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Intracellular Uptake and α-Amylase and Lactate Dehydrogenase Releasing Actions of the Divalent Cation Ionophore A23187 in Dissociated Pancreatic Acinar Cells

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Summary. Intracellular uptake of A23187 and the increased release of amylase and lactate dehydrogenase (LDH) accompanying ionophore uptake was studied using dissociated acinar cells prepared from mouse pancreas. Easily detected changes in the fluorescence excitation spectrum of A23187 upon transfer of the ionophore from a Tris-buffered Ringer's to cell membranes were used to monitor A23187 uptake. Uptake was rapid in the absence of extracellular Ca^{2+} and Mg^{2+} ($t^1/_2 = 1$ min) and much slower in the presence of Ca^{2+} or Mg^{2+} ($t^1/_2 = 20$ min). Cell-associated ionophore was largely intracellular as indicated by fluorescence microscopy, lack of spectral sensitivity to changes in extracellular Ca^{2+} and Mg^{2+}, and by equivalent interaction of ionophore with membranes of whole and sonicated cells.

A23187 (10 μM) increased amylase release 200% in the presence of extracellular Ca^{2+} and Mg^{2+}. In the absence of Ca^{2+} (but in the presence of Mg^{2+}) A23187 did not increase amylase release. A23187 (10 μM) also produced Ca^{2+}-dependent cell damage, as judged by increased LDH release, increased permeability to trypan blue, and by disruption of cell morphology. The cell damaging and amylase releasing properties of A23187 were distinguished by their time course and dose-response relationship. A23187 (1 μM) increased amylase release 140% without increasing LDH release or permeability to trypan blue.

Previous studies have shown that the divalent cation ionophore A23187 in the presence of extracellular calcium can produce increased amylase release from pancreatic acinar cells (Selinger *et al.*, 1974; Williams & Lee, 1974; Schreurs *et al.*, 1976). The ability of A23187 to stimulate secretion in the pancreas as well as in a number of other tissues (Foreman, Mongar, & Gomperts, 1973; Cochrane & Douglas, 1974; Russell, Hansen, & Thorn, 1974; Charles *et al.*, 1975; Cochrane *et al.*, 1975; Garcia, Kirpekar, & Prat, 1975; Wollheim *et al.*, 1975) has been thought to mean that the ionophore acts at the plasma membrane to increase influx of extracellular calcium and the resulting rise in cytoplasmic calcium activity is able to trigger exocytosis. Although the iono-

phore does increase calcium fluxes across the plasma membrane of whole cells (Reed & Lardy, 1972*a*; Foreman *et al.*, 1973; Hainaut & Desmedt, 1974; Williams & Lee, 1974; Kondo & Schultz, 1976), the assumption that A23187 acts exclusively at the plasma membrane is not well documented. Indeed, many compounds which incorporate into membranes such as ionophores (X-537A), mitochondrial uncouplers (DNP, FCCP), or fluorescent probes (ANS, chlortetracycline) have been localized or are known to act on intracellular organelles as well as the plasma membrane. No studies have dealt with intracellular uptake of A23187 directly, but the fact that A23187 can release calcium from sarcoplasmic reticulum vesicles and isolated mitochondria (Caswell & Pressman, 1972; Pressman, 1972; Reed & Lardy, 1972*b*; Scarpa, Baldassare, & Inesi, 1972; Wong *et al.*, 1973; Sordahl, 1975; Binet & Volfin, 1975) and the finding that A23187 can induce secretion or contraction in some cells in the absence of extracellular calcium (Hainaut & Desmedt, 1974; Schroeder & Strickland, 1974; Steinhardt & Epel, 1974; Ashby & Speake, 1975; Charles *et al.*, 1975; Karl *et al.*, 1975; Murray, Reed, & Fay, 1975) suggest that the ionophore is able to release intracellular calcium.

Calcium transport by A23187 at both plasma and intracellular membranes is of interest in the pancreatic acinar cell because extracellular and intracellular calcium may each play a role in stimulus-secretion coupling (Robberecht & Christophe, 1971; Case & Clausen, 1973; Chandler & Williams, 1974; Gardner *et al.*, 1975; Kondo & Schultz, 1976). In the present study we use fluorometry and fluorescence microscopy to demonstrate intracellular uptake of A23187 by dissociated pancreatic acinar cells. A23187 stimulates amylase release at low concentrations and produces cell damage at higher concentrations, both actions being dependent on extracellular calcium. Thus, at the plasma membrane ionophore-mediated entry of calcium is able to initiate exocytosis and cytoxic effects independently. Although A23187 is available at intracellular membranes, it does not appear to release intracellularly sequestered calcium at a rate sufficient to trigger secretion.

Materials and Methods

Tissue Preparation and Incubation

Dissociated pancreatic acinar cells were prepared by the method of Amsterdam and Jamieson (1972, 1974) with minor modifications (Williams, Cary, & Moffat, 1976). Pancreatic tissue (0.7 to 0.9 g) was obtained from 8 to 12 male Swiss white mice (18 to 24 g) which were fasted 16 hr prior to use. The dissociation procedure utilized a combination

of enzymatic digestion, divalent cation depletion and mechanical shearing. 100 U/ml of pure collagenase, 0.1 mg/ml chymotrypsin (both Worthington Biochemicals) and 2 mg/ml hyaluronidase (Sigma Type 1) were used in the digestion step. After dissociation cells were filtered through 75 μ mesh nylon cloth and centrifuged through a 4% albumin gradient to remove broken and undissociated cells.

Cell dissociation was carried out either in Krebs-Henseleit Bicarbonate buffer (KHB) containing per liter (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.56; $MgCl_2$, 1.13; $NaHCO_3$, 25; NaH_2PO_4, 1.15; and equilibrated with 95% O_2–5% CO_2, or in a Tris buffered Ringer's (TR) containing per liter (in mM): NaCl, 134; Tris HCl, 10; $CaCl_2$, 2.56; $MgCl_2$, 1.13; K_2HPO_4, 1.15; KCl, 2.6; at a pH of 7.38 and equilibrated with 100% O_2. Both KHB and TR contained 14 mM glucose, 1% bovine serum albumin (fraction V, Miles laboratories), 0.1 mg/ml soybean trypsin inhibitor (Sigman type 1-S), and Minimal Eagles Medium amino acid supplement. Media for tissue dissociation and all experiments were prepared with water distilled in stainless steel vessels and passed through a demineralizing column (Barnstead, Bantam Demineralizer).

After preparation dissociated cells were preincubated 60 min, centrifuged at $100 \times g$ for 5 min, washed once, and resuspended to a cell density of 1 to 2 mg dry wt per ml in the designated incubation medium. Incubations were carried out in 25 ml siliconized flasks at 37° in a Dubnoff type water bath shaken at 60 cycles/min unless noted otherwise.

Measurement of Amylase and Lactate Dehydrogenase (LDH) Release

At the start of the incubation period, 1 ml of the cell suspension was centrifuged at $8000 \times g$ for 20 sec in an Eppendorf microcentrifuge (Brinkman Inst., #3200) and the supernatant assayed for amylase and LDH activity. These initial values (usually about 0.1 U/mg dry wt amylase and 2% total LDH) were subtracted from values obtained after incubation to obtain the enzyme activity released during incubation. The pellets of these initial samples were resuspended in 1 ml distilled H_2O, transferred to a tared aluminum foil planchet, dried overnight at 80 °C and dry wt determined. Total cellular LDH activity was measured on a cell suspension frozen and thawed three times during a 45-min period. Amylase activity was measured by the method of Rinderknecht, Wilding and Haverback (1967) and is expressed as units per mg dry wt based on the reported activity of the amylase standard (Sigma type VI). LDH activity was measured spectrophotometrically by the method of Wacker, Ulmer and Vallee (1956).

Fluorometry and Fluorescence Microscopy

Fluorometric experiments were carried out with an Aminco-Bowman spectrofluorometer having a band pass of 15 nm. The instrument was modified to allow stirring and temperature control of the cuvette contents. Uncorrected spectra were recorded on an Esterline-Angus strip chart recorder with the wavelength scale marked manually via the event marker. Quinine sulphate dihydrate (1 mg/liter 0.1 N H_2SO_4) was used to verify the wavelength settings for the excitation monochronometer at 254 and 352 nm. Kinetic experiments were carried out in one of two ways. Whole cells, sonicated cells, or erythrocyte membranes were added as a concentrated suspension in a 0.15 or 0.30 ml volume, via syringe and 16 gauge needle to a cuvette containing 3 ml of ionophore-containing medium at a temperature of 37° and the fluorescence intensity of the suspension monitored contin-

uously on a strip chart recorder. Alternatively, cells or membranes were suspended in 3 ml of ionophore-containing medium, incubated in 25 ml siliconized flasks in a Dubnoff type water bath at 37°, and at designated times poured into a cuvette and the fluorescence intensity read.

The "concentrated" suspensions of cells or membranes added to the cuvette were prepared as follows. Whole cells (3 to 6 mg dry wt) were washed once and resuspended in 0.15 ml of the cuvette medium minus ionophore. Sonicated cell concentrates were prepared by suspending an equivalent amount of dissociated acinar cells in 0.15 ml of 7 mM EDTA (pH 7.4) and sonicating for 10 min with a bath sonicator (Laboratory Supplies Co., Hicksville, N.Y., Model #T-80-80-1-RS, 80 kHz 80/320 W). Unsealed human erythrocyte membranes were prepared by the method of Heinz and Hoffman (1965) using outdated blood bank blood. Erythrocyte membranes were suspended in 0.1 mM EDTA at a protein concentration of 2.8 mg/ml and 0.3 ml of this suspension added to the cuvette. The suspension medium for all fluorometric experiments was TR containing Ca^{2+}, Mg^{2+}, and EDTA as indicated but without albumin, trypsin inhibitor, or amino acid supplement in order to minimize background fluorescence.

Fluorescence, phase contrast, and darkfield microscopy were carried out with a Zeiss photomicroscope. Fluorescence microscopy utilized a 200 W mercury vapor lamp, oil immersion condenser, UG2 exciter filter, and a 410–650 bandpass barrier filter. Darkfield and phase contrast microscopy used a tungsten lamp without filters. Incubated cells and medium were applied to a glass slide and covered with a glass slip.

Light and Electron Microscopy

Dissociated acinar cells were fixed by adding an equal volume of 0.08 M sodium cacodylate, pH 7.4, containing 1.5% glutaraldehyde and 1% paraformaldehyde. Cells were then resuspended in full strength fixative and pelleted by centrifugation for one min at $8000 \times g$ in an Eppendorf microcentrifuge. Pellets were stored overnight at 4°, postfixed in 2% OsO_4 in 0.08 M sodium cacodylate buffer, rapidly dehydrated in ethanol and embedded in Araldite. Sections 0.5 μ thick were stained with 1% toludine blue for light microscopy. Silver thin sections were doubly stained with uranyl acetate and lead citrate and viewed in a JEM-100B electron microscope at 60 kV.

Quantitation of the fraction of dead cells in thick sections was carried out by scoring all cells as "dead" or "healthy". Cells designated as "dead" were obviously vesiculated such as those indicated by arrows in the section shown in Fig. 10*B*. For each experimental condition 2 to 8 thick sections were counted, representing 4000 to 8000 cells. Counting was done "blind" and repeated at least twice for each section; duplicate counts generally agreed to within 10%.

Trypan blue permeability was tested by mixing cells plus medium with an equal volume of 4% trypan blue in 0.9% NaCl and placing a drop of this suspension on a hemocytometer grid.

Ionophore Addition

A23187 was added to incubation media as a solution in ethanol such that the final ethanol concentration was 0.5% in most experiments. This concentration of ethanol produced small to negligible effects on amylase release; controls contain ethanol where noted. The A23187 used in these experiments was generously provided by Dr. Robert Hamill of Eli Lily Co.

Results

Excitation Spectra of A23187 in Ethanol and Tris Ringer's

It is well known that the fluorescence excitation spectrum of A23187 is sensitive to polarity of the medium and to chelation of divalent cations (Case, Vanderkooi, & Scarpa, 1974; Pfeiffer, Reed, & Lardy, 1974). Changes in position and intensity of peaks have been used to characterize ionophore interaction with mitochondria and sarcoplasmic reticular vesicles (Caswell & Pressman, 1972; Wong *et al.*, 1973; Case *et al.*, 1974), but corresponding studies with whole cells have not been reported. In order to study the interaction of A23187 with whole, dissociated pancreatic acinar cells we first examined the excitation spectra of A23187 in ethanol and in the Tris buffered Ringer's (TR)[1] used for cell suspension, as well as the effect of calcium on these spectra. A23187 fluorescence was monitored at 430 nm in all experiments since its emission spectrum consisted of one broad peak, 429–435 nm, that was relatively unaffected by the experimental conditions.

In ethanol the A23187 excitation spectrum (Fig. 1*A*, solid line) consisted of two peaks with maxima at 287 nm (peak I) and 378 nm (peak II). Upon addition of Ca^{2+} (Fig. 1*A*, dashed line) peak I shifted from 287 to 303 nm, both peaks I and II decreased in intensity, and the ratio of the intensities (II/I) decreased from 5.25 to 2.4. These spectra for A23187 in ethanol are similar to these obtained by Pfeiffer *et al.* (1974).

The excitation spectrum of A23187 in TR was considerably different. In EDTA TR, the spectrum consisted of one peak of low intensity at 335 nm (Fig. 1*B*, solid line; note change in ordinate scale and 15-fold increase in ionophore concentration). In the presence of Ca^{2+} and Mg^{2+}, the spectrum contained two peaks at 308 and 372 nm (Fig. 1*B*, dashed line) and the intensity of the spectrum was low compared to A23187 plus Ca^{2+} in ethanol and was variable from preparation to preparation.

Further experiments suggested that the spectral characteristics of A23187 in the presence of Ca^{2+} or Mg^{2+} could be attributed to A23187 being in the form of a particulate suspension. The fluorescence intensity of A23187 in TR was measured in control samples (Fig. 2, filled circles) and in the supernatant following centrifugation at $27{,}000 \times g$ for 5 min (open circles). As shown in Fig. 2*A*, the intensity of A23187 in TR

1 *Abbreviations used:* TR=Tris-buffered Ringer's (*see* Methods); OCa TR=TR without added Ca^{2+}; OCaOMgTR=TR without added Ca^{2+} or Mg^{2+}; EDTA TR=OCaOMgTR with 0.23 mM EDTA added.

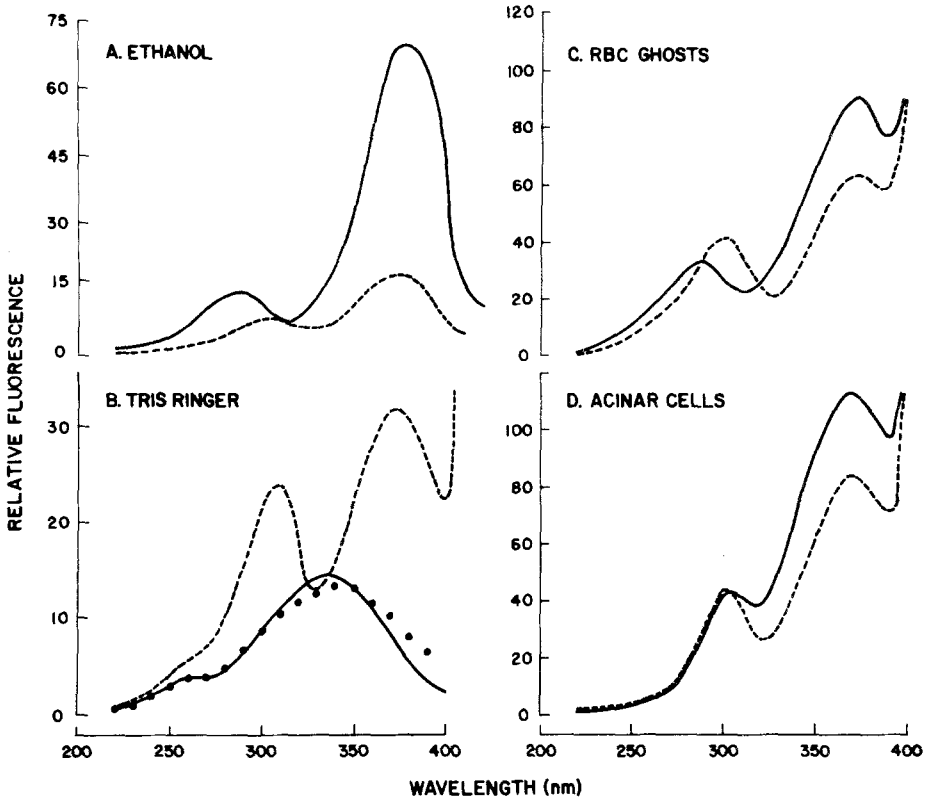

Fig. 1. Effect of Ca^{2+} and Mg^{2+} on the fluorescence excitation spectrum of A23187 in ethanol, Tris-buffered Ringer's (TR), erythrocyte membranes, and dissociated pancreatic acinar cells. (*A*) Spectra of 0.6 μM A23187 in ethanol with (----) or without (—) 2.56 mM $CaCl_2$ and 0.05% H_2O. (*B*) Spectra of 10 μM A23187 in TR (----), in EDTA TR (—), or in TR which had been centrifuged at 27,000 × *g* for 5 min (. . .). The intensity of the supernatant spectrum only has been increased 33-fold for illustration purposes. (*C*) Spectra of 10 μM A23187 in the presence of erythrocyte membranes. Spectra were recorded in either TR (----) or EDTA TR (—) 90 min after addition of membranes; final concn 0.25 mg protein per ml. (*D*) Spectra of 10 μM A23187 in pancreatic acinar cells. Acinar cells were incubated for 90 min at a cell density of 1.63 mg dry wt per ml in 3 ml of either TR (----) or EDTA TR (—) containing 10 μM A23187. Cells were washed once, resuspended in 3 ml of incubation medium minus ionophore, and their excitation spectra recorded. In all cases emission was monitored at 430 nm. Temperature was 30°. The baseline spectrum for buffer alone was arithmetically subtracted from Fig. 1*B*, . . . but not from other spectra. Light scattering and fluorescence of erythrocyte membranes or acinar cells alone contribute no greater than 7.5 and 4 units, respectively, to spectra in 1*C* and 1*D* over the range 200 to 370 nm; the contribution increases substantially above 370 nm

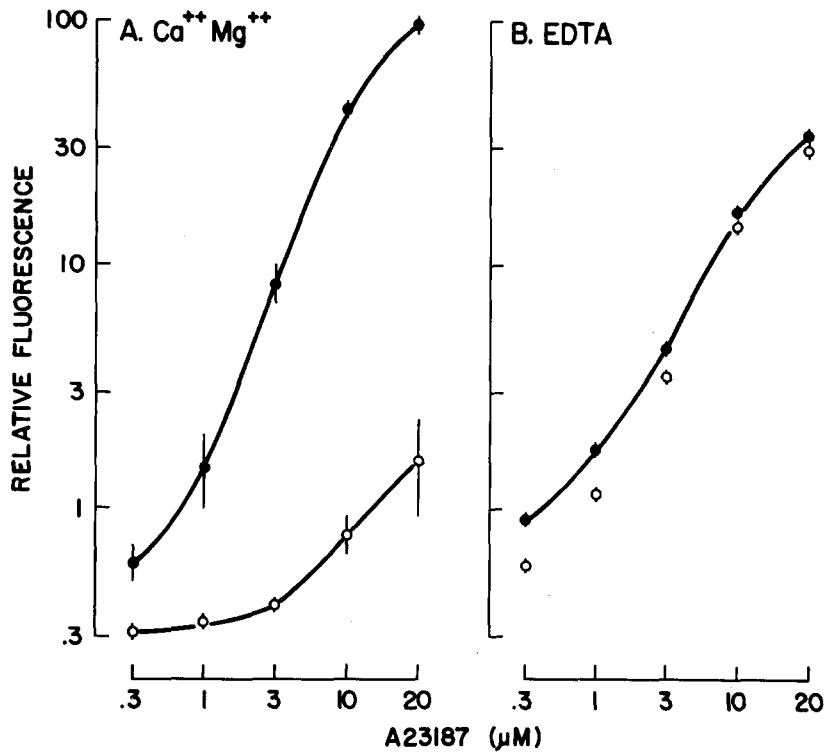

Fig. 2. Sedimentation of A23187 suspended in Tris buffered Ringer's. Samples containing the designated ionophore concentration and 1% ethanol were prepared by adding stock solutions of A23187 in ethanol to 4 ml aliquots of TR (*A*) or EDTA TR (*B*). Samples were mixed by inversion, allowed to stand for 30 min at room temperature, mixed again, and divided into two groups. One group (○) was centrifuged at 27,000 × *g* for 5 min at room temperature and the other (●) left undisturbed. Following centrifugation, the fluorescence intensities of both groups were read. Fluorescence was excited at 370 (*A*) or 340 nm (*B*) and emission read at 430 nm. Symbols are means ± SE for 3 to 7 samples

was decreased 60 to 98% by centrifugation. The excitation spectrum of the ionophore remaining after centrifugation (Fig. 1*B*, filled circles) was almost identical in form to that of ionophore in EDTA TR (Fig. 1*B*, solid line). In contrast, the fluorescence intensity of A23187 in EDTA TR was decreased only 5 to 20% (Fig. 2*B*) by centrifugation and the form of its excitation spectrum (Fig. 1*B*, solid line) was unchanged. These data suggest that in the presence of Ca^{2+} and Mg^{2+} A23187 is largely particulate and exhibits an excitation spectrum similar to that for ionophore in a less polar medium such as ethanol. Also present is a minor component, possibly ionophore in solution, having an excitation spectrum similar to ionophore in the absence of Ca^{2+} or Mg^{2+}.

Spectra of A23187 in the Presence of Erythrocyte Membranes or Dissociated Pancreatic Acinar Cells

Addition of unsealed erythrocyte membranes to A23187 in EDTA TR resulted in the A23187 excitation spectrum changing from one peak at 335 nm (Fig. 1*B*, solid line) to two peaks at 288 and 372 nm (Fig. 1*C*, solid line). In the presence of Ca^{2+} and Mg^{2+}, addition of erythrocyte membranes to A23187 produced a spectrum with two peaks at 302 and 372 nm (Fig. 1*C*, dashed line). These spectra were similar to those for A23187 in ethanol with respect to peak positions and alterations produced by divalent cations. In both cases the presence of Ca^{2+} and Mg^{2+} resulted in a shift of peak I from 288 to 302 nm, and a decrease in the ratio of peak II to peak I density. (II/I changed from 5.25 to 2.4 in ethanol and from 3.06 to 1.4 in the presence of erythrocyte membranes). These spectra indicated that the greater portion of the fluorescence signal was from ionophore which had been transferred from the aqueous medium to the less polar environment of the erythrocyte membrane.

Spectra of A23187 taken up by dissociated pancreatic acinar cells (Fig. 1*D*) were measured after washing and resuspending the cells with ionophore-free media and represent fluorescence of cellularly associated A23187 with no component from ionophore in the extracellular medium. Excitation spectra exhibited two peaks, at 303 and 369 nm both in the presence and absence of Ca^{2+} and Mg^{2+}. Again their resemblance to spectra for A23187 in ethanol suggested that the source of fluorescence was A23187 in a membrane environment.

Time Course of A23187 Uptake into Erythrocyte Membranes and Dissociated Acinar Cells

The striking changes in the excitation spectrum of A23187 upon its incorporation into membrane were used to study the time course of ionophore uptake by erythrocyte membranes and dissociated acinar cells. Fig. 3 shows that when either acinar cells or erythrocyte membranes were added to 10 μM A23187 in EDTA TR, fluorescence intensity in response to optimal excitation at 310 or 290 nm, respectively, rose to a plateau with a half time of less than one min. Membranes of acinar cells, although largely intracellular, took up ionophore almost as rapidly as the broken erythrocyte membrane. Separation of cells and medium after 5 min showed that by fluorescence, less than 25% of the A23187 remained in the medium. In contrast, when acinar cells or erythrocyte

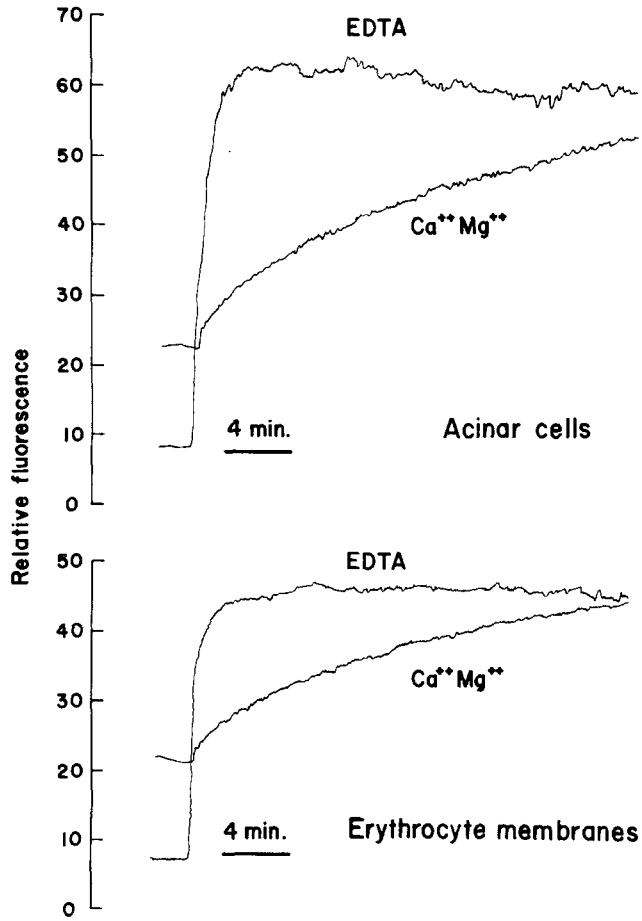

Fig. 3. Kinetics of fluorescence intensity changes during uptake of A23187 by dissociated pancreatic acinar cells and unsealed erythrocyte membranes. Acinar cells or erythrocyte membranes (RBC ghosts) were added by syringe to a cuvette containing 10 μM A23187 in 3 ml of either TR (designated $Ca^{++}Mg^{++}$) or EDTA TR (designated EDTA). Final cell density was 1.70 mg dry wt per ml and erythrocyte membrane concentration was 0.25 mg protein per ml. Cuvette contents were stirred and maintained at 37 °C. Fluorescence was excited at 310 nm except for erythrocyte membranes in EDTA TR where excitation was at 290 nm—the optimum for A23187 excitation under these conditions (*see* Fig. 1*C*, solid line). Emission was monitored continuously at 430 nm. The results are representative of 7 similar experiments

membranes were added to 10 μM A23187 in TR containing Ca^{2+} and Mg^{2+}, fluorescence intensity at 310 nm increased to a similar value but at a much slower rate. Experiments in which fluorescence was monitored for longer periods (not shown) indicated that uptake by acinar cells required 20 min to become half maximal and reached a plateau only after 90 min. Centrifugation of A23187 (27,000 × *g*, 5 min) further

reduced the rate of ionophore uptake into erythrocyte membranes by 90% suggesting that "particulate" A23187 contributed to uptake in the presence of divalent cations.

Intracellular Localization of A23187 in Dissociated Acinar Cells

The excitation spectrum of A23187 incorporated into acinar cells was different from that of A23187 in ethanol or erythrocyte membranes in one important aspect. Both the position and intensity of peak I were unaffected by the presence of Ca^{2+} and Mg^{2+} in the medium (compare Fig. 1*D* with Figs. 1*A* and 1*C*). This suggested that A23187 associated with acinar cells may be intracellular and therefore not sensitive to extracellular ionic changes. This point was investigated further by comparing A23187 uptake into whole cells and sonicated cells. If A23187 is intracellular, disruption of whole cells by sonication should increase the exposure of ionophore to extracellular divalent cations. Such differences in exposure were looked for by incubating equal amounts of whole or sonicated cells with A23187 in the absence of divalent cations for 15 to 20 min, then adding magnesium. Magnesium rather than calcium was used to avoid triggering secretion. Fig. 4 shows that uptake of A23187 from EDTA TR by both whole and sonicated cells was rapid and complete in five min. Addition of magnesium (final concn = 1.13 mM) to a cuvette containing A23187 and sonicated cells produced an immediate and large increase in intensity of fluorescence excited at 310 nm. Excitation spectra show that addition of magnesium shifts the position of peak I from 302 to 308 nm and increases its intensity (compare solid and dashed spectra, Fig. 5). This shift is similar, although not as large, as the shift of peak I in response to divalent cations seen in the spectra of A23187 in erythrocyte membranes (Fig. 1*C*). In contrast, addition of magnesium to A23187 in whole cells resulted in an immediate but much smaller increase in fluorescence intensity at 310 nm (*see* Fig. 4). Excitation spectra for A23187 in whole cells before and after addition of magnesium were nearly identical (*compare* open and closed circles in Fig. 5) and, in fact, were similar to the spectrum for A23187 in sonicated cells after magnesium addition. We interpret these changes in the excitation spectrum to mean that ionophore incorporated into membranes of broken acinar cells is exposed to extracellular ion changes and that peak I is a sensitive indicator of divalent cation chelation by membrane-associated ionophore. These changes are not due to transfer of A23187 from medium to membrane since this would increase the

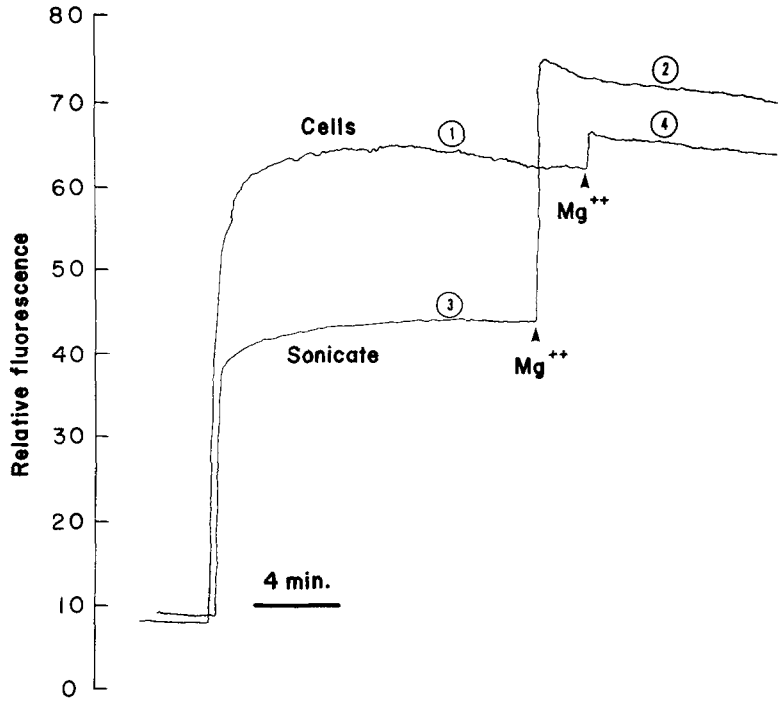

Fig. 4. Kinetics of fluorescence intensity changes during A23187 uptake by whole and sonicated pancreatic acinar cells in EDTA TR and subsequent addition of extracellular Mg^{2+}. Whole acinar cells or sonicated acinar cells were added by syringe to a cuvette containing 3 ml of 10 μM A23187 in EDTA TR. Final density of whole or sonicated cells was 1.67 mg dry wt per ml. Cuvette contents were stirred and maintained at 37 °C. $MgCl_2$ (final concn 1.13 mM) was added at the arrows. Fluorescence was excited at 310 nm and emission monitored at 430 nm continuously. The results are representative of six similar experiments

intensity of the entire spectrum. Rather, as shown in Fig. 5, peak I increased while peak II (373 nm) decreased in intensity. There was little or no contribution from ionophore not associated with membrane since identical spectral changes were observed with whole cells or sonicated cells which had been preincubated with A23187 then washed and resuspended in ionophore-free medium. The fact that the excitation spectrum of A23187 in whole cells was almost completely unaffected by Mg^{2+} addition and that this spectrum was similar to that for the ionophore in broken cells exposed to Mg^{2+} suggests that A23187 in whole cells is exposed to a finite but relatively constant divalent cation concentration in the face of large concentration changes in the extracellular medium. Because sonication resulted in spectral sensitivity of A23187 to extracellular cation addition, integrity of cell structure must be required to maintain this constant environment.

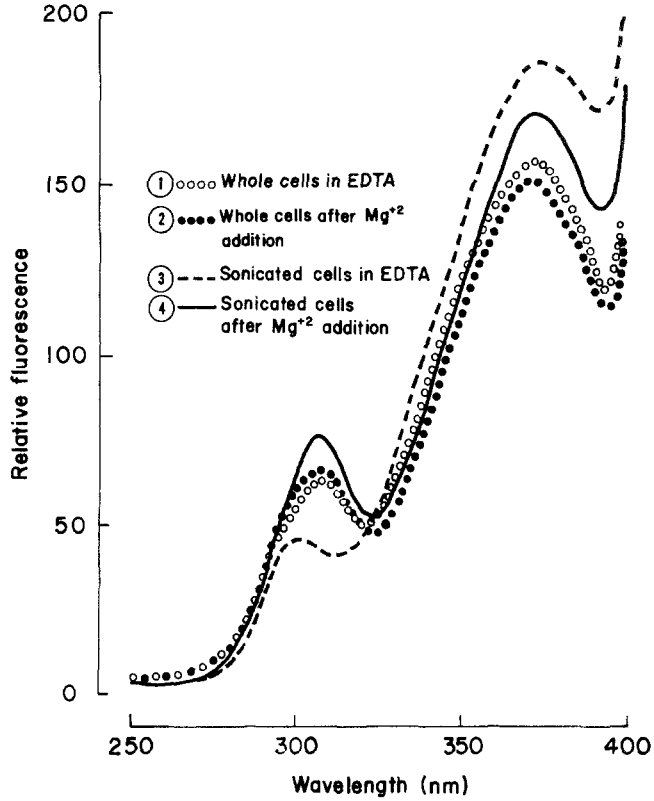

Fig. 5. Excitation spectra of A23187 incorporated into whole and sonicated pancreatic acinar cells in the absence and presence of extracellular Mg^{2+}. Excitation spectra were recorded during an experiment identical to that of Fig. 4 at the points indicated by circled numbers in Fig. 4

The protocol of the previous experiment (Fig. 4) was repeated for a 20-fold range of membrane concentrations. If ionophore incorporation were restricted to the plasma membrane of whole cells, an equal weight of cells broken up by sonication would present a many-fold greater amount of membrane in which A23187 could be incorporated. Therefore, A23187 incorporation in sonicated cells should reach a maximum at considerably lower membrane concentrations than would ionophore interaction with whole cells. However, Fig. 6 (filled squares and circles) shows that A23187 uptake by whole cells and uptake by sonicated cell membrane were similar in their dependence on membrane concentration: logarithmic functions of membrane concentration having similar slope in the range of 0.2 to 1 mg dry wt/ml, and reaching a maximum at 1.75 mg dry wt/ml. After addition of extracellular magnesium the fluores-

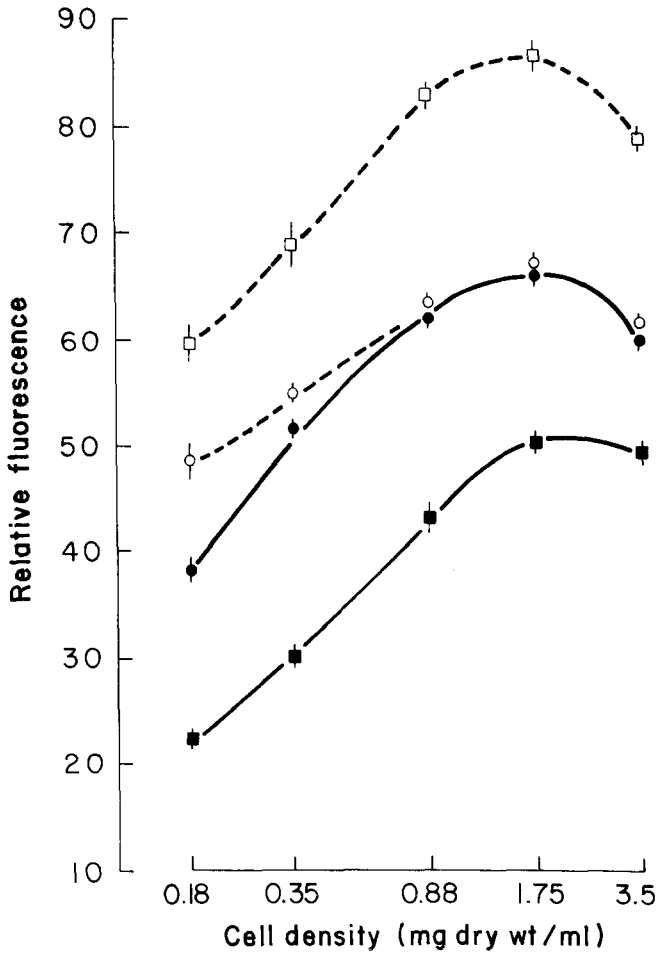

Fig. 6. Membrane concentration dependence of A23187 uptake by whole or sonicated pancreatic acinar cells. Whole or sonicated acinar cells were added to 25 ml flasks containing 10 μM A23187 in 3 ml of EDTA TR at the cell density (dry wt) specified. After 15 min incubation at 37 °C in a Dubnoff-type water bath, the fluorescence intensity of cells and medium were recorded, $MgCl_2$ added (final concn 1.13 mM), and fluorescence intensity read again 8 min later. Whole cells, before Mg^{2+} addition = ●; after Mg^{2+} addition = ○; sonicated cells, before Mg^{2+} addition = ■, after Mg^{2+} addition = □. Data has been corrected for light scattering of cells in the absence of A23187. Symbols are means ± SE of triplicate incubations. The results are representative of two similar experiments

cence intensity of A23187 in sonicated cell membrane (open squares) increased dramatically at all membrane concentrations. The intensity of A23187 in whole cells (open circles) was unchanged by magnesium addition except at low membrane concentrations. We conclude that a similar amount of membrane is available for A23187 interaction in whole and sonicated cells.

Fluorescence Microscopy

Dissociated pancreatic acinar cells incubated in 10 μM A23187 displayed a blue fluorescence which was absent in cells not incubated with ionophore. Fig. 7*A* shows that fluorescence was uniformly distributed in the cell with two exceptions. Nuclei (*N*) were nonfluorescent and appeared as black "holes". Areas containing zymogen granules (*Z*) frequently emitted light of lower intensity. Positions of nuclei were confirmed by phase contrast microscopy and the positions of granular areas confirmed by phase contrast and darkfield microscopy. Fig. 7*B* and *C* shows fluorescence and darkfield images, respectively, of the same pair of cells. In Fig. 7*C* the zymogen granule areas (*Z*) scatter light intensely and correspond in position to areas of lower fluorescence intensity in Fig. 7*B*. As in Fig. 7*A*, the rest of the cells fluoresce except for the nuclear "holes". The uniform cytoplasmic fluorescence is observed within five min of incubation in 10 μM A23187 with Ca^{2+} and Mg^{2+} either present or absent. When the medium contained Ca^{2+} and Mg^{2+} large (~1–5 μ), highly fluorescent particles were present in the background, and appeared to adhere to some cells. Other cells, such as those in Fig. 7*D* and the cell on the left in Fig. 7*B*, displayed uniformly small point sources of emission. This occurred frequently in OCa TR and only rarely in OCaOMg TR. As suggested by Fig. 7*D*, a portion of the cell population often contained punctate fluorescence while neighboring cells had none. We could not detect whether these sources of cellular emission were intracellular or extracellular. When 10 μM A23187 in TR was centrifuged at 27,000 × *g* for 5 min and then used to incubate cells, the cells again exhibited the pattern of emission seen in Fig. 7*A*. Although the fluorescence appeared within several minutes, the intensity was low and there was a complete absence of particulate or pointlike sources in both cells and background. These observations are consistent with the fluorometric data showing that medium fluorescence and membrane uptake of A23187 is sharply reduced after removal of "particulate" A23187 by centrifugation.

Ionophore Mediated Release of Amylase from Dissociated Pancreatic Acinar Cells: Calcium Dependence

A23187 (10 μM) in the presence of extracellular Ca^{2+} and Mg^{2+} increased amylase release from dissociated pancreatic acinar cells by 201% ± 28% (mean ± SE, n = 14) during a 30 min incubation. Fig. 8 (com-

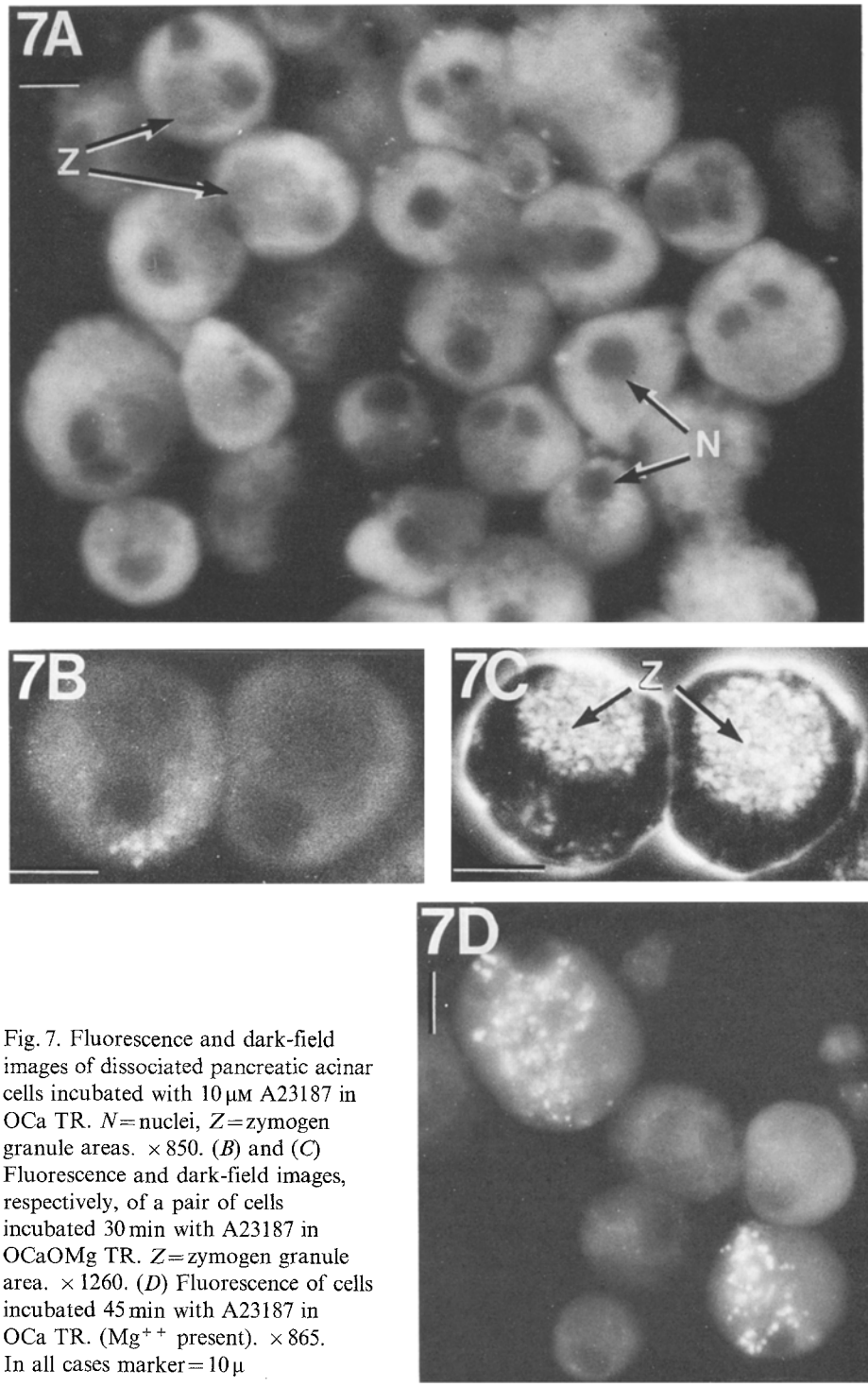

Fig. 7. Fluorescence and dark-field images of dissociated pancreatic acinar cells incubated with 10 μM A23187 in OCa TR. *N*=nuclei, *Z*=zymogen granule areas. ×850. (*B*) and (*C*) Fluorescence and dark-field images, respectively, of a pair of cells incubated 30 min with A23187 in OCaOMg TR. *Z*=zymogen granule area. ×1260. (*D*) Fluorescence of cells incubated 45 min with A23187 in OCa TR. (Mg^{++} present). ×865. In all cases marker=10 μ

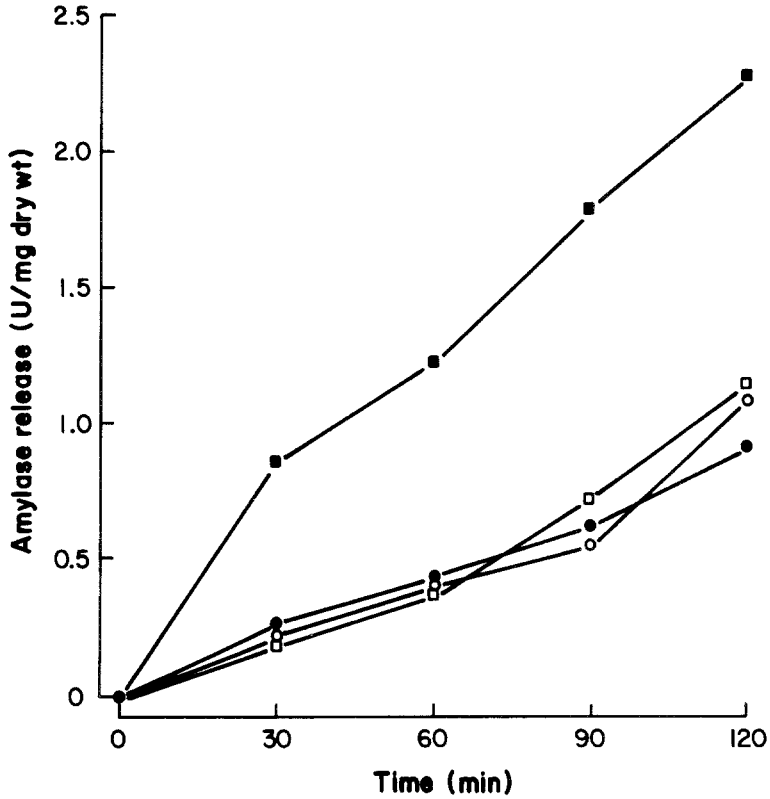

Fig. 8. Time course and calcium dependence of basal and A23187-stimulated amylase release from dissociated pancreatic acinar cells. Acinar cells were incubated at a density of 1.33 mg dry wt per ml in 4 ml of medium as follows: TR, no A23187 = ●; TR, 10 μM A23187 = ■; OCa TR, no A23187 = ○; OCa TR, 10 μM A23187 = □. 0.5 ml aliquots of cells and medium were removed at the designated times and amylase release determined. Symbols are means of closely agreeing (within 10%) duplicate incubations. The results are representative of three similar experiments

pare filled circles and squares) shows that this ionophore-mediated release of amylase was well maintained over a 2-hr period with a 150 to 220% increase in release at each time point. In contrast, 10 μM A23187 in the absence of extracellular Ca^{2+} (but with Mg^{2+} present) had no effect on the rate of amylase release over the same 2-hr period (Fig. 8, compare open circles and squares) in 2 out of 3 experiments. In the third experiment, ionophore increased amylase release but only by 30% at 90 and 120 min.

Ionophore Mediated Cell Damage and Release of Lactate Dehydrogenase

Further experiments showed that cytoplasmic proteins such as lactate dehydrogenase (LDH) as well as secretory proteins such as amylase

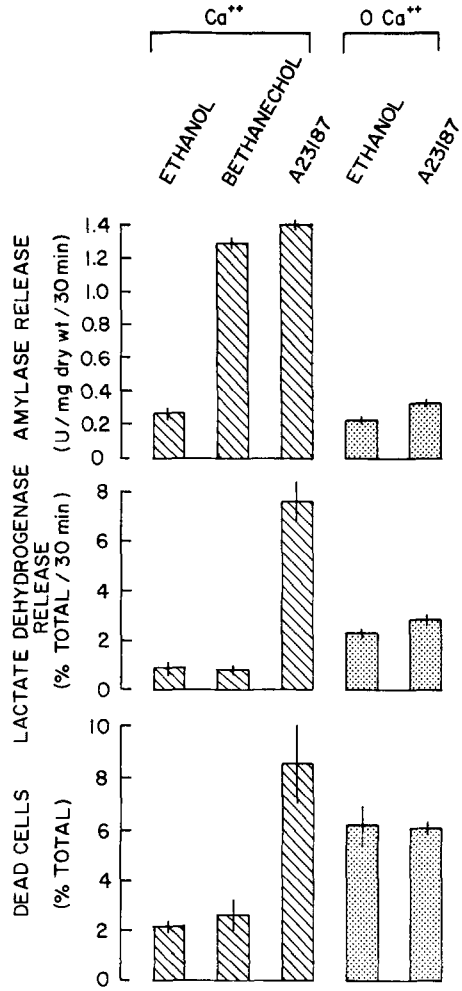

Fig. 9. Calcium dependence of amylase release, lactate dehydrogenase release, and structural damage produced by 10 μM A23187 in dissociated pancreatic acinar cells. Acinar cells, suspended at a density of 1.78 mg dry wt per ml, were incubated for 30 min in 2 ml of TR (hatched bars) or OCa TR (dotted bars) containing 0.5% ethanol, 150 μM bethanechol, 10 μM A23187 as designated. Cells and medium were then centrifuged and the medium assayed for amylase and LDH activities. Other cells, incubated in an identical manner, were fixed, embedded, and sectioned and "dead" cells quantitated by the manner described in the *Materials and Methods*. Amylase and LDH activities are means ± SE of triplicate incubations from a single experiment representative of three similar experiments. Values for "dead" cells are means ± SE of four experiments

were released by A23187. In the experiment shown in Fig. 9 (top) 10 μM A23187 increased amylase release by 414% in the presence of calcium but had almost no effect in the absence of calcium. In the same experiment, LDH release (Fig. 9, middle) was increased 740% in the presence of calcium. In the absence of extracellular calcium, LDH release was

elevated, but the addition of ionophore had no additional effect. Comparable stimulation of amylase release (370%) by the cholinergic agonist bethanechol was not accompanied by increased LDH release. These data indicate that ionophore mediated LDH release in the presence of Mg^{2+} is calcium dependent and that such release is pathological in the sense that it does not occur during stimulation of secretion by normal secretagogues.

Because leakage of a cytoplasmic protein could result from compromise of the plasma membrane as a permeability barrier, ionophore mediated cell damage was further assessed by light and electron microscopic appearance of fixed cells and exclusion of the dye trypan blue by live cells. Structural damage was quantitated by light microscopy of cells incubated with and without ionophore for 30 min, then fixed, embedded and sectioned. Fig. 10*A* shows a representative field of cells incubated in the absence of A23187. The cells are well stained, contain easily recognizable nuclei and zymogen granules, and the preparation contains only a small amount of debris. In contrast, Fig. 10*B* shows a typical field of a cell preparation which had been exposed to 10 μM A23187. Such preparations contained a large population of cells which were nearly normal in appearance and a smaller population of total disrupted cells (arrows, Fig. 10*B*). Quantitation of disrupted cells (Fig. 9, bottom) showed that A23187 in the presence of extracellular calcium increased the dead cell count four-fold—from 2.2% in ethanol controls to 8.7%. In the absence of calcium the count was elevated to 6.2% but ionophore had no additional effect. The cholinergic stimulant, bethanechol, produced no increase in disrupted cells. In two experiments, trypan blue exclusion by live cells gave results consistent with those from light microscopy of fixed cells. Only 2% of control cells failed to exclude the dye. In the presence of 10 μM A23187 and calcium, 15 to 20% of cells became permeable to the dye.

Ultrastructure of cells exposed to 10 μM A23187 ranged from relatively normal (Fig. 11, cells labeled *A*) to the completely disrupted cells previously observed by light microscopy (Fig. 11, cells labeled *B*). Disrupted cells consisted of zymogen granules and swollen mitochondria randomly spaced in a matrix of vesiculated membrane. A larger proportion (30 to 50%) of intact cells exhibited swollen endoplasmic reticulum and/or large vesicles in the golgi area (Fig. 11, cells labeled *C*). Although control cells often contained some vacuoles, the high frequency and great extent of cell swelling in the presence of ionophore suggested that these were intermediate stages of cell disruption.

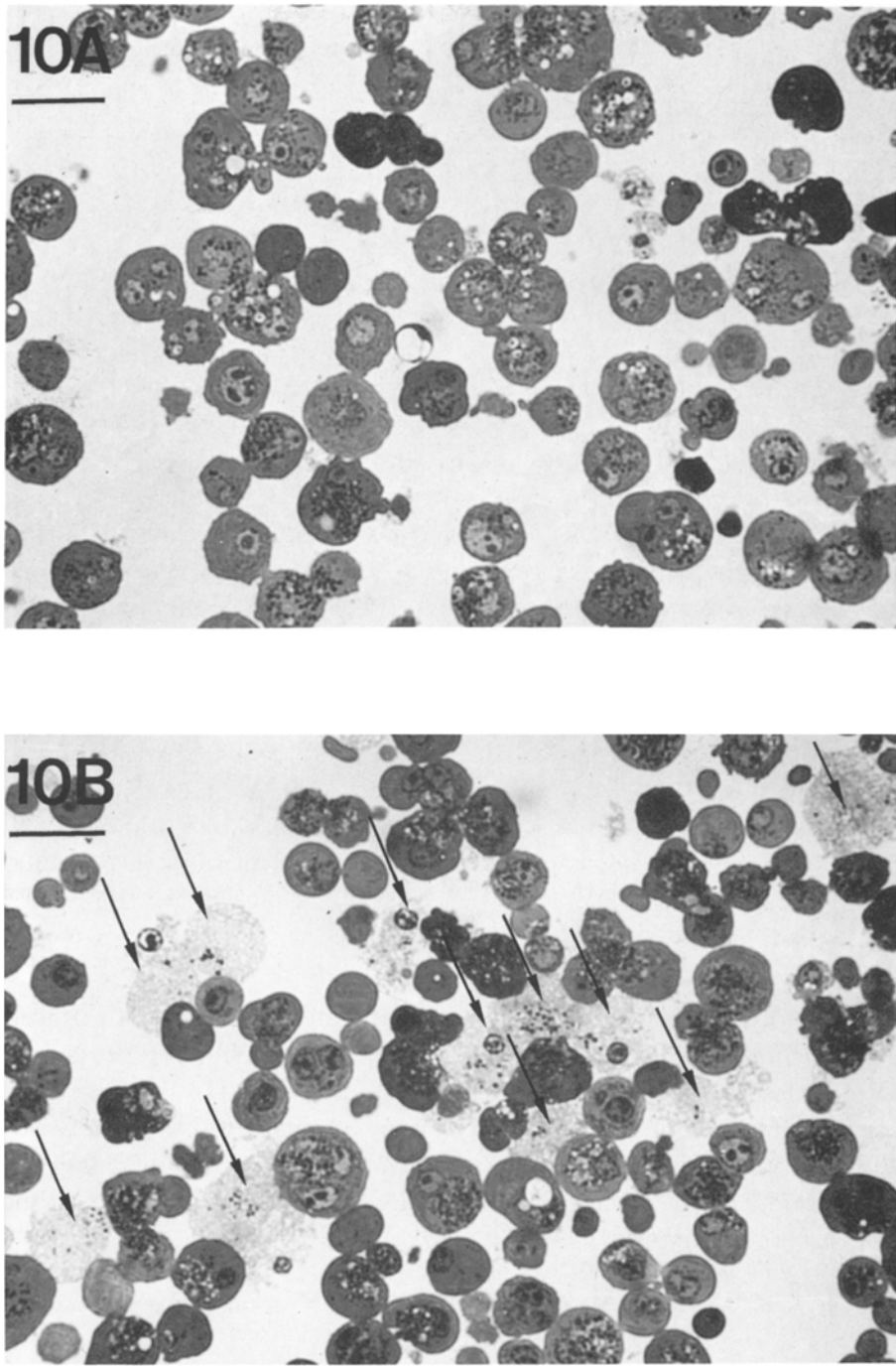

Fig. 10. (*A*) Representative field of dissociated pancreatic acinar cells incubated 30 min in TR. (*B*) Representative field of acinar cells incubated 30 min in TR containing 10 μM A23187. Arrows denote cells scored as "dead". Both *A* and *B* ×665, marker=20 μ

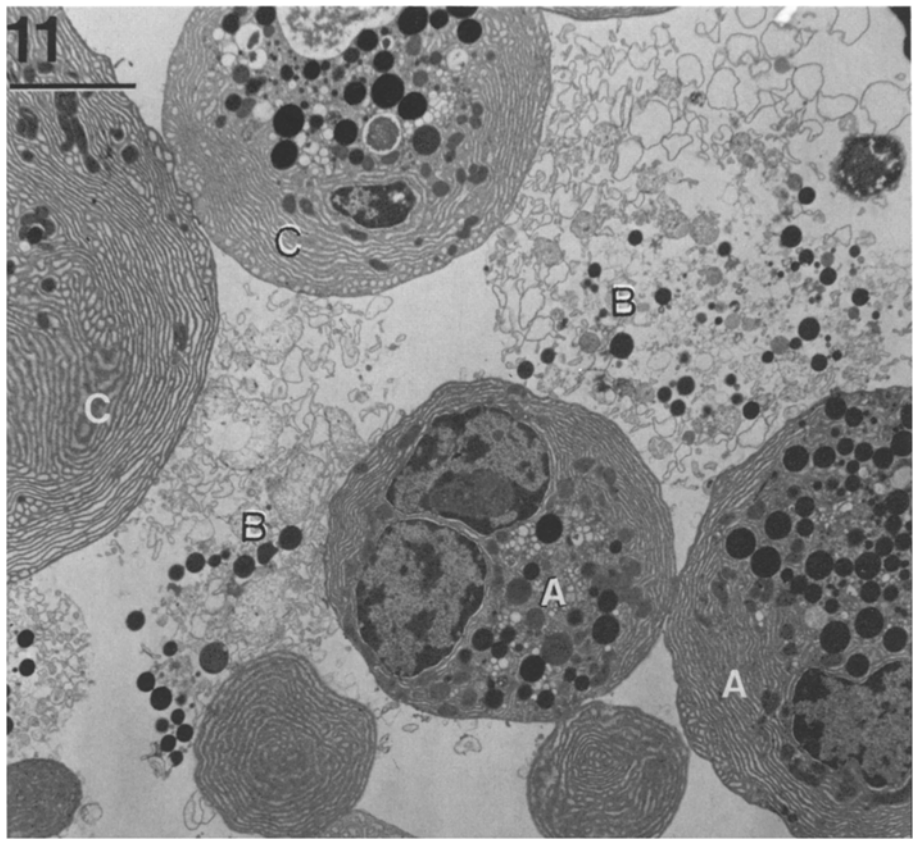

Fig. 11. Ultrastructure of dissociated pancreatic acinar cells exposed to 10 μM A23187 in TR for 30 min. *A*=normal cells, *B*=totally disrupted cells, *C*=swollen and vacuolated cells. ×3500, marker=5 μm

Therefore, we conclude that 10 μM A23187 in the presence of extracellular calcium produces structural damage in a portion of the acinar cell population ranging from leakiness to total disruption. LDH release, trypan blue exclusion, and light microscopy all indicate that when Mg^{2+} is present such damage requires extracellular calcium and that damage is not produced by stimulation of amylase secretion by the cholinergic agonist bethanechol.

Differentiation of Amylase Release Due to Cell Damage and Calcium Influx

Complete structural disruption of at least a partion of the cell population by 10 μM A23187 suggested that amylase release by A23187 could

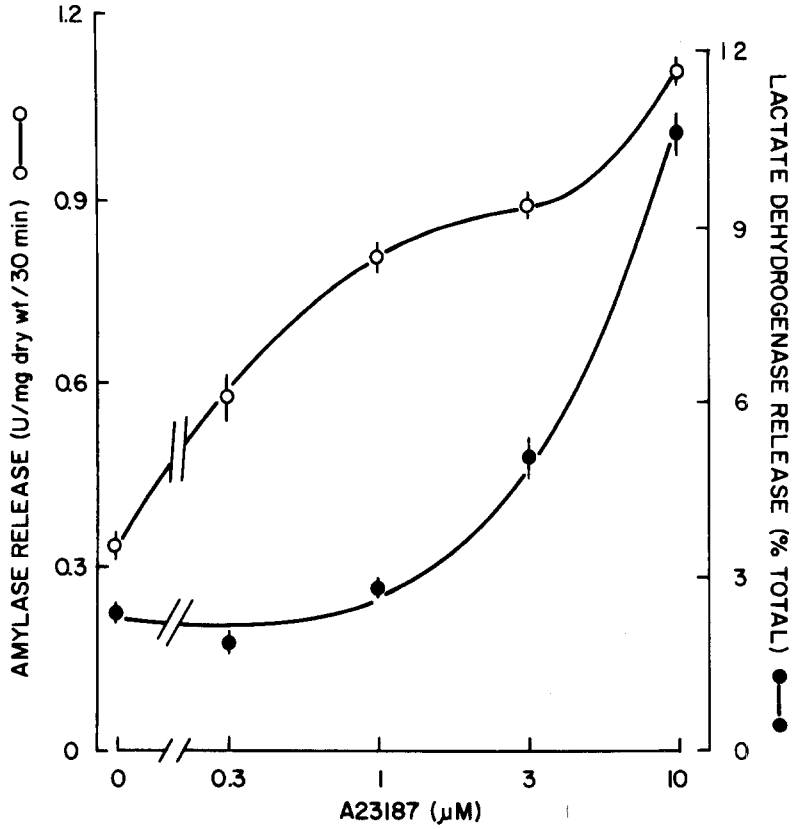

Fig. 12. Dose-response relationship for amylase and lactate dehydrogenase releasing actions of A23187 on dissociated pancreatic acinar cells. Acinar cells were incubated for 30 min in 2 ml of TR containing the designated concentration of A23187. Cells and medium were then centrifuged and assayed for amylase and LDH activities. Symbols are means ± SE of triplicate incubations. Results are representative of three similar experiments

result from cell damage as well as triggering of normal secretory processes by influx of extracellular calcium. To differentiate between these two pathways, ionophore provoked amylase and LDH release were compared in their dose-response relationship and time course. Fig. 12 shows that the dose-response relationship for release of amylase and LDH by A23187 differed substantially. Amylase release was increased by lower concentrations of ionophore than was LDH release. A23187, at 1 μM, increased amylase release 140% without increasing LDH release while at higher concentrations (3 and 10 μM) A23187 increased release of both amylase and LDH.

Amylase and LDH release by A23187 also differed in time course

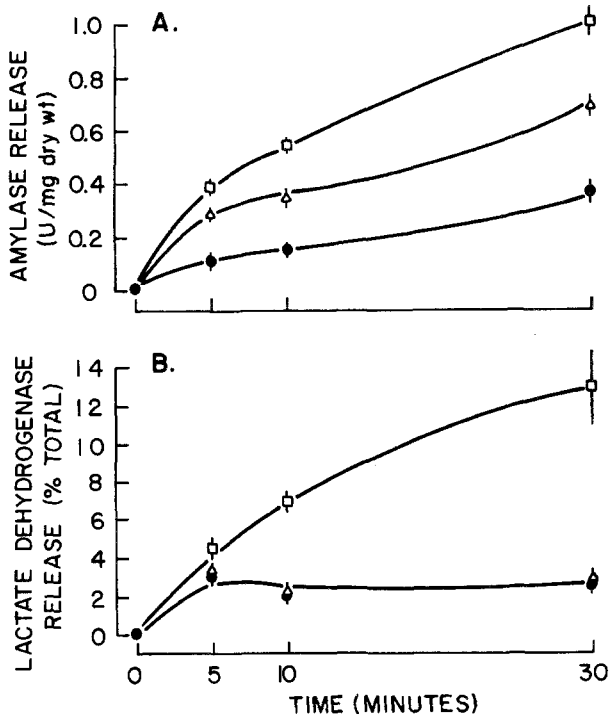

Fig. 13. Time course of amylase and lactate dehydrogenase release from dissociated pancreatic acinar cells by A23187. Acinar cells were incubated for the designated length of time in 2 ml of TR containing 0.5% ethanol (●), 1 μM A23187 (△) or 10 μM A23187 (□). Cells and medium were then centrifuged and assayed for amylase and LDH activities. Symbols are means ± SE of triplicate incubations. Results are representative of two similar experiments

during the first 30 min. As shown in Fig. 13, amylase release in the presence of 1 μM A23187 (open triangles) was increased 158, 154, and 83% over control values (filled circles), at 5, 10 and 30 min, respectively. In contrast, LDH release was identical to control values at each time point. Although 10 μM A23187 (Fig. 13, open squares) released both amylase and LDH, these actions greatly differed in extent during the first 5 min of incubation. At this time, amylase release increased 268% (mean of two experiments) while LDH release increased only 25% (mean of two experiments).

We conclude, on the basis of the dose-response relationship and time course, that A23187 is able to release amylase by an action other than cell damage – most likely, by increasing cytoplasmic calcium activity through increased influx of extracellular calcium.

Table 1. Amylase and lactate dehydrogenase release from dissociated pancreatic acinar cells in OCaOMg Tris buffered Ringer's

Medium	Amylase release (U/mg dry wt/30 min)	LDH release (% total/30 min)
Ethanol, 0.5%	0.46 ± 0.04 (4)	2.9 ± 0.25 (3)
A23187, 10 μM	1.10 ± 0.04 (4)	8.4 ± 0.88 (3)
Bethanechol, 300 μM	1.32 (2)	2.6 (1)

Acinar cells were incubated for 30 min in 2 ml of OCaOMg TR containing 0.1 mM EGTA and ethanol, A23187, or bethanechol as designated. Cells and medium were centrifuged and medium assayed for amylase and LDH activities. Values are means ± SE for the number of experiments shown in parentheses.

Ionophore Mediated Amylase Release in the Absence of Extracellular Ca^{2+} and Mg^{2+}

A23187, 10 μM, increased amylase release from dissociated pancreatic acinar cells 136 ± 27% (mean ± SE, n = 4) in the absence of both calcium and magnesium in the extracellular medium (Table 1). However, the ionophore also increased LDH release 189% ± 15% (mean ± SE, n = 3) under these conditions. Further experiments (not shown) indicated that ionophore-mediated amylase and LDH release in the absence of both calcium and magnesium could not be distinguished on the basis of their dose response relationships; both increased as the A23187 concentration was raised from 0.3 to 3 μM.

Discussion

This report considers questions which are fundamental to the use of the divalent cation ionophore A23187 in secretory tissues. Is A23187 taken up intracellularly? Do its uptake kinetics bear a relationship to its actions? Does release of secretory protein by ionophore and physiological secretagogues occur by the same pathway?

Our results indicate that A23187 is taken up intracellularly. Fluorescence microscopy shows that A23187 emission is distributed throughout the cell, but punctuated by the negative images of nuclei and zymogen granule areas. This emission pattern is similar to that for chlortetracycline (Chandler, 1976), a molecule known to incorporate into intracellular membrane, and resembles the distribution of intracellular membrane. The acinar cell is packed with endoplasmic reticular, golgi, and mitochon-

drial membrane except for the nucleus and zymogen granules. The latter are bounded by membrane, but contain large volumes of protein, nucleic acids, salts and water which could account for their nonfluorescence. Negative contrast of internal organelles is not observed when the fluorescence is restricted to the surface of cells as exemplified by fluorescent antibody or concanavalin A binding to lymphocytes (Unanue, Perkins, & Karnovsky, 1972; de Petris, 1975).

Fluorometric data also point to an intracellular location for A23187. The excitation spectrum of A23187 associated with intact cells (but not sonicated cells) indicates that the ionophore is exposed to a finite concentration of divalent cations which is relatively unaffected by changes in extracellular Mg^{2+} concentration. These results can be explained by the hypothesis that the majority of A23187 is incorporated into intracellular membrane which is exposed to cytoplasm having a magnesium activity of about 1 mM and that the integrity of the plasma membrane is required for maintenance of this concentration. Intracellular uptake of A23187 by intact cells also accounts for the observation that disruption of cell structure by sonication does not increase the amount of membrane available for interaction with A23187 (*see* Fig. 6).

The effect of extracellular Ca^{2+} and Mg^{2+} on the kinetics of A23187 uptake is striking. The rate of uptake is reduced 20-fold in the presence of these ions. The rate limiting step probably comes at or before the plasma membrane in whole cells since rate of A23187 incorporation into erythrocyte membranes which are completely exposed to the medium is also reduced by the presence of divalent cations. In addition, breaking of intact cells by sonication greatly increases the rate and extent of A23187 incorporation in the presence of Ca^{2+} and Mg^{2+} (D. Chandler, *unpublished observation*). These observations also suggest that divalent cation effects on the kinetics of A23187 uptake are not due to A23187 mediated cell damage. Additional results making this unlikely are that A23187 in the presence of Mg^{2+} alone does not cause cell damage, yet its uptake kinetics are similar to those observed when both Ca^{2+} and Mg^{2+} are present. In the absence of Ca^{2+} and Mg^{2+}, readmission of Mg^{2+} (Fig. 4) produces only a small fluorescence increase showing that the majority of A23187 is in cells which maintain a permeability barrier to extracellular Mg^{2+}.

Although the effect of divalent cations on A23187 uptake is clear the underlying mechanism is not obvious. The heterogenous physical state of A23187 in divalent cation containing media makes it possible that A23187 uptake occurs by more than one route. The sedimentation

and microscopic observations presented suggest that the bulk of ionophore is present as particles up to 5 μ in diameter and spectral data indicate that these particles coexist with a much lower concentration of soluble ionophore. The intense punctate fluorescence seen in a portion of the cell population (*see* Fig. 7*D*) may represent either A23187 particles taken up directly, for example by phagocytosis or simple adhesion, or soluble ionophore taken up and then concentrated by an intracellular organelle such as mitochondria. The diffuse fluorescence seen in the membrane-filled areas of acinar cells (*see* Fig. 7*A*) can be attributed to uptake of soluble ionophore. Such a diffuse fluorescence pattern is seen in the virtual absence of the punctate pattern when soluble ionophore is taken up under two different circumstances—from EDTA TR and from regular TR which has been centrifuged at $27{,}000 \times g$ to remove particulate A23187.

Fluorometric measurements show that divalent cations must limit the uptake of soluble ionophore since the changes in fluorescence intensity of A23187 that are monitored are a result of incorporation of ionophore molecules into the hydrophobic interior of membranes. Uptake could be limited by the rate that A23187 particles dissolve to form soluble ionophore. In fact, the observation that removal of A23187 particles by centrifugation reduces the rate of ionophore uptake into erythrocyte membranes by 90% suggests that particulate A23187 does act as a reservoir for soluble ionophore. Of greater interest is that A23187 uptake by erythrocyte membrane even after removal of particulate by centrifugation is slow (time of half maximal uptake greater than 15 min). This is not due to the low ionophore concentration since A23187 at concentrations as low as 10^{-7} M is taken up rapidly from EDTA TR. It suggests that chelation with Ca^{2+} or Mg^{2+} *per se* reduces the rate of A23187 incorporation into membrane.

These divalent cation effects on the time course of A23187 uptake may, in some cases, determine the time course of ionophore actions. Schreurs *et al.* (1976) have shown that application of A23187 to fragments of rabbit pancreas in KHB results in a 100% increase in rate of amylase release but only after a lag of 30 min. In contrast, when fragments were superfused with A23187 in the absence of both Ca^{2+} and Mg^{2+} and Ca^{2+} then readmitted, amylase release was increased 300% within several min. Comparable results have been obtained in mouse pancreatic fragments (Poulsen & Williams, 1976). The much faster uptake of A23187 in the absence of divalent cations may also account for the fact that in some tissues, notably posterior pituitary, addition of ionophore to

a complete medium does not produce secretion (*see* discussion after Selinger *et al.*, 1974) while preincubation with A23187 in the absence of Ca^{2+} and Mg^{2+} followed by Ca^{2+} readmission produces an immediate, large release of secretory product (Russell *et al.*, 1974).

It is also clear, however, that some actions of A23187 are immediate even if the ionophore is added to a divalent cation containing medium. Data in this report show that amylase release from isolated cells is increased 268% within 5 min. In pancreatic fragments membrane depolarization of surface cells occurs within minutes of A23187 application (Poulsen & Williams, 1976). Similarly, other actions such as calcium uptake and triggering of exocytosis in mast cells (Foreman *et al.*, 1973; Cochrane & Douglas, 1974), contraction of isolated smooth muscle cells (Murray *et al.*, 1975), activation of sea urchin eggs (Steinhardt & Epel, 1974), and insulin release by perifused islets (Karl *et al.*, 1975) are initiated within seconds to minutes of ionophore addition. Because almost all of these rapid actions involve isolated cells or superficially located cells, it is tempting to speculate that the availability of ionophore to interior cells of tissue fragments is limited by physical exclusion of ionophore particles as well as slower membrane uptake of chelated A23187.

Data presented in this report shows that ionophore induced release of secretory protein by a nonphysiological process is a real possibility. The increased release of lactate dehydrogenase (LDH), increased permeability to the dye trypan blue, and disruption of cell morphology produced by high A23187 concentration (10 μM) all suggest that amylase could be released from structurally compromised cells. Although cells isolated by enzymatic hydrolysis may be more fragile than cells in intact tissues, reexamination of previously published data (Selinger *et al.*, 1974) indicates the A23187 also increased LDH release from pancreatic fragments. Evidence of a different nature suggests that A23187 can release norepinephrine from adrenergic nerve terminals by a nonexocytic process. In vas deferens, Thoa and coworkers (Thoa *et al.*, 1974) have shown that 100 μM A23187 releases deaminated metabolites of norepinephrine as well as norepinephrine. This suggests that part of the norepinephrine was released into the cytoplasm and metabolized rather than being released directly to the extracellular space via exocytosis. These results emphasize that one must interpret release of secretory products by high concentrations of A23187 with caution. This is particularly true in view of the fact that ionophore mediated cell damage (in the presence of Mg^{2+}) is Ca^{2+} dependent just as are the secretory processes one is likely to be studying.

The ionic dependence of A23187 mediated cell damage requires that there be at least two mechanisms of action. That A23187 in the presence of Mg^{2+} requires extracellular Ca^{2+} to produce cell damage (*see* Fig. 9) suggests that increased Ca^{2+} influx is responsible. That A23187 also causes comparable cell damage in the absence of divalent cations (*see* Table 1) suggests that loss or redistribution of intracellular divalent cations or a direct membrane action of the ionophore may contribute to cell damage. Though the mode of action of ionophore in the absence of divalent cations is not clear it is remarkable that damage can be entirely prevented by the presence of extracellular Mg^{2+}.

The present study by ascertaining the time course and dose-response relationship of amylase release has been able to show that 1 μM A23187 plus extracellular calcium increases amylase release from dissociated acinar cells without producing cell damage. When extracellular calcium is absent but magnesium present, there is no evidence that ionophore produces amylase release even after 2 hr. This is in spite of the fact that ionophore is available intracellularly as shown by fluorescence microscopy and that uptake of ionophore reaches a plateau within 90 min. Release of intracellular Ca^{2+} must be slow enough to avoid triggering of secretion. In fact, application of A23187 to fragments of rabbit pancreas in KHB lacking Ca^{2+} and Mg^{2+} does not noticeably increase efflux of Ca^{2+} from slowly exchanging pools (Schreurs *et al.*, 1976). This situation is not unknown in other tissues. Although A23187 increases Ca^{2+} efflux from skeletal muscle, for example, resting tension is not affected (Hainaut & Desmedt, 1974).

This study has also shown that A23187 increases amylase release in the absence of both Ca^{2+} and Mg^{2+}—an observation of potentially great interest since physiologic secretagogues can cause release of cellular calcium and trigger secretion in the absence of extracellular calcium (Williams & Chandler, 1975; Williams *et al.*, 1976). In this case, however, amylase release and LDH release induced by A23187 cannot be differentiated on the basis of the dose-response relationship. Therefore, amylase release could be an artifact resulting from cell damage and further interpretation is unfeasible.

The usefulness of A23187 as a tool for increasing intracellular calcium lies in its ease of application. At present, the only alternative is ionotophoretic application of Ca^{2+} ions through an intracellular microelectrode—a technique that is limited to large, whole cells and whose results can be technically difficult to observe. However, use of A23187, though technically simple, has its own complexities. Uptake of ionophore and

kinetics of its actions may be dependent on whether divalent cations are present during application. Calcium-dependent release of secretory protein resulting from cell damage is possible. Finally, the isolubility of A23187 in divalent cation containing media poses a real difficulty in determining what the effective concentration of ionophore is and by what route it is being delivered to cell membranes. While these findings do not invalidate the use of A23187, they suggest that care must be taken in the interpretation of such data.

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References

Amsterdam, A., Jamieson, J.D. 1972. Structural and functional characterization of isolated pancreatic exocrine cells. *Proc. Nat. Acad. Sci. USA* **69**:3028

Amsterdam, A., Jamieson, J.D. 1974. Studies on dispersed pancreatic exocrine cells. I. Dissociation technique and morphologic characteristics of separated cells. *J. Cell Biol.* **63**:1037

Ashby, J.P., Speake, R.N. 1975. Insulin and glucagon secretion from isolated islets of langerhans. Effects of calcium ionophores. *Biochem. J.* **150**:89

Binet, A., Volfin, P. 1975. Effect of the A23187 ionophore on mitochondrial Mg^{2+} and Ca^{2+}. *FEBS Lett.* **49**:400

Case, R.M., Clausen, T. 1973. The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas. *J. Physiol. (London)* **235**:75

Case, G.D., Vanderkooi, J.M., Scarpa, A. 1974. Physical properties of biological membranes determined by the fluorescence of the calcium ionophore A23187. *Arch. Biochem. Biophys.* **162**:174

Caswell, A.H., Pressman, B.C. 1972. Kinetics of transport of divalent cations across sarcoplasmic reticulum vesicles induced by ionophores. *Biochem. Biophys. Res. Commun.* **49**:292

Chandler, D.E. 1976. Stimulus-secretion coupling in the exocrine pancreas: Use of the fluorescent probe chlorotetracycline to detect divalent cation redistribution. *Fed. Proc.* **35**:603 (*Abstr.*)

Chandler, D.E., Williams, J.A. 1974. Pancreatic acinar cells: Effects of lanthanum ions on amylase release and calcium ion fluxes. *J. Physiol. (London)* **243**:831

Charles, M.A., Lawecki, J., Pictet, R., Grodsky, G.M. 1975. Insulin secretion. Interrelationships of glucose, cyclic adenosine 3′:5′-monophosphate and calcium. *J. Biol. Chem.* **250**:6134

Cochrane, D.E., Douglas, W.W. 1974. Calcium induced extrusion of secretory granules exposed to 48/80 or the ionophores A23187 and X-537A. *Proc. Nat. Acad. Sci. USA* **71**:408

Cochrane, D.E., Douglas, W.W., Mouri, T., Nakazato, Y. 1975. Calcium and stimulus-secretion coupling in the adrenal medulla: Contrasting stimulating effects of the ionophores X-537A and A23187 on catecholamine output. *J. Physiol. (London)* **252**:363

Foreman, J.C., Mongar, J.L., Gomperts, B.D. 1973. Calcium ionophores and movement

of calcium ions following the physiological stimulus to a secretory process. *Nature (London)* **245**:249

Garcia, A.G., Kirpekar, S.M., Prat, J.C. 1975. A calcium ionophore stimulating the secretion of catecholamines from the cat adrenal. *J. Physiol. (London)* **244**:253

Gardner, J.D., Conlon, T.P., Klaeveman, H.L., Adams, T.D., Ondetti, M.A. 1975. Action of cholecystokinin and cholingergic agents on calcium transport in isolated pancreatic acinar cells. *J. Clin. Invest.* **56**:366

Hainaut, K., Desmedt, J.E. 1974. Calcium ionophore A23187 potentiates twitch and intracellular calcium release in single muscle fibers. *Nature (London)* **252**:407

Heinz, E., Hoffman, J.F. 1965. Phosphate incorporation and Na, K-ATPase activity in human red blood cell ghosts. *J.Cell Comp. Physiol.* **65**:31

Karl, R.C., Zawalich, W.S., Ferrendelli, J.A., Matschinsky, F.M. 1975. The role of Ca^{2+} and cyclic adenosine 3′:5′-monophosphate in insulin release induced in vitro by the divalent cation ionophore A23187. *J. Biol. Chem.* **250**:4575

Kondo, S., Schulz, I. 1976. Calcium ion uptake in isolated pancreas cells induced by secretagogues. *Biochim. Biophys. Acta.* **419**:76

Murray, J.J., Reed, P.W., Fay, F.S. 1975. Contraction of isolated smooth muscle cells by ionophore A23187. *Proc. Nat. Acad. Sci. USA* **72**:4459

Petris, S. de 1975. Concanavalin A receptors, immunoglobulins, and θ-antigen of the lymphocyte surface. *J.Cell Biol.* **65**:123

Pfeiffer, D.R., Reed, P.W., Lardy, H.A. 1974. Ultraviolet and fluorescent spectral properties of the divalent cation ionophore A23187 and its metal ion complexes. *Biochemistry* **13**:4007

Poulsen, J.H., Williams, J.A. 1976. Pancreatic acinar cell depolarization and amylase release evoked by Ca^{2+}-ionophore A23187 and bethanechol. *Fed. Proc.* **35**:603 (*Abstr.*)

Pressman, B.C. 1972. Carboxylic ionophores as mobile carriers for divalent ions. *In:* The Role of Membranes in Metabolic Regulation M.A. Mehlman and R.W. Hanson, editors. p. 149. Academic Press, New York

Reed, P.W., Lardy, H.A. 1972*a*. Antibiotic A23187 as a probe for the study of calcium and magnesium in biological systems. *In:* The Role of Membrane in Metabolic Regulation, A.M. Mehlman and R.W. Hanson, editors. p. 111. Academic Press, New York

Reed, P.W., Lardy, H.A. 1972*b*. A23187: A divalent cation ionophore. *J. Biol. Chem.* **247**:6970

Rinderknecht, H., Wilding, P., Haverback, B.J. 1967. A new method for the determination of α-amylase. *Experientia* **23**:805

Robberecht, P., Christophe, J. 1971. Secretion of hydrolases by perfused fragments of rat pancreas: Effects of calcium. *Am. J. Physiol.* **220**:911

Russell, J.T., Hansen, E.L., Thorn, N.A. 1974. Calcium and stimulus-secretion coupling in the neurohypophysis. III. Ca^{2+} ionophore (A23187)-induced release of vasopressin from isolated rat neurohypophysis. *Acta Endocrinol. (Copenhagen)* **77**:443

Scarpa, A., Baldassare, J., Inesi, G. 1972. The effect of calcium ionophores on fragmented sarcoplasmic reticulum. *J. Gen. Physiol.* **60**:735

Schreurs, V.V.A.M., Swarts, H.G.P., dePont, J.J.H.M., Bonting, S.L. 1976. Role of calcium in exocrine pancreatic secretion. II. Comparison of the effects of carbachol and the ionophore A23187 on enzyme secretion and calcium movements in rabbit pancreas. *Biochim. Biophys. Acta* **419**:320

Schroeder, T.E., Strickland, D.L. 1974. Ionophore A23187, calcium and contractility in frog eggs. *Exp. Cell Res.* **83**:139

Selinger, Z., Eimerl, S., Savion, N., Schramm, M. 1974. A Ca^{++} ionophore (A23187) simulating hormone and neurotransmitter action in the rat parotid and pancreas glands. *In:* Secretory Mechanisms of Exocrine Glands, N.A. Thorn and O.H. Petersen, editors. p. 68. Munksgaard, Copenhagen

Sordahl, L.A. 1975. Effects of magnesium, ruthenium red and the antibiotic ionophore A23187 on initial rates of calcium uptake and release by heart mitochondria. *Arch. Biochem. Biophys.* **167**:104

Steinhardt, R.A., Epel, D. 1974. Activation of sea-urchin eggs by a calcium ionophore. *Proc. Nat. Acad. Sci. USA* **71**:1915

Thoa, N.B., Costa, J.L., Moss, J., Kopin, I.J. 1974. Mechanism of release of norepinephrine from peripheral adrenergic neurones by the calcium ionophore X-537A and A23187. *Life Sci.* **14**:1705

Unanue, E.R., Perkins, W.D., Karnovsky, M.J. 1972. Ligand induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* **136**:885

Wacker, W.E.C., Ulmer, D.D., Vallee, B.L. 1956. Metalloenzymes and myocardial infarction. II. Malic and latic dehydrogenase activities and zinc concentrations in serum. *N. Engl. J. Med.* **255**:449

Williams, J.A., Cary, P.C., Moffat, B. 1976. Effects of ions on amylase release by dissociated pancreatic acinar cells. *Am. J. Physiol.* **231**:1562

Williams, J.A., Chandler, D. 1975. Ca^{++} and pancreatic amylase release. *Am. J. Physiol.* **228**:1729

Williams, J.A., Lee, M. 1974. Pancreatic acinar cells: Use of a Ca^{++} ionophore to separate enzyme release from the earlier steps in stimulus-secretion coupling. *Biochem. Biophys. Res. Commun.* **60**:542

Wollheim, C.B., Blondel, B., Trueheart, P.A., Renold, A.E., Sharp, G.W.G. 1975. Calcium-induced insulin release in monolayer culture of the endocrine pancreas. Studies with ionophore A23187. *J. Biol. Chem.* **250**:1354

Wong, D.T., Wilkinson, J.R., Hamill, R.L., Horng, J.-S. 1973. Effects of antibiotic ionophore, A23187, on oxidative phosphorylation and calcium transport of liver mitochondria. *Arch. Biochem. Biophys.* **156**:578