in Results. Reaction was started by adding ATP or ADP to give a final concentration of 5 mM. Incubation was for 10 min. Duplicate aliquots were filtered and washed with 10 ml of 75 mM KCl/50 mM Tris-HCl, pH 7.5, dried and counted in a $\beta$-spectrometer.

*ATPase assay.* The splitting of ATP was assayed by the release of $^{32}P$ from [$\gamma$-$^{32}P$]ATP as described in Ref. 13. Digitonin-disrupted cells were incubated in a medium consisting of 50 mM NaCl/5 mM KCl/90 mM saccharose/6 mM $MgCl_2$/0.5 mM EGTA/5 mM [$\gamma^{32}P$]ATP/50 mM imidazole, pH 7. The incubation volume was 0.1 ml, containing 30–60 $\mu$g of cellular protein.

*Chemicals*

ATP, ADP, GTP, ITP, UTP, CTP, ouabain and oligomycin were obtained from Boehringer Mannheim; EGTA, FCCP and digitonin from Sigma; Ruthenium red and azide from Merck. $^{45}CaCl_2$ and [$\gamma$-$^{32}P$]ATP from the Radiochemical Centre, Amersham.

## Results

*Calcium transport in digitonized cells*

*Effect of azide.* In order to compare the azide-sensitive and azide-insensitive components of calcium transport, supported by ATP or ADP together with inorganic phosphate, experiments were designed keeping in mind the effect of azide and oxalate. It should be noted that 3 mM Tris-succinate was present overall (see Materials and Methods). In the case of respiration-driven calcium uptake, the incubation medium was supplemented up to 6 mM succinate. With succinate alone i.e., in the absence of adenine nucleotides, there was an initial burst of calcium transport within the first 5 min, followed by a decrease back to control values. For this reason cells were preincubated for 5 min. In this way the net effect of adenine nucleotides on calcium transport could be clearly established.

Figs. 1 and 2 show the time course of ATP- or

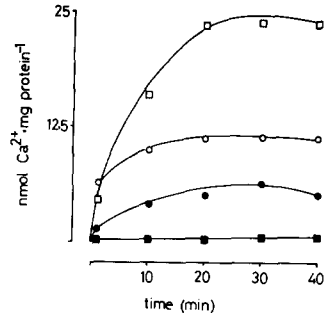


Fig. 1. Time course of ATP-dependent calcium uptake. Effect of azide. The uptake of calcium was assayed at 37°C by the centrifugation procedure (see Materials and Methods). The incubation medium was supplemented as follows: ○, 3 mM ATP; ●, 3 mM ATP plus 3 mM sodium azide; □ 3 mM ATP plus 3 mM sodium azide and 6 mM Tris-oxalate; ■ 3 mM sodium azide and 6 mM Tris-oxalate. Controls without any effector were processed in parallel and the corresponding values, quite similar to those obtained in the presence of azide and oxalate, were subtracted. The uptake of calcium was started by adding $^{45}CaCl_2$ to give a free calcium concentration of $3.6 \cdot 10^{-7}$ M. Results are the mean of five separate experiments. Standard error was less than 10% of the mean.

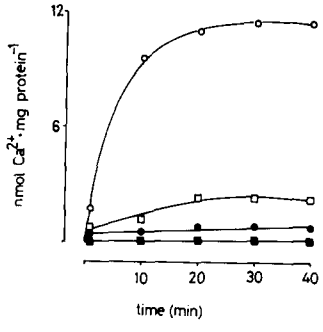


Fig. 2. Time course of the ADP-dependent calcium uptake. Effect of azide. Conditions and symbols are as in Fig. 1 except that 3 mM ADP was added instead of ATP. The main incubation medium was supplemented with up to 6 mM Tris-succinate. The final free calcium concenration was $3.6 \cdot 10^{-7}$ M. Results are the mean of three separate experiments.

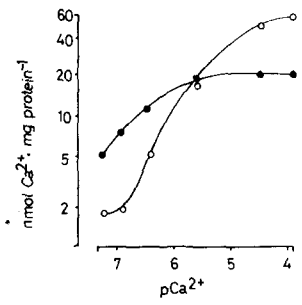


Fig. 3. Calcium dependence of azide-sensitive and azide-insensitive calcium transport. The uptake of calcium was assayed at 37°C during 10 min incubation, at free calcium concentrations indicated as the $pCa^{2+}$ (−log of free calcium). The values for azide-sensitive (○) calcium uptake were obtained by subtracting the values obtained in the presence of 3 mM ATP together with 3 mM azide to those obtained with 3 mM ATP alone. Azide-insensitive calcium uptake (●) represents the values obtained in the presence of 3 mM ATP together with 3 mM azide and 6 mM oxalate. Values represent the mean of three – eight separate experiments.

ADP-supported calcium transport, at a free calcium concentration of $3.6 \cdot 10^{-7}$ M. The shape of calcium transport was quite similar for both ATP and ADP (note the difference in the ordinate scale), with an apparent saturation obtained after 10 min. Azide clearly inhibited the transport of calcium supported by ADP, while the ATP-supported calcium transport was reduced to 50%. In the presence of azide, oxalate stimulated the ATP-supported uptake of calcium several-fold, in contrast to a minor effect when ADP was used instead of ATP.

Experiments similar to those shown in Fig. 1 were performed over a wide range of free calcium concentrations. The azide-sensitive and azide-insensitive components of calcium transport, as a function of $pCa^{2+}$, are plotted in Fig. 3. The activity represents the values obtained after 10 min incubation, when an apparent saturation of the azide-sensitive component was attained. The azide-sensitive calcium transport was maximal at approx. $10^{-4}$ M calcium, with half-maximal activity at nearly $9 \cdot 10^{-6}$ M. The azide-insensitive calcium transport was maximal at $5 \cdot 10^{-6}$ M calcium. We may observe from the relative activity at submicromolar calcium concentrations that the highest affinity for calcium transport corresponds to the azide-insensitive component.

TABLE I

NUCLEOTIDE DEPENDENCE OF CALCIUM TRANSPORT IN DIGITONIZED CELLS. EFFECT OF AZIDE AND OXALATE

The transport of calcium was assayed at 37°C by the centrifugation procedure (see Materials and Methods). The values represent nmol $Ca^{2+}$/mg proptein at 10 min after addition of nucleotides. Free calcium concentration was $3.6 \cdot 10^{-7}$ M. Nucleotide concentration was 3 mM. Where indicated, the medium was supplemented with Tris-succinate up to 6 mM. Results are the mean of triplicate aliquots from a single experiment performed with the same batch of cells.

| Nucleotide | Control | +5 mM $NaN_3$ | +6 mM oxalate +5 mM $NaN_3$ |
|---|---|---|---|
| None | 0.8 | 0.6 | 0.6 |
| ATP | 9.8 | 4.7 | 12.6 |
| ADP + succinate | 7.9 | 1.2 | 1.6 |
| CTP | 4.2 | 1.3 | 1.3 |
| ITP | 1.3 | 0.7 | 1.2 |
| UTP | 3.4 | 0.9 | 1.1 |
| GTP | 2.8 | 1.2 | 1.2 |

The azide-insensitive component of calcium uptake in digitonized cells was enhanced by the presence of the calcium-trapping agent, oxalate, and this effects was quite specific for ATP. Among other nucleotides tested (see Table I), only ATP was able to support the effect of oxalate on azide-insensitive calcium transport.

*Effect of Ruthenium red.* In a set of experiments, calcium uptake was assayed at 25°C, a more convenient temperature for mitochondrial calcium transport, keeping in mind the effect of Ruthenium red, an specific inhibitor of mitochondrial calcium transport. The time course of calcium transport is shown in Fig. 4. Ruthenium red at the concentration tested, 10 μM, inhibited 50% of the ATP-dependent calcium uptake. In the presence of Ruthenium red, oxalate stimulated the ATP-dependent calcium transport.

The effect of Ruthenium red on calcium transport was assayed at a wide range of free calcium concentration (Fig. 5). The Ruthenium red-sensitive calcium transport showed a maximal capacity at nearly $10^{-4}$ M calcium, with an apparent half-maximal activity at $5 \cdot 10^{-6}$ M calcium. The Ruthenium red-insensitive calcium transport had an apparent half-maximal activity at $0.19 \cdot 10^{-6}$ M

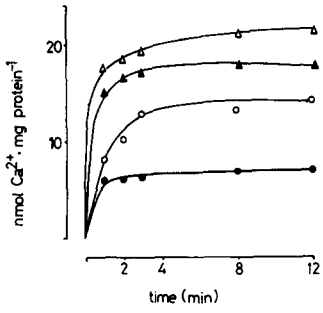


Fig. 4. Time course of ATP-dependent calcium uptake in digitonized cells. Effect of Ruthenium, red. The uptake of calcium was assayed at 25°C by the filtration procedure (see Materials and Methods). Cells were preincubated for 10 min in the main incubation medium supplemented as follows: ○ none; ● 10 μM Ruthenium red; △ 6 mM oxalate; ▲ 10 μM Ruthenium red plus 6 mM oxalate. After 10 min (zero time) uptake was started by adding ATP, 3 mM final concentration. Free calcium concentration was $3.2 \cdot 10^{-7}$ M. Duplicate aliquots of 75 μl were removed at the indicated times and processed as described in Materials and Methods. Results are from a single experiment, representative of two others.

calcium. Oxalate stimulated up to three times the maximal capacity of the Ruthenium red-insensitive component of calcium transport.

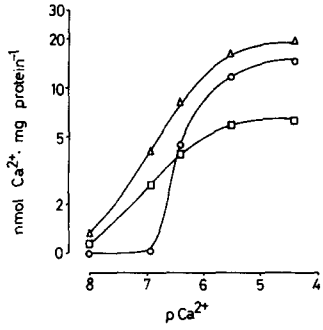


Fig. 5. Calcium dependence of Ruthenium red-sensitive and Ruthenium red-insensitive calcium transport. Digitonized cells were preincubated for 10 min and the main incubation was started by adding ATP, 3 mM final concentration. 3 min later incubation was stopped by filtering duplicate aliquots of 75-μl. The incubation temperature was 25°C. Ruthenium red-sensitive (○) calcium uptake refers to values obtained by subtracting the values obtained in the presence of 3 mM ATP plus 10 μM Ruthenium red from those obtained in the presence of ATP alone. Ruthenium red-insensitive calcium uptake was measured in the presence of 3 mM ATP and 10 μM Ruthenium red with (△) or without (□) 6 mM oxalate. Results are the mean of four separate experiments. Standard error was less than 10% of the mean.

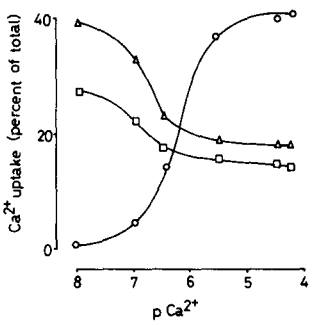


Fig. 6. Calcium dependence of the inhibitory effect of azide and Ruthenium red on calcium transport in digitonized cells. Calcium uptake, as percent of total calcium added, was plotted as a function of $pCa^{2+}$. The Ruthenium red-insensitive (□) and Ruthenium red-sensitive (○) calcium transport were assayed as described in Fig. 5. Azide-insensitive (△) calcium uptake refers to the values obtained in the presence of 3 mM ATP and 5 mM azide. Oxalate was omitted overall in this experiment. Results are of a single experiment representative of five others for the Ruthenium red-sensitive and -insensitive calcium transport.

As shown in Fig. 6, experiments were designed in order to compare the azide-insensitive and the Ruthenium red-insensitive transport of calcium. The azide-insensitive component was also assayed in these experiments. The uptake of calcium, expressed as a percentage of total calcium added, indicated a slightly higher inhibition of calcium uptake by Ruthenium red. Fig. 6 also illustrates the higher calcium affinity for the inhibitors-insensitive components of cellular calcium transport.

Table II summarizes the effect of several inhibitors on ATP-dependent calcium uptake in digitonized cells. The proton ionophore, FCCP, was the most potent inhibitor, reaching up to 75% inhibition of maximal transport. The lack of effect of oxalate in the presence of FCCP was very significant, in contrast to the activation observed in the presence of azide or Ruthenium red.

*Calcium transport in the mitochondrial fraction obtained from digitonin-disrupted pancreatic acinar cells.* A crude mitochondrial fraction could be prepared from the digitonized cells (Table III). The uptake of calcium was supported by ATP or by ADP together with phosphate and succinate. Maximal uptake obtained by ADP after 5 min incubation reached approximately 50% of total calcium added. The uptake of calcium was inhibited up to 90% of the maximal capacity by both

TABLE II

EFFECT OF INHIBITORS ON ATP-DEPENDENT $^{45}Ca^{2+}$ UPTAKE IN DIGITONIZED CELLS

The incubation medium contained 3 mM ATP, except where indicated. Cells were preincubated for 5 min at 25°C. Calcium uptake was started by adding $^{45}CaCl_2$ to give a free calcium concentration of $3.6 \cdot 10^{-7}$ M. After 10 min at 25°C duplicate aliquots were processed as described for the filtration procedure (see Materials and Methods). Results are the mean ± S.,E. of three – five separate experiments.

| Additions | Calcium uptake (nmol/mg protein) | |
|---|---|---|
| | Without oxalate | With 6 mM oxalate |
| None | 11.1 ± 0.9 | 14.5 ± 1.2 |
| $NaN_3$ (5 mM) | 5.1 ± 0.4 | 11.5 ± 1.1 |
| Ruthenium red (10 μM) | 4.2 ± 0.3 | 11.2 ± 0.8 |
| FCCP (30 μM) | 3.2 ± 0.1 | 3.9 ± 0.1 |
| Oligomycin (30 μg/ml) | 10.6 ± 0.8 | 13.5 ± 0.9 |
| ATP omitted | 1.2 ± 0.07 | 1.5 ± 0.1 |

TABLE III

CALCIUM UPTAKE BY MITOCHONDRIA ISOLATED FROM DIGITONIN-DISRUPTED CELLS

The uptake of calcium was assayed at 25°C for 5 min. The incubation medium contained 5 mM succinate and 5 mM ATP or ADP. Results are the mean ± S.E. of three separate experiments. Free calcium concentration was $5 \cdot 10^{-7}$ M.

| Additions | Calcium uptake (nmol/mg protein) | |
|---|---|---|
| | Without oxalate | With 5 mM oxalate |
| None | 2.5 ± 0.09 | 2.3 ± 0.1 |
| ATP | 22.9 ± 1.7 | 20.4 ± 1.9 |
| ATP + 3 μM Ruthenium red | 3.9 ± 0.12 | 5.5 ± 0.2 |
| ATP + 5 mM $NaN_3$ | 4.4 ± 0.17 | 5.6 ± 0.3 |
| ADP | 27.5 ± 1.9 | 25.4 ± 1.7 |
| ADP + 3 μM Ruthenium red | 3.8 ± 0.1 | 4.5 ± 0.2 |
| ADP + 5 mM $NaN_3$ | 4.6 ± 0.2 | 3.9 ± 0.16 |

Ruthenium red and azide. The degree of inhibition was quite similar for the ATP-dependent calcium transport and for the respiration-driven (ADP plus succinate) calcium transport. It was also noteworthy that oxalate did not significantly modify the transport of calcium.

*Microsomal calcium uptake.* The uptake of calcium by a microsomal fraction obtained from guinea pig pancreas tissue (see Materials and Methods) was stimulated by ATP and enhanced by the calcium trapping agent, oxalate. It was not inhibited by either azide or Ruthenium red at the concentrations indicated in Table IV. ADP, together with phosphate and succinate, was not able to support the uptake of calcium in the microsomal fraction of the guinea pig pancreas.

*ATPase activity.* The splitting of ATP by digitonin-disrupted cells was linear over a period of

TABLE IV

CALCIUM UPTAKE BY GUINEA-PIG PANCREAS MICROSOMES

Calcium uptake was assayed as described in Materials and Methods. Nucleotide concentration was 5 mM. The tubes containing ADP were supplemented with 4 mM succinate and 1.2 mM inorganic phosphate. The incubation medium contained 5 mM oxalate, except where indicated. Values are the mean ± S.E. of the number of experiments given in parentheses. Where no S.E. is given, values are averages.

| Additions | Calcium uptake (nmol/mg protein) |
|---|---|
| ATP | 31 ± 2.8 (5) |
| ATP + 5 mM $NaN_3$ | 27 ± 1.7 (5) |
| ATP + 3 μM Ruthenium red | 33 (2) |
| ATP + 10 μM Ruthenium red | 31 (2) |
| ATP without oxalate | 8.2 ± 0.6 (4) |
| ATP omitted | 3.3 ± 0.2 (5) |
| ADP | 5.5 (2) |
| ADP + 5 mM $NaN_3$ | 5.9 (2) |

TABLE V

ATPase ACTIVITY IN DIGITONIN-DISRUPTED GUINEA-PIG PANCREATIC ACINAR CELLS

ATPase was assayed as described in Materials and Methods. The main incubation medium contained 0.5 mM ouabain, except where indicated. Incubation took place for 20 min at 25°C. Digitonized cells were used just after resuspension or following three times freezing and thawing. No differences were observed between both procedures. Results are the mean ± S.E. of five separate experiments.

| Additions | μmol $P_i$/mg protein per 20 min |
|---|---|
| None | 0.66 ± 0.05 |
| FCCP (10 μM) | 1.2 ± 0.09 |
| $NaN_3$ (5 mM) | 0.34 ± 0.03 |
| $NaN_3$ (ouabain omitted) | 0.71 ± 0.05 |

30 min (data not shown). Maximal activity approximated the hydrolysis of 25–30% of total ATP added, after 30 min incubation, and was attained at equimolar concentrations of Mg and ATP. Azide inhibited the $Mg^{2+}$-ATPase by 50%. In the presence of azide a $(Na^{+} + K^{+})$Mg-ATPase which was totally inhibited by ouabain was detected. A $Mg^{2+}$-dependent, $Ca^{2+}$-stimulated ATPase, but with very low activity, could only be detected when the inhibitors azide and ouabain were present in the incubation medium. The interest of these experiments was focused on the possible accessibility of ATP to digitonized cells. Because of the fact that no differences were observed in ATPase activity of cells broken by repeated freezing and thawing as compared to unbroken cells, the results obtained by both procedures were pooled, and these are summarized in Table V.

## Discussion

The treatment of isolated cells with digitonin or saponin has been described as a method for the separation of mitochondria and other particulate components from the soluble cytoplasm [5–7,14], and this method has been applied to rat pancreatic acinar cells [4,10]. By using this procedure in guinea pig pancreatic acinar it was possible both to permeabilize the plasma membrane and to maintain unaltered the mitochondrial matrix compartiment, as evidenced by the release of lactate deshydrogenase and amylase in the first case and the release of glutamate deshydrogenase in the second.

Our procedure to permeabilize acinar cells with digitonin allowed us to study the transport of calcium during incubation periods of up to 40 min, showing a good stability of the possible vesicular spaces where calcium is accumulated. The washing out of digitonin in our experiments could explain the differences in the stability of the calcium transport compared to that described by Wakasugi et al. [10]. The possibility that the washing out of digitonin could lead to a resealing of cells, as pointed out by Schulz et al. [4], was contrasted by the accessibility of ATP as substrate for intracellular ATPase. No differences were observed in the ATPase activity of cells broken by repeated freezing and thawing procedures as compared to unbroken cells. Another feature, in view of this possibility, was the failure of these cells to maintain a potassium gradient (data not shown).

Mitochondria obtained from digitonin-disrupted acinar cells, maintained the ability to take up calcium (see Table III), as did mitochondria obtained from a guinea pig pancreas homogenate [3]. Azide inhibited the uptake of calcium by mitochondria of digitonized cells independently of whether the uptake of calcium was supported by ATP or by ADP together with phosphate and succinate. The degree of inhibition was similar so that obtained with Ruthenium red, a well-known inhibitor of mitochondrial calcium transport, which has been used to distinguish non-mitochondrial calcium transport in subcellular fractions [15]. In contrast, neither azide nor Ruthenium red were capable of inhibiting the uptake of calcium by a microsomal fraction obtained from guinea pig pancreas (see Table IV). This microsomal fraction was enriched in endoplasmic reticulum membrane and a $Mg^{2+}$-dependent, $Ca^{2+}$-stimulated ATPase could be detected [16] (unpublished data). It is also noteworthy that ADP was unable to support the uptake of calcium by pancreatic microsomes. Calcium uptake by pancreatic microsomes could be stimulated several-fold by oxalate when ATP was present. These results agree rather well with those previously reported [3,17] in guinea pig pancreatic microsomes and in mouse pancreatic microsomes. The stimulation by oxalate of calcium transport in pancreatic microsomes contrasts with the lack of effect on mitochondrial calcium transport (see Table III). Oxalate, a calcium-trapping agent, has been widely used for the sequestration of calcium in sarcoplasmic reticulum and also in microsomes from non-muscular tissue [18,19].

In the present study, using acinar cells treated with digitonin, it was possible to distinguish two different pools of calcium accumulation. One was sensitive to azide and Ruthenium red and depended on the presence of ATP or ADP together with phosphate, succinate providing the respiratory substrate. Maximal uptake in this pool was obtained at micromolar calcium concentrations, while the uptake of calcium was negligible below 0.3 $\mu$M calcium. These results agree rather well with the data reported by Schulz et al. [4] on digitonin-disrupted rat pancreatic acini. The other component

of calcium transport was insensitive to Ruthenium red or azide; it was supported only by ATP and was several-fold stimulated by oxalate. The uptake of calcium in this pool was inhibited by the proton ionophore FCCP. A similar effect of FCCP has been described by Wakasugi et al. [10] in digitonin-disrupted rat pancreatic acini. The effect of FCCP is not easy to explain unless a proton gradient controls calcium transport in the Ruthenium red-insensitive pool. In sarcoplasmic reticulum, it has been proposed by Shosan et al. [20], that a proton gradient controls a calcium-release channel, and Madeira [21] has reported that formation of a transmembrane proton gradient is an early event in calcium transport by sarcoplasmic reticulum. The higher effect of FCCP, as compared to other mitochondrial inhibitors, could be due to increased mitochondrial ATPase activity, and therefore increased breakdown of ATP, as described by Wakasugi et al. [10]. Although the ATPase activity in the presence of the uncoupler FCCP was approximately three times higher than the azide-sensitive ATPase (Table V), it can be estimated that the hydrolysis of ATP, less than 15% after 20 min, is not the limiting factor for the uptake of calcium in the presence of FCCP (Table II). Moreover, the degree of inhibition of calcium uptake by FCCP at shorter incubation periods was quite similar (data not shown).

In contrast with data previously reported in pancreatic acinar cells and pancreatic mitochondria [3], oligomycin alone, i.e., in the absence of inhibitors of electron transport, did not inhibit the ATP-dependent calcium uptake. Although similar results were obtained by Wakasugi et al. [10] at calcium concentrations below $10^{-6}$ M, we failed to find a satisfactory explanation for the lack of effect of oligomycin on ATP-dependent calcium transport sensitive to azide and Ruthenium red (see Table II).

When the inhibitor-insensitive (Ruthenium red or azide) and the Ruthenium red-sensitive components of calcium transport were compared and the relative activity was plotted as a function of free calcium concentrations (see Fig. 6), a shift in the efficiency could be observed, i.e., the affinity for calcium transport below 1 $\mu$M calcium was higher in the inhibitor-insensitive component. These results correlate rather well with those reported by Schulz et al. [4] for mitochondrial and non-mitochondrial calcium pools in digitonized rat pancreatic acini.

The Ruthenium red-insensitive calcium transport in digitonized cells resembles the properties of the microsomal calcium transport as follows: (a) it was ATP-dependent; (b) ADP was ineffective; (c) it was not affected by azide or Ruthenium red; (d) it was several-fold stimulated by oxalate. In contrast, the Ruthenium red-sensitive component of calcium transport in digitonized cells has characteristics resembling those of calcium transport by mitochondria obtained from digitonized cells, as follows: (a) it could be supported by ATP or by ADP together with phosphate and succinate; (b) it was inhibited by azide or by Ruthenium red; (c) it was not affected by oxalate. All these data suggest that mitochondrial and non-mitochondrial calcium-buffering systems are present in guinea pig pancreatic acinar cells the latter being more effective at submicromolar calcium concentrations.

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