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Calcium Uptake into Acini from Rat Pancreas: Evidence for Intracellular ATP-Dependent Calcium Sequestration

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Summary. Intracellular ATP-dependent Ca^{2+} sequestration mechanisms were studied in isolated dispersed rat pancreatic acini following treatment with saponin or digitonin to disrupt their plasma membranes. In the presence of $^{45}Ca^{2+}$ concentrations $< 10^{-6}$ mol/liter, addition of 5 mmol/liter ATP caused a rapid increase in $^{45}Ca^{2+}$ uptake exceeding the control by fivefold. ADP mimicked the ATP effect by 50 to 60%, whereas other nucleotides such as AMP-PNP, AMP-PCP, CTP, UTP, ITP, GTP, cAMP and cGMP did not. Maximal ATP-promoted Ca^{2+} uptake was obtained at 10^{-5} mol/liter Ca^{2+}. Inhibition of Ca^{2+} uptake by mitochondrial inhibitors was dependent on the Ca^{2+} concentration, indicating the presence of different Ca^{2+} storage systems. Whereas the apparent half-saturation constant found for mitochondrial Ca^{2+} uptake was $\sim 4.5 \times 10^{-7}$ mol/liter, in the presence of antimycin and oligomycin (nonmitochondrial uptake) it was $\sim 1.4 \times 10^{-8}$ mol/liter. In the absence of Mg^{2+} both ATP- and ADP-promoted Ca^{2+} uptake was nearly abolished. The Ca^{2+} ionophore and mersalyl blocked Ca^{2+} uptake. Electron microscopy showed electron-dense precipitates in the rough endoplasmic reticulum of saponin-treated cells in the presence of Ca^{2+}, oxalate and ATP, which were absent in intact cells and in saponin-cells without ATP or pretreated with A23187. The data suggest the presence of mitochondrial and nonmitochondrial ATP-dependent Ca^{2+} storage systems in pancreatic acini. The latter is likely to be located in the rough endoplasmic reticulum.

Key words pancreas · Ca^{2+} pools · Ca^{2+} pump · acini · saponin · secretagogue

Introduction

Calcium ions play a critical role in stimulation of enzyme secretion from the exocrine pancreas. It is the general hypothesis that secretagogue-induced rise of cytosolic free Ca^{2+} concentration triggers enzyme release from pancreatic cells [16, 22, 31, 35, 36, 52, 56, 58, 65]. Experiments in which isolated acinar cells had been preincubated with $^{45}Ca^{2+}$ until steady state had been reached have shown biphasic $^{45}Ca^{2+}$ movements following stimulation with secretagogues of enzyme secretion. An initial phase of $^{45}Ca^{2+}$ release is followed by $^{45}Ca^{2+}$ reuptake that can exceed the control value [16, 26, 55, 60, 62]. Addition of an antagonist such as atropine following carbachol caused a further rapid $^{45}Ca^{2+}$ uptake with subsequent decline back to the control. It was then possible to induce $^{45}Ca^{2+}$ release a second time by another secretagogue, whereas without an interposed step of the antagonist it was not [62]. Unidirectional Ca^{2+} flux measurements showed increased $^{45}Ca^{2+}$ exchange at steady state and zero net flux and increased $^{45}Ca^{2+}$ pool size in the presence of secretagogues [35, 36]. These observations were interpreted to mean that in the initial phase of secretagogue action $^{45}Ca^{2+}$ was released from a "trigger" pool that could be refilled by the antagonist, whereas $^{45}Ca^{2+}$ reuptake during the second phase was due to increased $^{45}Ca^{2+}$ influx into the cell and uptake into one or more intracellular pools other than the "trigger" pool [62].

Convincing evidence for the localization of these Ca^{2+} stores has not yet been obtained. Possible candidates for the site of Ca^{2+} release and reuptake are plasma membrane, endoplasmic reticulum and mitochondria and these cell sites had been considered as Ca^{2+} stores by different investigators [17, 20, 51–53, 62]. To investigate Ca^{2+} uptake into intracellular Ca^{2+} stores we treated isolated acinar cells with saponin and digitonin to disrupt plasma membranes. In smooth muscle this treatment makes the plasma membrane permeable to small ions and molecules, leaving the sarcoplasmic reticulum functionally intact [23]. Both saponin and digitonin also have been shown to disrupt synaptosomes [8, 9, 47] and plasma membranes of hepatocytes [6, 50]. By electron microscopy on Rous sarcoma virus and cell membranes, pits of 80–100 Å were observed following treatment with saponin or digitonin [5, 21, 27]. The number of these holes increased with increasing saponin concentrations [21], and it was assumed that this selective

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action of saponin results from the fact that the lipophilic heads of saponin interact with cholesterol in a 1:1 molar ratio forming a ring with a central hydrophilic hole in a micellar-type arrangement [27]. It therefore may be expected that the properties of cholesterol-rich membranes, such as plasma membranes, are primarily affected [8, 37, 63], whereas cholesterol-poor membranes, such as those of the endoplasmic reticulum and mitochondria [37], should be much less affected.

The data to be presented show that in saponin-treated cells Ca^{2+} uptake can be induced with ATP, whereas in untreated cells this effect is absent or very small. Using inhibitors of known action we have tried to distinguish between intracellular Ca^{2+} stores such as mitochondria and endoplasmic reticulum. ATP-induced Ca^{2+} uptake was inhibited but not abolished by a variety of mitochondrial inhibitors such as atractyloside, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CFCCP) and combinations of redox and mitochondrial ATPase inhibitors. Nonmitochondrial inhibitors known to inhibit the Ca^{2+} sequestration mechanism in sarcoplasmic reticulum such as quercetin and caffeine [61] inhibited ATP-dependent Ca^{2+} uptake by 40%, whereas mersalyl and the Ca^{2+} ionophore A23187 abolished it completely. Micrographs of sections through cells treated with saponin and incubated with Ca^{2+}, oxalate and ATP showed Ca^{2+} precipitations in the rough endoplasmic reticulum and occasionally in mitochondria, whereas no electron-dense deposits were seen when cells were incubated in ATP-free medium. The presence of calcium in the area of oxalate precipitates has been demonstrated by mass spectroscopy. The data therefore suggest that both nonmitochondrial and mitochondrial Ca^{2+} storage sites are involved in ATP-dependent Ca^{2+} uptake. We assume that they might play a role in Ca^{2+} reuptake following carbachol-induced Ca^{2+} release.

Materials and Methods

Materials

Reagents were obtained from the following sources. Collagenase (from *Cl. histolyticum*) type CLSPA 395-593 U/mg, and type III 110 U/mg from Worthington (Freehold, N.J.); ATP, ITP, UTP, CTP, GTP, AMP-PNP, AMP-PCP, ADP, cAMP, cGMP, carbonylcyanid-*p*-trifluoromethoxyphenylhydrazone (CFCCP), phosphoenolpyruvate, pyruvate kinase (200 U/mg), creatinphosphate and creatin kinase (25 U/mg) from Boehringer (Mannheim, Germany); chromatographically purified soybean trypsin inhibitor, oligomycin, rotenone and antimycin A from Serva (Heidelberg, Germany); ruthenium red, azide, cyanide, caffeine and the LDH Merckotest (No. 3339) for determination of lactate dehydrogenase from Merck (Darmstadt, Germany). Saponin, essentially fatty acid-free bovine plasma albumin, atractyloside and quercetin were obtained from Sigma (St. Louis, Mo.); synthetic octapeptide of cholecystokinin-pancreozymin, A23187 and N,N′-dicyclohexylcarbodiimide (DCC) from Calbiochem (Giessen, Germany). Trifluoperazine dihydrochloride (TFP) was a gift from Roehm Pharma (Darmstadt, Germany). Nucleopore filters (∅25 mm with 3 and 5-μm pore size) were obtained from Biorad (Munich, Germany), and $^{45}Ca^{2+}$ (4-30 Ci/g calcium) from NEN Chemicals (Dreieich, Germany). The luciferin-luciferase test kit was purchased from Abimed (Duesseldorf, Germany; distributor for Lumac, Florida).

Preparation of Acini

Acini were prepared from pancreatic tissue (1.2 to 1.8 g wet weight) from 1 to 2 Wistar rats (180–220 g) that had been fasted overnight similar to the method described for acinar cells [1] but omitting a step in which divalent cations were removed with EGTA [57]. The procedure involves digestion for 15 min with 1539 U of collagenase per 15 ml of a Krebs-Ringer's-Hepes buffer containing (in mmol/liter): 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 Hepes adjusted with NaOH to pH 7.4, 15 glucose, 0.1 mg/ml trypsin inhibitor and 0.2% albumin. In a second step tissue was digested with 2461 U of collagenase per 15 ml for 45 to 60 min when type III and 70 to 90 min when type CLSPA of Worthington collagenase was used. Acini were then mechanically dissociated by sequential passage through pipettes of 3 mm and 1.5-mm bore size. The suspension was filtered through a double layer of gauze, it was then layered over 15 ml of buffer containing 4% albumin and centrifuged for 3 min at 800 rpm. The pellet was washed 3 times with the same buffer containing 120 mmol/liter KCl, 4.9 mmol/liter NaCl and 10 mmol/liter Hepes, adjusted with KOH to pH 7.4 and calcium as indicated. Other constituents were the same as in the Krebs-Ringer's buffer mentioned above. In some experiments it also contained phosphoenolpyruvate (2 mmol/liter) and pyruvate kinase (10 U/ml) or creatinphosphate (2 mmol/liter) and creatin kinase (5 U/ml). Since these ATP-generating systems had no effect on ATP-induced Ca^{2+} uptake they were later omitted. The final pellet was taken up in 36 ml of incubation medium and aliquots of 9 ml cell suspension were transferred to four plastic vials. Cells were gassed continuously with 100%O_2 and shaken at ~80 oscillations/min in a water bath at 37 °C.

Treatment of Acini with Saponin and Digitonin and Tests for Estimation of the Plasma Membrane Permeability to Trypan Blue and Lactate Dehydrogenase

Acini were preincubated in the presence of saponin or digitonin for 10 min prior to the start of the experiment. In each experiment uptake of trypan blue (0.5%) was measured at the end of each experiment to judge the relative permeability of saponin-treated and untreated cells. Cells were counted in a "Neuberger" cell counting chamber and those which had taken up trypan blue were estimated in percent of total cells. In two experiments trypan blue uptake was 30% when incubated with 20 μg of saponin per ml and 40% at 20 μg/ml of digitonin. It was 95 and 80% at 45 μg/ml of saponin and digitonin, respectively, and 100% for both saponin and digitonin at 60 μg or at 100 μg/ml. In the following $^{45}Ca^{2+}$ uptake was measured in acini treated with 45μg saponin/ml and trypan blue uptake was 90 to 100% in all experiments, whereas in untreated cells it was 3 to 5%. Another way to assess the permeability of the plasma membrane to high molecular substances was estimation of release of the cytosolic enzyme lactatehydrogenase (LDH) from acini treated with saponin or digitonin. In one experiment LDH release from acini at 20 or 45 μg of saponin and 45 μg of digitonin per ml of incubation medium and in another one 20, 40, and 100 μg saponin/ml were tested. At different times 300 μl of cell suspension were removed from the incubation medium and centrifuged for one min at 14,000 × *g* in an Eppendorf

microfuge. LDH was measured in the pellet as well as in the supernatant. LDH release expressed in percent of total activity present in cells at time 0 (i.e. before adding saponin or digitonin), steadily increased with time in both control and saponin- or digitonin-pretreated acini. After 100 min of incubation LDH release was 27 and 15% from control acini. It was 57% and 30% with 20 μg of saponin, 69 and 40% with 45 and 40 μg saponin and 80% with 100 μg saponin in both experiments. With 45 μg digitonin 74% was released. Trypan blue uptake in the same acini was 5% in control, 70 and 60% with 20 μg saponin and 100% with 45 and 100 μg of saponin as well as with 45 μg of digitonin.

$^{45}Ca^{2+}$ Uptake Measurements

Acini were preincubated in the presence of saponin (45 μg/ml) for 10 min and further incubated with $^{45}Ca^{2+}$. In most experiments $^{45}Ca^{2+}$ (2 μCi/ml) was added without EGTA. This gave a total calcium concentration of about 10^{-6} to 10^{-5} mol/liter. Where indicated, free Ca^{2+} concentration was adjusted with EGTA. Since EGTA might have unspecific effects on membrane proteins, 1 mmol/liter of EGTA was added at all Ca^{2+} concentrations used even at 10^{-4} mol/liter of free Ca^{2+} at which a buffering effect of EGTA could not be expected. Acini were separated from the incubation medium by suction of 200 μl aliquots through Nucleopore filters (⌀25 mm, 3 or 5 μm pore size) followed by a single wash with 10 ml of ice-cold buffer of the same composition as the incubation medium but without glucose, trypsin-inhibitor and KH_2PO_4 and with 10^{-4} mol/liter $LaCl_3$. La^{3+} in the wash solution was used since it was expected to prevent Ca^{2+} efflux from compartments into which Ca^{2+} had been taken up before. Although it was found, however, that in the presence of 10^{-4} mol/liter La^{3+} in the wash solution $^{45}Ca^{2+}$ uptake was only insignificantly higher than without La^{3+} (~8%), the use of La^{3+} was continued throughout the experiments. Addition of 2 mmol/liter of EGTA in the wash solution had no effect, whereas 10^{-3} mol/liter of Ca^{2+} decreased uptake by 20%. The washing procedure took 10 sec. For protein determination 1 ml of cell suspension was removed from each incubation vial, centrifuged in an Eppendorf centrifuge at 14,000 rpm for 2 min and the pellet was taken up in 1 ml of water. For estimation of protein that remained on the filters, each filter was placed into a vial, containing 0.5 ml of aqua bidest. The samples were ultrasonicated for 20 sec and 100 μl was removed for the assay according to Lowry et al. [43] using bovine serum albumin as standard. Three ml of Picofluor TM 15 Packard was then added to each vial and the radioactivity was counted in a Packard liquid scintillation counter using the preset ^{14}C channel for counting $^{45}Ca^{2+}$. The mean protein concentration in acini dispersed in incubation medium from 60 samples in 15 experiments was 3.42 mg/ml ± 0.14 SE. The mean protein that remained on the filter after washing was 0.3 mg/filter ± 0.01 SE as calculated from 409 filters (5 experiments). Thus only 45% of the cell protein that had been transferred onto the filter was recovered from the filter. In most experiments protein per filter was calculated from the protein concentration in the incubation medium. The $^{45}Ca^{2+}$ uptake was referred to mg protein. Although there was a small difference, i.e. ~20% less protein/filter in saponin-treated as compared to untreated acini, this difference could not account for the increased $^{45}Ca^{2+}$ uptake/mg protein at steady state in saponin-treated acini (*see* Fig. 2). The same conclusion was drawn from separate experiments in which $^{45}Ca^{2+}$ uptake into "saponintreated" and untreated acini was measured using the "oil method" and ^3H_2O for determination of intracellular water space as had been described by us previously [62]. Briefly it involves incubation of acini in the presence of $^{45}Ca^{2+}$ ($<10^{-6}$ mol/liter, no EGTA) and ^3H_2O (1 μCi/ml, each) for different periods and quick centrifugation of aliquots through silicone oil in order to separate acini from the incubation medium. The extracellular space of the pellet was determined using [methoxy-^{14}C] inulin. The $^{45}Ca^{2+}$ content of the pellet is expressed per μl of intracellular H_2O after correction of $^{45}Ca^{2+}$ within the inulin space. The results obtained by this method were, in principle, the same as those obtained with the "filter" method; but the absolute effects of ATP-promoted $^{45}Ca^{2+}$ uptake (at 2 or 5 mmol/liter ATP), although significant ($p<0.01$), were much smaller. We assumed that removal of $^{45}Ca^{2+}$ from watery phases especially in saponin-treated acini was not as effective as compared to the filter wash procedure. All subsequent experiments were therefore done with the "filter" method.

Electron Microscopy

The site of intracellular Ca^{2+} uptake was monitored by electron microscopy using oxalate to precipitate Ca^{2+} at the site of accumulation.

Acini were incubated as usual in the presence or absence of saponin (45 μg/ml), fixed with an ice-cold solution of 3–4% glutaraldehyde, buffered with 0.1 mol/liter of sodium cacodylate (pH 7.2). Samples were then washed with 0.1 mol/liter cacodylate buffer, postfixed with 1% OsO_4 in 0.05 mol/liter cacodylate buffer (pH 7.2) and dehydrated in a series of alcohols for the final embedding in Spurr's resin. All steps, except embedding, were performed on ice. To minimize loss of calcium precipitates all solutions, except the resin, were supplemented with 20 mmol/liter K^+ oxalate. Thin sections were cut with glass knives in an ultramicrotome ("Ultracut", Reichert, Austria). The trough fluid was either water containing 5% K^+-oxalate or anhydrous glycerol. Sections cut in glycerol were shortly washed in acetone. Lead citrate stained and unstained sections were examined in a Philips 300 electron microscope.

Mass Spectroscopy

Laser microprobe mass analysis (LAMMA) was performed as described previously [33] using a LAMMA 500 (Leybold Heraeus, Koeln, Germany). Sections, about 150 nm thick, were chosen by electron microscopy to select areas for LAMMA analysis. A pilot laser beam was then focused on the area of interest and a high energy laser shot was performed. The ionized part of the evaporated area was immediately analyzed by a time-of-flight (TOF) mass spectrometer. The mass spectrum was monitored on a pen recorder.

ATP Determination

Determination of ATP was made using the luciferin-luciferase test kit (Abimed). Measurements were carried out in a Bioluminescence Analyzer XP 2000 (Sean AG, Switzerland).

Results

Effect of ATP and Other Nucleotides on $^{45}Ca^{2+}$ Uptake

As shown in Fig. 1 in cells pretreated with saponin or digitonin, $^{45}Ca^{2+}$ uptake increased rapidly after addition of ATP. Maximal uptake was obtained at 45 μg/ml of saponin or digitonin within 10–20 min, whereas at 20 μg saponin/ml $^{45}Ca^{2+}$ uptake was slower and still increased within the period observed. At 60 and 100 μg saponin/ml, however, ATP-promoted $^{45}Ca^{2+}$ was smaller as compared to 45 μg/ml. The mean maximal $^{45}Ca^{2+}$ uptake after ATP addition

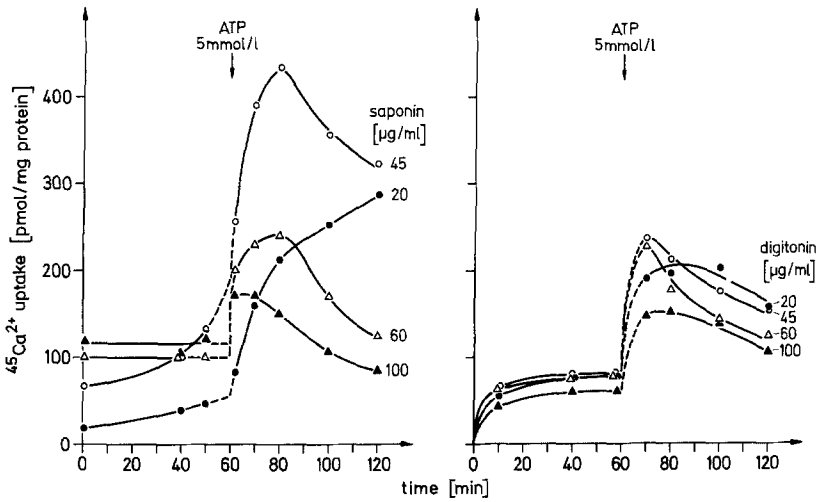

Fig. 1. Effect of saponin and digitonin on ATP-promoted $^{45}Ca^{2+}$ uptake. Acini were incubated with saponin (*left*) or digitonin (*right*) at indicated concentrations. At time 0 $^{45}Ca^{2+}$ (10^{-6} mol/liter) and 60 min later ATP (5 mmol/liter) was added. The Figure shows a single experiment with saponin, whereas that with digitonin is one out of two similar ones

Table 1. $^{45}Ca^{2+}$ uptake (pmoles/µl intracellular H_2O) in acini using the "oil method"[a]

	With saponin (45 µg/ml)				Without saponin			
Incubation (min)	60	66	90	120	60	66	90	120
$^{45}Ca^{2+}$ (pmol/µl H_2O) mean ± SE	with ATP				with ATP			
	4.94±0.19	7.93±0.68	7.54±0.50	6.62±0.26	2.62±0.16	3.59±0.42	3.96±0.32	4.21±0.22
	without ATP				without ATP			
	5.05±0.12	5.37±0.17	6.37±0.37	6.61±0.46	2.67±0.26	2.74±0.22	3.15±0.19	3.88±0.17

[a] ATP (2 mmol/liter) was added after 60 min incubation. The data show mean values ± SE from 5 experiments, each value obtained in duplicate

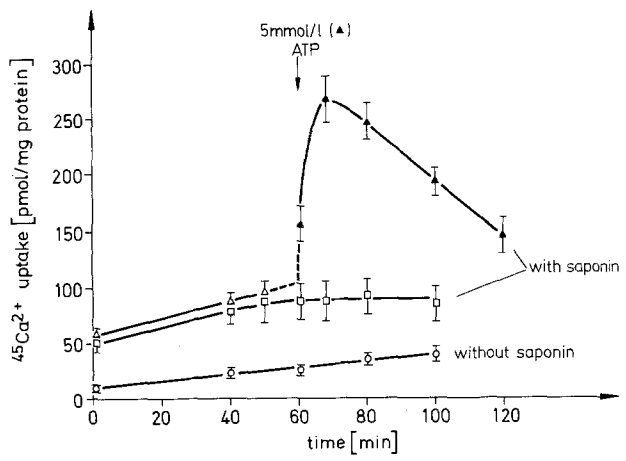

Fig. 2. Effect of ATP on $^{45}Ca^{2+}$ uptake. Acini were preincubated for 10 min with (△, □) or without (○) saponin (45 µg/ml). At time 0 $^{45}Ca^{2+}$ (10^{-6} mol/liter) was added. Sixty min later ATP (▲) was added to saponin-treated acini. □, ○ indicate that no ATP was added. Experiments show the mean ± SE from 13 experiments (each point is taken in triplicate)

in 13 experiments was 266±22 SE pmol/mg protein. Within 10 min threefold higher $^{45}Ca^{2+}$ uptake than before ATP addition was reached followed by subsequent decline. In acini that had not been treated with saponin or digitonin $^{45}Ca^{2+}$ uptake within the first 50 min of incubation with $^{45}Ca^{2+}$ was approximately one-third of the $^{45}Ca^{2+}$ uptake of saponin-treated acini (Fig. 2). The maximal effective ATP concentration to promote $^{45}Ca^{2+}$ uptake within 10–20 min after ATP addition was 5 mmol/liter (apparent K_m 2×10^{-3} mol/liter). At higher concentrations (7 and 10 mmol/liter of ATP) the effect on Ca^{2+} uptake persisted longer (up to 60 min) but the maximal Ca^{2+} uptake was the same (*data not shown*). Comparison of all data presented are therefore made for highest Ca^{2+} uptake obtained using 5 mmol/liter of ATP. For comparison $^{45}Ca^{2+}$ uptake obtained with the "oil"-method and referring to intracellular ^3H_2O space are shown in Table 1.

Addition of 5 mmol/liter ADP to saponin-treated

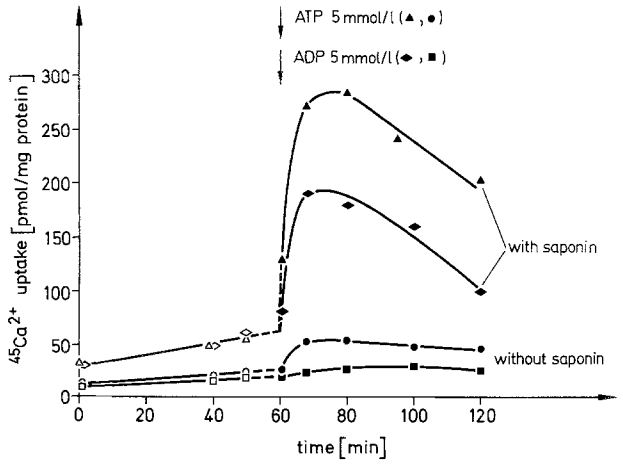

Fig. 3. Effect of ATP and ADP on $^{45}Ca^{2+}$ uptake into acini with (▲, ◆) or without (●, ■) saponin (45 μg/ml). Inubation conditions were as described in Fig. 2. One experiment out of three similar ones

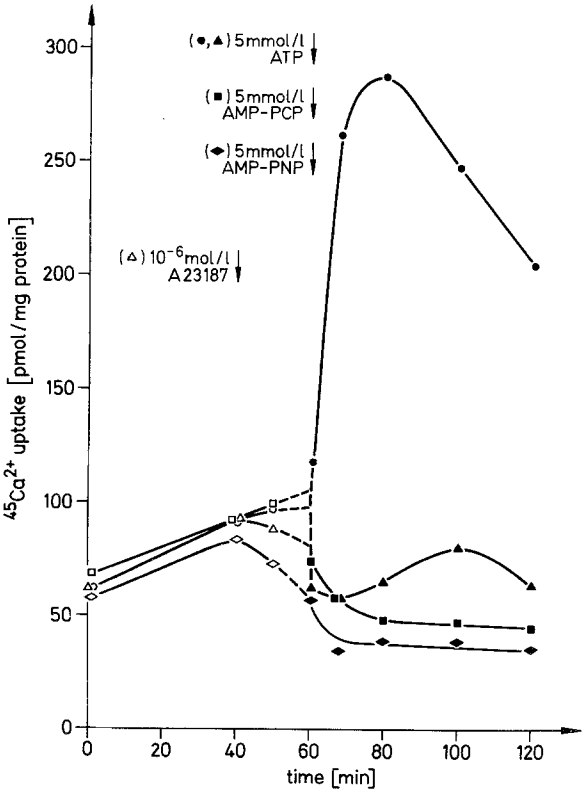

Fig. 4. Effect of A23187 on ATP-promoted $^{45}Ca^{2+}$ uptake and of nonhydrolyzable ATP analogs on $^{45}Ca^{2+}$ uptake. Cells were pretreated for 10 min with saponin (45 μg/ml) and at 0 time $^{45}Ca^{2+}$ (10^{-6} mol/liter) was added. ● addition of Na_2ATP (5 mmol/liter) at 60 min; △ addition of A23187 (10^{-6} mol/liter) at 40 min and subsequent addition of Na_2ATP (▲, 5 mmol/liter) at 60 min; ◆ addition of adenylyl-imidodiphosphate (tetralithium salt), AMP-PNP; and ■ of adenylyl (β,γ-methylen)-diphosphonate (tetralithium salt), AMP-PCP, at 60 min. One experiment

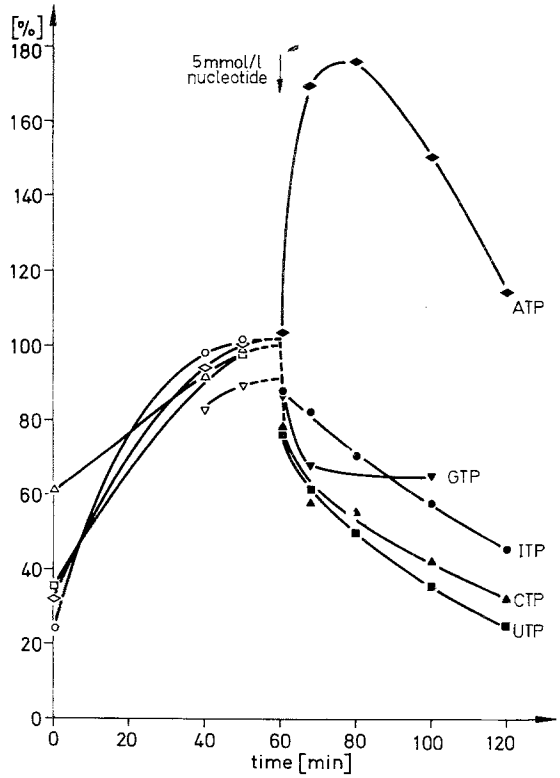

Fig. 5. Effect of Na_2ATP and its analogs guanosin-5′-triphosphate (Na_2GTP), inosin-5′-triphosphate (Na_2ITP), cytidin-5′-triphosphate (Na_2CTP) and uridin-5′-triphosphate (Na_3UTP) on $^{45}Ca^{2+}$ uptake in saponin-treated acini. Incubation conditions were similar to those described in Fig. 2. One out of two similar experiments

acini also showed increased $^{45}Ca^{2+}$ uptake which was about 60% of the value obtained with ATP (Fig. 3), whereas in cells without saponin both ATP and ADP effects were considerably smaller. To assess whether ADP stimulates Ca^{2+} uptake directly, or indirectly as a consequence of conversion to ATP via adenylate kinase, diadenosylpenta-phosphate (DPP), a potent inhibitor of adenylate kinase [9, 41] was added 20 min before addition of ATP or ADP or together with the nucleotides. At 10^{-5} mol/liter no effect on ATP-stimulated uptake was observed, whereas the ADP effect was abolished. At 10^{-4} mol/liter, however, the ATP effect was inhibited by 50% and at 10^{-3} mol/liter of DPP it was abolished (*data not shown*).

Other nucleotides such as the ATP analogs AMP-PNP and AMP-PCP of which the γ-phosphate groups are blocked for hydrolysis had no effect on $^{45}Ca^{2+}$ uptake and rather decreased cellular $^{45}Ca^{2+}$ within the first 15 sec after addition (Fig. 4). Similarly, ITP, UTP, CTP and GTP decreased rather than increased cellular $^{45}Ca^{2+}$ (Fig. 5). Cyclic nucleotides, such as cAMP and cGMP neither affected ATP-promoted

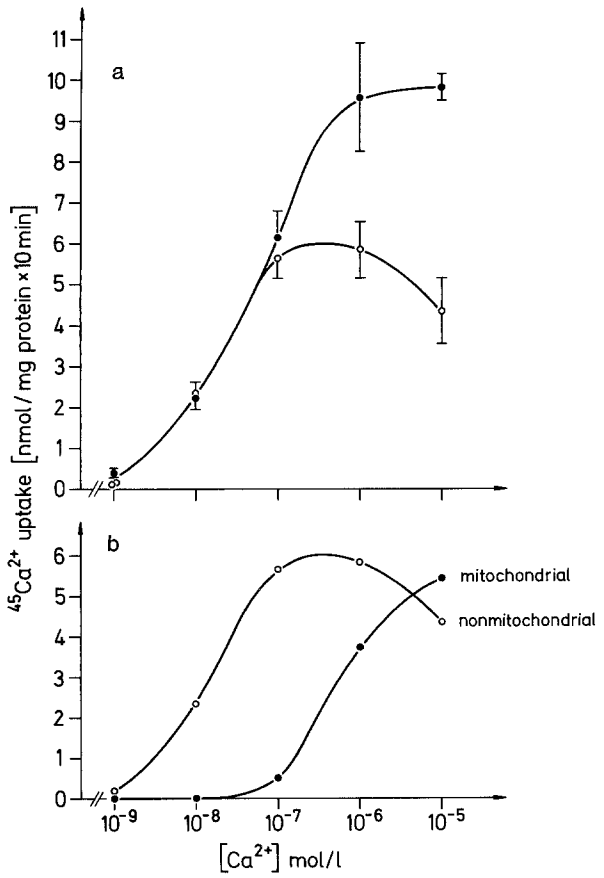

Fig. 6. (*a*) Effect of different Ca^{2+} concentrations on ATP-promoted $^{45}Ca^{2+}$ uptake. Free Ca^{2+} concentration was adjusted with EGTA as described in the text. The data are values at 10 min after addition of ATP. ● — ● without mitochondrial inhibitors. ○ — ○ in the presence of oligomycin and antimycin A (10^{-5} mol/liter each). Values show the mean ±SE of 3 to 6 experiments. (*b*) Replot of curves from Fig. 6*a*. Curve for "mitochondrial Ca^{2+} uptake" was obtained by subtracting the Ca^{2+} uptake in the presence of inhibitors (○ — ○) from the Ca^{2+} uptake in the absence of inhibitors (● — ●)

Ca^{2+} uptake nor did they influence $^{45}Ca^{2+}$ uptake when given in the absence of ATP (*data not shown*).

Effect of Inorganic Ions on ATP-Promoted $^{45}Ca^{2+}$ Uptake

Effect of Ca^{2+}. Figure 6 shows the effect of ATP on $^{45}Ca^{2+}$ uptake into acini at different free Ca^{2+} concentrations in the presence or absence of mitochondrial inhibitors (antimycin 10^{-5} mol/liter + oligomycin 10^{-5} mol/liter). Free Ca^{2+} concentrations were adjusted by adding different amounts of calcium to a constant ethyleneglycol-*bis*(β-aminoethyl ether)-N,N′ tetraacetic acid (EGTA) concentration of 1 mmol/liter, pH 7.4. True binding constants of log K 10.97 for CaEGTA and log K 5.20 for MgEGTA

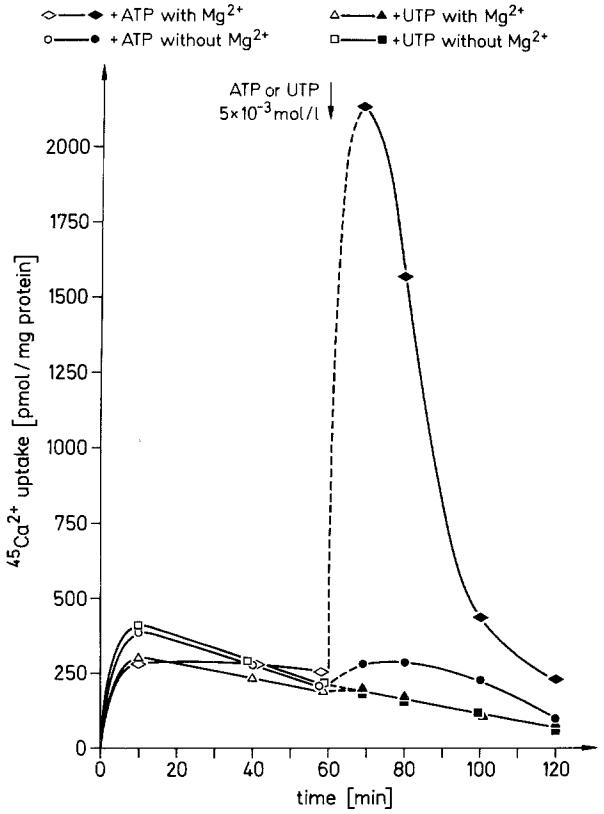

Fig. 7. Effect of Mg^{2+} on ATP-promoted $^{45}Ca^{2+}$ uptake. Acini were preincubated in incubation medium with or without Mg^{2+} for 10 min in the presence of saponin (45 μg/ml). At time 0 $^{45}Ca^{2+}$ (10^{-5} mol/liter) and 60 min later Na_2ATP (5 mmol/liter) or Na_3UTP (5 mmol/liter) was added. One experiment out of two similar ones

and respective pK values for protonated ligand were used to calculate free Ca^{2+} concentrations [2]. CaATP and MgATP binding constants were log K 3.6 and log K 4, respectively [10]. To assure a constant free Ca^{2+} concentration before and after addition of ATP, MgATP (1 Mg^{2+} per 1 ATP) was added. The calculated free Mg^{2+} concentration, however, varied a little (e.g. from 0.8 mmol/liter without MgATP to 1.22 mMol/liter after addition of MgATP at 10^{-6} mol/liter of Ca^{2+}) and remained relatively constant at different Ca^{2+} concentrations in the presence of ATP (i.e. 1.14–1.24 mmol/liter at 10^{-8} to 10^{-5} mol/liter of free $[Ca^{2+}]$).

Mg^{2+} Omission. Since there is evidence for the presence of a Mg^{2+}-dependent Ca^{2+}-ATPase in cell membranes of the exocrine pancreas [42, 44, 53] we tested the effect of omitting Mg^{2+} from the incubation medium on Na_2ATP-induced $^{45}Ca^{2+}$ uptake in saponin-treated cells. As can be seen from Fig. 7, omission of Mg^{2+} inhibited $^{45}Ca^{2+}$ uptake nearly completely. Since the sodium salt of ATP had to be used, we have compared the effect of Na_3UTP

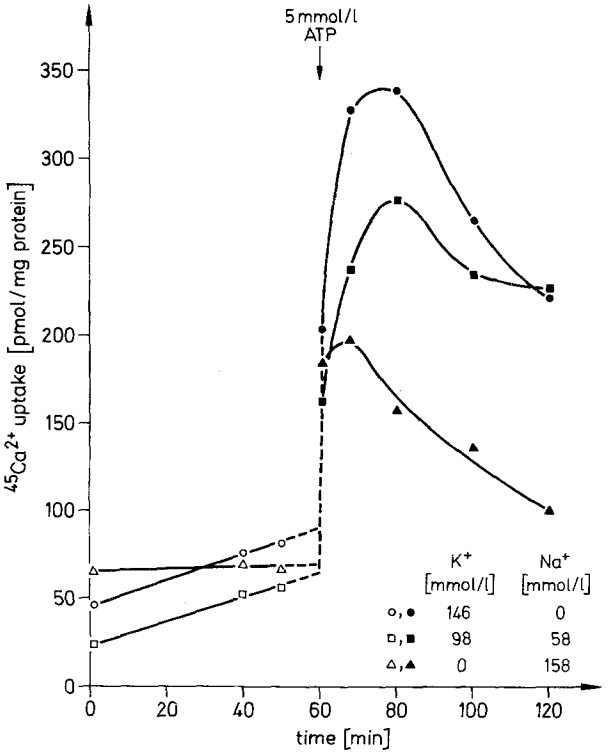

Fig. 8. Effect of Na^+ and K^+ on $^{45}Ca^{2+}$ uptake into "saponin-treated" acini. Incubation conditions were as described in Fig. 2. K^+ of the incubation medium was successively replaced by Na^+, the final Na^+ and K^+ concentrations were determined by flame photometry (Hitachi)

in the presence and absence of Mg^{2+} to account for possible chelation of the nucleotide with $^{45}Ca^{2+}$. In addition, we increased the $^{45}Ca^{2+}$ concentration (10^{-5} mol/liter) in this experiment. Similarly, the effect of ADP on $^{45}Ca^{2+}$ uptake was completely abolished in the absence of Mg^{2+} (*data not shown*).

Replacement of K^+ by Na^+. As shown in Fig. 8 replacement of K^+ by Na^+ decreased ATP-induced $^{45}Ca^{2+}$ uptake considerably. With increasing K^+ concentrations $^{45}Ca^{2+}$ uptake became higher, and the effect on Ca^{2+} uptake seemed to persist longer.

Effect of Phosphate. Since mitochondrial Ca^{2+} uptake can be stimulated by phosphate that causes Ca^{2+} accumulation by precipitation of calcium phosphate in mitochondria, we have tested its influence on $^{45}Ca^{2+}$ uptake. When 5 mmol/liter KH_2PO_4/K_2HPO_4 (pH 7.4) was added no increase in $^{45}Ca^{2+}$ uptake was observed (*data not shown*).

Inhibitors of ATP-Induced $^{45}Ca^{2+}$ Uptake

Mitochondrial Inhibitors. Inhibitors of electron transport such as antimycin A, rotenone, cyanide and azide, and those of oxidative phosphorylation like oligomycin had no effect on ATP-induced Ca^{2+} transport when given alone at Ca^{2+} concentrations lower than 10^{-6} mol/liter (i.e. addition of tracer without EGTA [Table 2], or adjusted with EGTA [Fig. 6]. At 10^{-5} mol/liter of Ca^{2+} the redox inhibitors antimycin and rotenone inhibited ATP-promoted Ca^{2+} uptake by 20% and the ATPase inhibitor oligomycin by 60% (Table 2). When given in combination of an electron transport inhibitor and oxidative phosphorylation inhibitor, however, ATP-promoted $^{45}Ca^{2+}$ uptake was reduced by 30 to 40% at low Ca^{2+} concentrations (Table 2 and Fig. 6) and by 95% at 10^{-5} mol/liter of Ca^{2+} (Table 2) or 50 to 60% (Fig. 6), suggesting that significant mitochondrial Ca^{2+} uptake does not occur below 10^{-7} mol/liter of free Ca^{2+}. Ruthenium red, considered to inhibit specifically $^{45}Ca^{2+}$ uptake into mitochondria [49] did not affect ATP-induced Ca^{2+} at low Ca^{2+} concentrations. However, the uncoupler of oxidative phosphorylation, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (CFCCP), inhibited ATP-induced $^{45}Ca^{2+}$ uptake by 60% (Table 2) and atractyloside, the inhibitor of mitochondrial ATP/ADP exchange [34] by 30% at low $^{45}Ca^{2+}$ concentrations (Table 2).

Nonmitochondrial Inhibitors. Mersalyl, caffeine and quercetin have been shown to inhibit Ca^{2+} transport in sarcoplasmic reticulum [30, 46, 61]. In synaptosomes a nonmitochondrial Ca^{2+} sequestration mechanism has been demonstrated that could be inhibited by A23187, tetracaine, and mersalyl [8]. Mersalyl (10^{-3} mol/liter) abolished $^{45}Ca^{2+}$ uptake at low $^{45}Ca^{2+}$ concentration (Table 2). It even caused a rapid $^{45}Ca^{2+}$ release from saponin-treated cells. The following addition of ATP did not enhance $^{45}Ca^{2+}$ uptake (*time course not shown*). A similar observation was made with the Ca^{2+} ionophore A23187 that completely abolished the ATP effect when added to acini 20 min before ATP addition (Table 2). During this time $^{45}Ca^{2+}$ content even decreased rapidly to about 50% of the value before addition of A23187 (Fig. 4). Although this ionophore also uncouples mitochondria, its 100% inhibitory effect at low [Ca^{2+}] suggests that it also acts on nonmitochondrial Ca^{2+} uptake. Caffeine at a relatively high concentration inhibited ATP-induced $^{45}Ca^{2+}$ uptake by 36% at low Ca^{2+} concentrations and had no effect at 10^{-5} mol/liter [Ca^{2+}], whereas quercetin inhibited it by 10 to 30% (Table 2). The effects of both, however, were not higher than a combination of redox- and ATPase-inhibitors on Ca^{2+} uptake at low [Ca^{2+}]. Furthermore, the effects of both mitochondrial inhibitors and caffeine or quercetin, respectively, were not clearly additive (*data not shown*). It is therefore likely that in our preparation both substances affect mitochon-

Table 2. Effect of inhibitors on ATP-promoted $^{45}Ca^{2+}$ uptake into saponin-treated acini[a]

Inhibitor	mol/liter	Inhibition (%) (mean ± SE)			
		$[^{45}Ca^{2+}]$ $<10^{-6}$ mol/liter	n	$[^{45}Ca^{2+}]$ 10^{-5} mol/liter	n
ruthenium red (RR)	10^{-5}	no effect	1	–	
oligomycin	10^{-5}	no effect	1	58, 59	2
cyanide	10^{-5}	no effect	1	–	
antimycin A (amA)	10^{-5}	no effect	1	20, 24	2
azide	10^{-4}	no effect	2	–	
	10^{-6}	no effect	2	–	
	10^{-8}	no effect	2	–	
rotenone	10^{-5}	no effect	1	24	1
N,N-dicyclohexylcarbodiimide (DCC)	10^{-5}	no effect	1	–	
RR + DCC	10^{-5}, 10^{-5}	no effect	1	–	
calmodulin	10^{-9}	no effect	2	–	
CFCCP	4×10^{-4}	60 ± 8	6	–	
KCN + oligomycin	10^{-5}, 10^{-5}	39	1	–	
rotenone + amA + oligomycin	10^{-5} each	37 ± 2	4	–	
amA + oligomycin	10^{-5} each	–		95	1
atractyloside	10^{-5}	31 ± 3	5	–	
caffeine	5×10^{-3}	36 ± 6	3	no effect	2
quercetin	2.5×10^{-5}	no effect	1	–	
	5×10^{-5}	no effect	1		
	10^{-4}	14	1	24	1
	10^{-3}	27	1	–	
mersalyl	10^{-3}	100	1	–	
A23187	10^{-6}	100	2	–	
trifluoperazinedihydrochloride	10^{-4}	66, 42	2	–	

[a] Inhibitors were added to saponin-treated acini at indicated concentrations 20 min before addition of ATP (5 mmol/liter). The data are values at 10 min after addition of ATP. Ca^{2+} concentrations were not adjusted with EGTA. Either only $^{45}Ca^{2+}$ (2 μCi/ml) was added resulting in a free Ca^{2+} concentration of $\sim10^{-6}$ mol/liter or lower (left column), or 10^{-5} mol/liter of Ca^{2+} was added (right column). n = number of experiments.

dria. Trifluoperazine dihydrochloride that antagonizes the action of the calcium-binding protein calmodulin, inhibited ATP-stimulated $^{45}Ca^{2+}$ uptake by 66 and 42% at low $[Ca^{2+}]$, whereas the activator calmodulin had no effect.

Effect of pH on ATP-Promoted $^{45}Ca^{2+}$ Uptake

In three experiments maximal ATP effects were obtained around pH 7.8. At higher pH values the effect on $^{45}Ca^{2+}$ uptake rapidly decreased (*data not shown*).

Electron Microscopy and Mass Spectroscopy

Figure 9 shows the presence of electron-dense precipitates in the rough endoplasmic reticulum and mitochondria of acinar cells incubated with saponin (45 μg/ml) and in the presence of ATP (5 mmol/liter), K^+-oxalate (20 mmol/liter) and Ca^{2+} (10^{-4} mol/liter), whereas in the absence of ATP no precipitates were seen (Fig. 10). Oxalate precipitates were observed in 70% out of 600 cells, when the incubation medium contained ATP, whereas in the absence of ATP and an ATP generating system, precipitates were observed in only 5% of 600 cells. Figure 11 shows the result of laser microprobe mass analysis in areas of section through acini using the LAMMA 500. It illustrates increased calcium content in areas of dark precipitates as compared to those without precipitates.

Discussion

In the exocrine pancreas Ca^{2+} ions are considered to be the second messenger for most secretagogues that stimulate enzyme secretion. Its trigger function for exocytosis has often been compared with that in muscle contraction. Much of the muscle Ca^{2+} is stored in the sarcoplasmic reticulum and contraction is triggered when some of the stored Ca^{2+} is released into the sarcoplasmic space after depolarization of the muscle plasma membrane [18, 24]. Relaxation occurs when released Ca^{2+} is reaccumulated by the sarcoplasmic reticulum [28, 29]. Studies on $^{45}Ca^{2+}$ movements in isolated pancreatic acinar cells and acini have shown that Ca^{2+} is initially released from

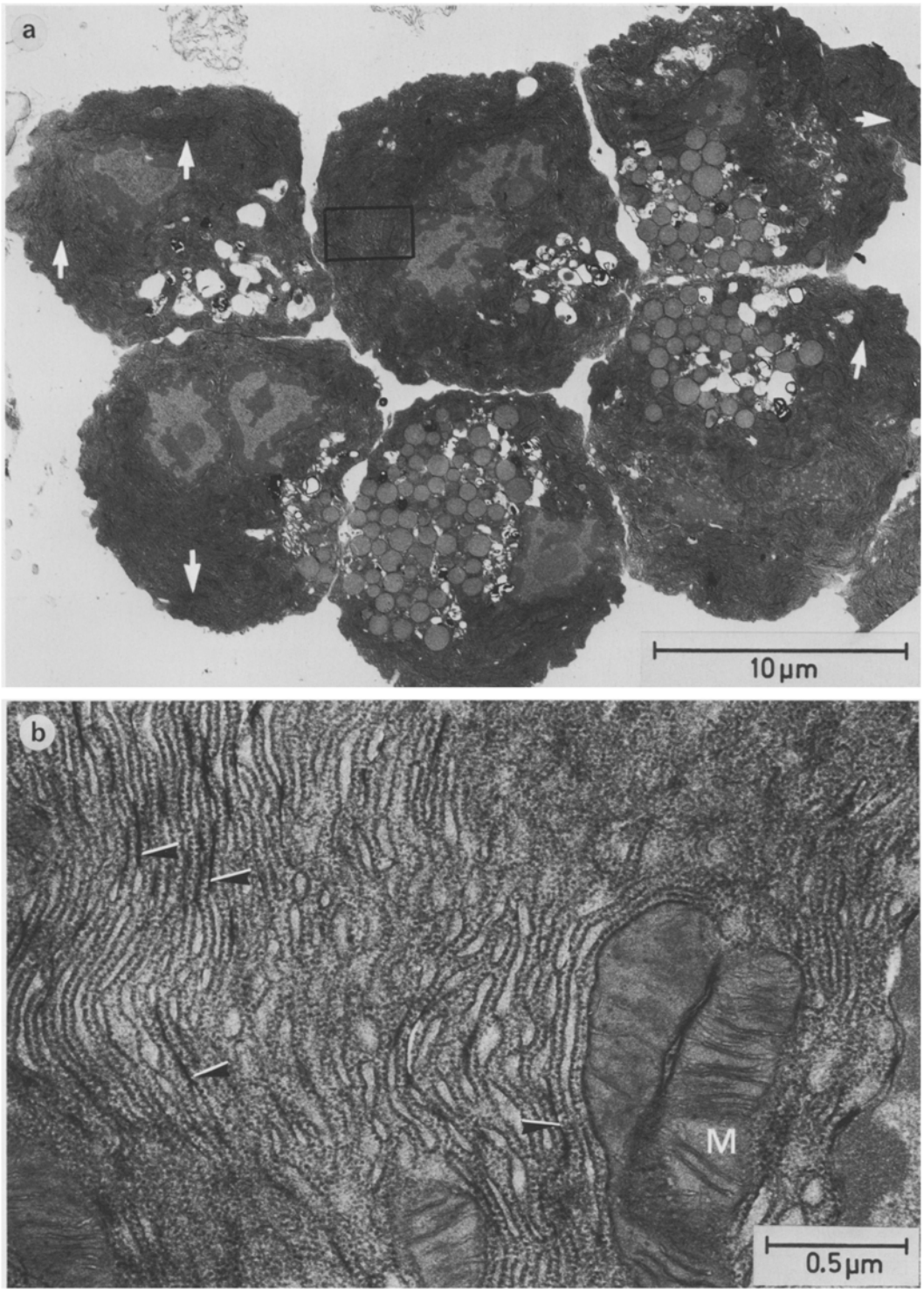

Fig. 9. (*a*) Electron micrograph of a section through several acinar cells from rat pancreas. Cells were incubated in a medium containing saponin (45 µg/ml), calcium (10^{-4} mol/liter), K^+-oxalate (20 mmol/liter), ATP (5 mmol/liter) and an ATP-generating system (phosphoenol-pyruvate, 2 mmol/liter) and pyruvate kinase (10 U/ml). Unspecific extracellular precipitates are seen at the cell peripherie. Occasionally also dark precipitates are found in the supranuclear region of the cell (in secondary lysosomes, and to a smaller degree in the golgi apparatus and condensing vacuoles). These precipitates, however, were also seen in the absence of ATP (Fig. 10). In the basal part of the cells dark areas (arrows) are seen in the rough endoplasmic reticulum. (*b*) Higher magnification of the indicated area in Fig. 1. Electron dense precipitates can be seen in the lumen of the cistern of the rough endoplasmic reticulum (arrowheads) and in mitochondria (*M*)

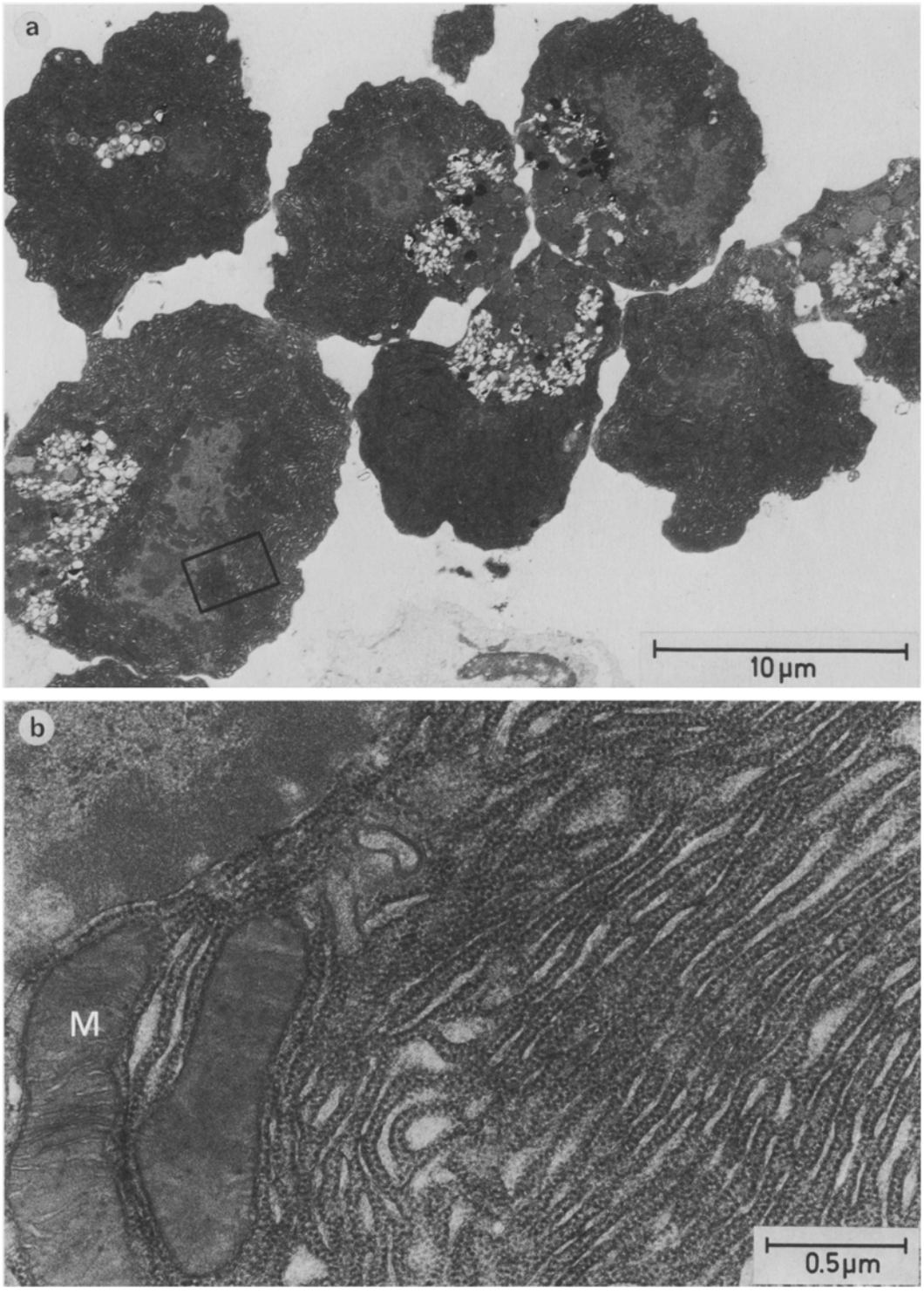

Fig. 10. (*a*) Micrograph of acinar cells from rat pancreas which were incubated as control in the same medium as above but without ATP and an ATP-regenerating system. Only lysosomal, golgi elements and sometimes condensing vacuoles appear to be black. No precipitates are found in the cisterns of the rough endoplasmic reticulum. (*b*) Higher magnification of the indicated area in *a*

the cells following stimulation by secretagogues [26, 62]. In a second phase calcium is taken up again from the cell, the amount of which can exceed the unstimulated control value [62]. The sites of Ca^{2+} release and Ca^{2+} reuptake have not yet been identified. In order to obtain some insight into the nature of intracellular Ca^{2+} pools, we have performed experiments in which the ATP dependency of Ca^{2+} uptake into intracellular Ca^{2+} stores could be directly studied. Saponin and digitonin have previously been used

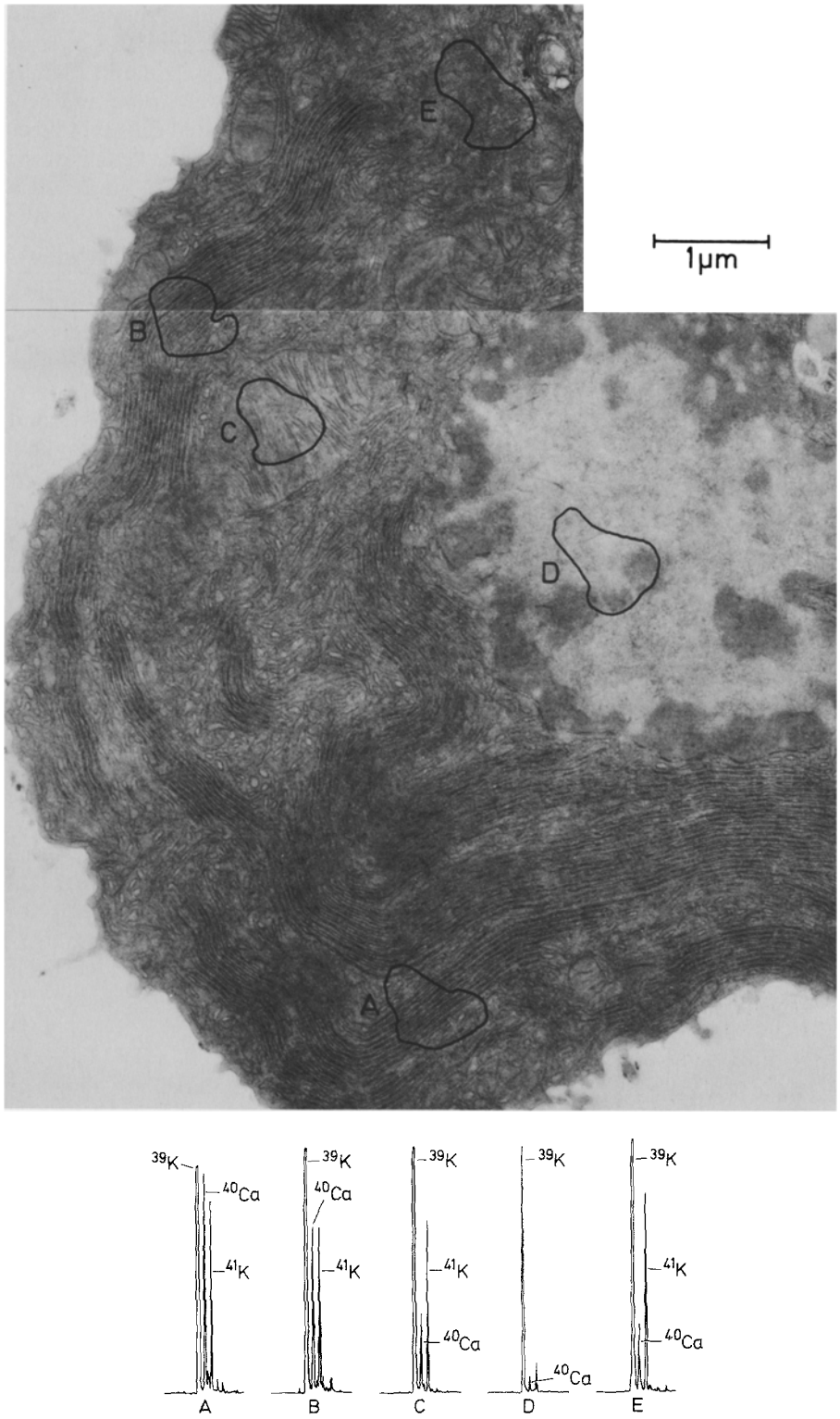

Fig. 11. Laser microprobe mass analysis of calcium precipitates. The micrograph depicts the basal part of an exocrine pancreatic cell, containing precipitates in the rough endoplasmic reticulum. The encircled areas mark sites that had been analyzed by mass sepctroscopy. In the lower panel the corresponding LAMMA-spectra are given. Dark areas containing precipitates are sites of calcium accumulation, whereas small amounts of calcium were present in areas without precipitates

to "chemically skin" muscle fibers [25] and to disrupt synaptosomes [8]. It was assumed that saponin interacts with cholesterol and therefore should affect primarily cholesterol-rich membranes such as plasma membranes [37, 63], whereas cholesterol-poor membranes, such as those of endoplasmic reticulum and mitochondria [37] should be less or not affected. A similar effect has been ascribed to digitonin. Indeed, Ca^{2+} uptake into isolated skeletal muscle sarcoplasmic reticulum vesicles was unaffected by a 5- to 6-hr

exposure to low concentrations of digitonin or saponin [45]. Using digitonin it became possible to determine the cytosolic free Ca^{2+} concentration and intracellular Ca^{2+} buffering in isolated permeabilized hepatocytes [6, 50].

In the present study trypan blue uptake into isolated acini could be increased by incubating acini with saponin or digitonin. At 45 μg of saponin or digitonin per ml of incubation medium, trypan blue uptake was 90 to 100% and ATP-induced $^{45}Ca^{2+}$ uptake was maximal. At higher concentrations the ATP effect was smaller suggesting that intracellular membranes were affected too. In untreated cells trypan blue uptake was only 3 to 5%. Similarly release of cytoplasmatic lactate dehydrogenase (LDH) from saponin- and digitonin-treated cells was increased. Remarkably LDH release was the same in control acini and those treated with 20 μg of saponin/ml up to 80 min. Then it increased to double the control in saponin acini within the following 20 min, whereas at higher saponin concentrations LDH release immediately increased (*not shown*). Similarly, ATP-induced $^{45}Ca^{2+}$ uptake slowly increased following 70 to 80 min of incubation with 20 μg/ml of saponin (Fig. 1, left). This lets us assume that at low saponin concentrations relatively more acini stay intact for a longer time as compared to higher concentrations or that a critical number of pores had to be formed in the plasma membrane before the cell became leaky. Access of ATP to intracellular sites and ATP breakdown due to the presence of a variety of ATPases and adenylcyclases should therefore be slower at low concentrations of saponin as compared to higher concentrations that caused 100% leaky cells. Indeed, when ATP breakdown was measured with the luciferin-luciferase method, we observed that within 5 min 40% of 5 mmol/liter ATP added to intact acini had disappeared, whereas in saponin-treated cells it was 65% (*data not shown*). It was therefore necessary to use relatively high concentrations of ATP at which maximal Ca^{2+} uptake was observed (i.e. 10–20 min following addition of 5 mmol/liter of ATP). The late decline of Ca^{2+} uptake even at higher ATP concentrations suggests leakiness of the membrane over which ATP-dependent Ca^{2+} transport occurs. Thus, following breakdown of ATP, return of the Ca^{2+} distribution to the level before addition of ATP could take place (Figs. 1–5, 7, 8). In acini treated with 45 μg and higher concentrations of saponin or digitonin intracellular ions and smaller molecules should rapidly equilibrate with the external medium and it can be assumed that K^+, Ca^{2+} and Na^+ concentration differences over the plasma membrane could not be maintained. We therefore used an incubation medium of high potassium, low sodium and low Ca^{2+} concentrations to mimic intracellular conditions. When ATP was added to cells treated with 45 μg saponin, a rapid uptake of $^{45}Ca^{2+}$ occurred exceeding the control value by three- and fourfold (Fig. 1, left, and Fig. 2). At 20 μg saponin $^{45}Ca^{2+}$ uptake was slower and persisted longer (Fig. 1, left). In untreated cells this effect was also there, probably due to some leakiness of isolated acini. However, it was only about 20% as compared to saponin-treated cells (Fig. 3).

Effect of Other Nucleotides

When ADP was added, $^{45}Ca^{2+}$ uptake was increased, too; however, it was only 60% of the ATP effect (Fig. 3). Since the ADP effect could be due to conversion to ATP via adenylate- or myokinases we used the adenylate kinase inhibitor diadenosylpentaphosphate (DPP) [41]. The ADP- but not the ATP-induced Ca^{2+} uptake was inhibited by this inhibitor at 10^{-5} mol/liter, a concentration shown to inhibit adenylate kinase effectively [41]. The data therefore suggest that the ADP effect was due to conversion to ATP. At higher concentrations, however, the ATP effect was inhibited by 50% (at 10^{-4} mol/liter) or abolished (at 10^{-3} mol/liter). Since DPP contains moieties like ATP and binds to ATP- and AMP-binding sites that are located in close neighborhood on the adenylate kinase unspecific effects of DPP, for instance binding to two closely located ATP-binding sites on the ATPase might be possible. Recently a Ca^{2+} (Mg^{2+}-independent) ATP-dephosphohydrolase has been described in membranes from the exocrine pancreas that also hydrolyzes ADP. Since in our experiments the effect of ADP was abolished by DPP and was Mg^{2+} dependent (*see below*) it does not seem to be likely that this enzyme is involved in the observed ADP-promoted Ca^{2+} uptake.

Other nucleotides such as UTP, CTP, ITP and GTP did not increase $^{45}Ca^{2+}$ uptake. As shown in Fig. 5 these nucleotides even caused a rapid $^{45}Ca^{2+}$ release. This effect is most likely due to chelation of extracellular Ca^{2+} with a consequent decrease in free $[Ca^{2+}]$ followed by Ca^{2+} efflux from the Ca^{2+} store. It could, however, also be interpreted to mean that these nucleotides displaced residual ATP from its binding sites. Since both Ca^{2+} influx into and Ca^{2+} efflux from a Ca^{2+} sequestering compartment could be considered to be equal at steady state, removal of ATP from its binding site at the Ca^{2+} pump should result in decrease in the Ca^{2+} influx component and a transient predominance of the Ca^{2+} efflux component.

Two analogs of ATP with blocked γ-phosphate groups, AMP-PCP and AMP-PNP similarly decreased $^{45}Ca^{2+}$ uptake (Fig. 4). This experiment therefore indicates that a Ca^{2+} ATPase is involved in ATP-promoted Ca^{2+} uptake.

Cation Dependence

Ca^{2+}. ATP-induced Ca^{2+} uptake was tested at different Ca^{2+} concentrations in the incubation medium (Fig. 6). The maximal effect of ATP-promoted Ca^{2+} uptake was observed at 10^{-5} mol/liter of Ca^{2+}. However, in the presence of the mitochondrial blockers, oligomycin and antimycin, maximal ATP-promoted Ca^{2+} uptake was only 50% of that without inhibitors and the Ca^{2+} uptake curve was shifted to the left with a half-maximal Ca^{2+} uptake between 10^{-8} and 10^{-7} mol/liter, a Ca^{2+} concentration close to intracellular free [Ca^{2+}] measured in other cells [3, 7, 19]. We can conclude from these data that at low Ca^{2+} concentration up to 10^{-7} mol/liter mitochondrial Ca^{2+} uptake is very small and that the intracellular free Ca^{2+} concentration is regulated by a Ca^{2+} sequestration mechanism other than mitochondria, whereas at higher Ca^{2+} concentrations mitochondria take up Ca^{2+} too. From the amount of $^{45}Ca^{2+}$ EGTA-buffered Ca^{2+} concentrations (Fig. 6) it can be estimated that the total $^{45}Ca^{2+}$ concentration of 10^{-6} mol/liter without EGTA which we used throughout this study results in a free Ca^{2+} concentration that is far less than 10^{-6} mol/liter. At this concentration mitochondrial uptake was negligible and could be partly inhibited by a combination of redox and ATPase inhibitors but not with one of the inhibitors alone (Table 2). Since EGTA might have unspecific effects on membrane proteins, we preferred to use unbuffered $^{45}Ca^{2+}$, although the free Ca^{2+} concentration was not exactly known.

Mg^{2+}. A Ca^{2+}-stimulated Mg^{2+}-dependent ATPase has been described in microsomes from rat pancreas [42, 44]. On the other hand, the presence of a Ca^{2+}-stimulated Mg^{2+}-independent ATPase has been demonstrated in pancreatic membranes, too [39, 48]. We therefore tested if the presence of Mg^{2+} was required for the ATP- and ADP-promoted $^{45}Ca^{2+}$ uptake in our system. As illustrated in Fig. 7 omission of Mg^{2+} from the incubation medium inhibited Ca^{2+} uptake. Similarly, the Na_2ADP effect was completely abolished (*data not shown*). This agrees with our assumption that a Ca^{2+} (Mg^{2+}-dependent) ATPase is involved in ATP-promoted $^{45}Ca^{2+}$ uptake. It further suggests that the ADP-dephosphohydrolase present in the pancreas as described by Le Bel et al. [40] is not involved in the Ca^{2+} uptake induced by ATP or ADP as observed in the present study.

Na^+ and K^+. Electrophysiological measurements have shown that secretagogues of enzyme secretion depolarize acinar cells by increasing the plasma membrane permeability to Na^+ [32]. Studies with $^{22}Na^+$ showed increased Na^+ uptake into pancreatic acinar cells in the presence of secretagogues [54]. Increased Na^+ uptake into acinar cells might be necessary for NaCl and fluid secretion that is evoked by secretagogues of enzyme secretion in herbivores [59]. However, a role of Na^+ in enzyme secretion has also been considered [12]. Similarly as had been described for liver mitochondria [11], increased intracellular Na^+ concentration could inhibit Ca^{2+} uptake into intracellular stores, thereby releasing Ca^{2+}, which in turn increases its free concentration in the cytosol. Since it is assumed that increased cytosolic Ca^{2+} concentrations trigger enzyme secretion, Na^+ would be indirectly a promoter for this process. As shown in Fig. 8 Na^+ inhibits ATP-promoted Ca^{2+} uptake, an effect that should lead to raised cytosolic free Ca^{2+} concentrations in intact cells. The significance of this observation, however, is not clear, since ouabain that should raise intracellular Na^+ and consequently [Ca^{2+}] in the cytosol did neither increase enzyme secretion in pancreatic acini nor in the intact organ [12].

Inhibitors of Ca^{2+} Uptake

As mentioned above, at low Ca^{2+} concentrations ($< 10^{-6}$ mol/liter) mitochondrial uptake can be assumed to be small since it was partly inhibited by mitochondrial redox- and ATPase inhibitors only when given together (Table 2). We explain this observation with the capability of mitochondria to use the energy for Ca^{2+} transport from the electrochemical H^+ ion gradient as well as from ATP. If one of the pathways for Ca^{2+} uptake is blocked by either redox or ATPase inhibitors, the other pathway is used. At higher Ca^{2+} concentrations mitochondrial uptake predominated and could be inhibited by either inhibitor alone or in combination up to 70 and 95% (Fig. 6 and Table 2, respectively). CFCCP, an uncoupler of oxidative phosphorylation inhibited Ca^{2+} uptake by $\sim 60\%$ at low Ca^{2+} concentrations. The higher effect of CFCCP as compared to other mitochondrial inhibitors could be due to increased mitochondrial ATPase activity and therefore increased breakdown of ATP. It could also mean, however, that the nonmitochondrial Ca^{2+} uptake was reduced by increased H^+ permeability of the membrane over which Ca^{2+} transport takes place.

The Ca^{2+} ionophore A23187 abolished Ca^{2+} uptake completely; moreover, A23187 rapidly released previously accumulated Ca^{2+} from these storage sites (Fig. 4). It therefore appears that the observed ATP-promoted Ca^{2+} uptake occurs into a vesicular space and that the membrane over which Ca^{2+} is taken up can be made permeable to Ca^{2+} by this ionophore. From these observations Ca^{2+} uptake could be divided operationally into two distinct processes, both

requiring ATP but only one that is inhibited by mitochondrial blockers. Two forms of Ca^{2+} uptake had also been described for isolated axoplasm from giant axons. However, only one of these required ATP and was blocked by cyanide and oligomycin, whereas the other form was energy-independent Ca^{2+} binding [4]. Electron-microscopic pictures showed the presence of Ca^{2+} oxalate precipitates in the rough endoplasmic reticulum in saponin-treated cells in the presence of ATP (Figs. 9 and 11). These precipitates were absent without ATP (Fig. 10) and when A23187 was added to the ATP-containing incubation medium (*not shown*). We therefore assume that A23187 affects a Ca^{2+} storage pool located in the rough endoplasmic reticulum. These observations are similar to those obtained in presynaptic nerve terminals treated with saponin [47]. Using ultrastructural and electron microprobe analysis, these authors found electron dense Ca^{2+} oxalate deposits in intraterminal mitochondria and smooth endoplasmic reticulum cisterns. In the presence of either the Ca^{2+} ionophore A 23187 or the Ca^{2+} chelator EGTA electron dense deposits were very rarely observed within the intraterminal organelles [47]. The mercurial compound mersalyl that had been shown to inhibit the Ca^{2+} sequestration in sarcoplasmic reticulum [30, 46] abolished nonmitochondrial Ca^{2+} uptake in our system, whereas the effect of caffeine and quercetin [23, 61] was less clear (Table 2). Trifluoperazine, a specific calmodulin antagonist that inhibits calmodulin-dependent stimulation of Ca^{2+}-dependent enzymes such as Ca^{2+} ATPases, cyclic nucleotide phosphodiesterases and protein kinases [38, 64] decreased ATP-dependent Ca^{2+} uptake (Table 2). This therefore suggests that the Ca^{2+}-regulator protein is involved in the Ca^{2+} uptake mechanism of pancreatic acini. Since calmodulin itself had no effect when added to acini (Table 2) we assume that sufficient amounts of endogenous calmodulin were present in saponin-treated acini.

pH Effect

As could be expected from other Ca^{2+} transporting systems and Ca^{2+} ATPase in a variety of tissues, the pH optimum for ATP-induced Ca^{2+} uptake was found to be around 8.0 (*data not shown*). This indicates an influence of pH on the transport system, or on the membrane permeability for Ca^{2+} over which transport takes place.

This study was performed to obtain insight into intracellular Ca^{2+} pools and mechanisms involved in uptake and release of Ca^{2+} from these pools. The use of substances that selectively increase plasma membranes to high molecular substances, made it possible to study ATP-promoted Ca^{2+} uptake in isolated acini and inhibitors on it. The present data give evidence for ATP-promoted Ca^{2+} uptake into mitochondria and the rough endoplasmic reticulum. It is likely that these Ca^{2+} pools play a major role in Ca^{2+} uptake during stimulation with secretagogues of enzyme secretion. Indeed with different methods both mitochondria and endoplasmic reticulum have been suggested as candidates for Ca^{2+} pools involved in secretagogue-induced Ca^{2+} release and reuptake [13–15, 17, 20, 53]. We assume that during stimulation with secretagogues Ca^{2+} is taken up into intracellular ATP-dependent Ca^{2+} pools [62]. Probably these stores are identical to those described in the present study. However, direct evidence for this assumption is still lacking. We hope that the method to make intracellular organelles directly accessible to high molecular substances will be useful in further investigation of these problems.

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