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The IP3-sensitive calcium store of HIT cells is located in a surface-derived vesicle fraction

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Electron microscopic and biochemical techniques were used to study the cellular localization of the ATP-dependent, IP3-sensitive, Ca^{2+} store in the glucose- and phosphatidylinositol(PI) agonist-sensitive hamster insulinoma cell line HIT-T15. Scanning electron microscopy revealed conspicuous shape changes of the microvilli following stimulation of these cells with bombesin or thapsigargin. These changes closely resemble those previously shown to accompany stimulation of hexose transport in adipocytes with insulin [J. Cell. Physiol. 142 (1990) 1–14]. Using a hydrodynamic shearing technique for the isolation of microvilli, two cell surface-derived vesicle fractions were prepared containing 80% of the total cellular Ca^{2+}-storing activity. In contrast, subcellular fractionation using normal homogenization with a glass/teflon homogenizer yielded the well-known distribution of the Ca^{2+}-storing activity which is then predominantly recovered within the microsomal fraction. The surface-derived vesicle fraction was clearly distinguished from the microsomal fraction by its high content of Na^+/K^+-ATPase and an immunoreactive fragment of the GluT-1 glucose transporter isoform which both are not detectable in the microsomal fraction isolated from homogenates from sheared cells The Ca^{2+} uptake properties of the cell surface-derived vesicle fractions including the vanadate, A23187, and thapsigargin sensitivity were found to be identical with those described for the microsomal Ca^{2+} stores of various cell types. Inositol 1,4,5-trisphosphate (IP3) at 1 μM induced a maximal release of 35–40% of the stored Ca^{2+} from these vesicles.

Calcium ion store (HIT cells); Localization, Inositol Tris-phosphate; Ultrastructure; Localization

1. INTRODUCTION

In a previous study, the concept of the 'receptor-operated entrance compartment' has been put forward [1–4] which was designed to explain the rapid stimulatory effect of insulin on glucose transport as well as the other known forms of hexose transport regulation by a common cellular mechanism acting on the structure of cell surface microvilli. This concept was based on the finding that the total insulin-sensitive pool of glucose transporters of 3T3-L1 adipocytes is located in a microvillar membrane portion isolated from the cell surface of intact cells by a hydrodynamic shearing technique [3]. Using normal homogenization and fractionation techniques, this transporter pool is always found within the microsomal fraction.

Apart from their implication for hexose transport regulation, these findings point to the general failure of conventional cell fractionation to distinguish between membrane vesicles of intracellular origin and those formed by vesiculation of specialized plasma membrane regions during the homogenization procedure. We, therefore, suspected that there may still be other relevant microsomal systems for which this shortcoming of cell fractionation techniques could have relevance.

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One of these systems appears to be the ATP-dependent Ca^{2+} store which has been detected in the microsomal fractions of a great variety of cells. Although this store was shown by different criteria to be located in the immediate vicinity of the plasma membrane [5–9] or even associated with a plasma membrane fraction in hepatocytes [10], it could not yet be identified as a distinct morphological structure.

In the present study, we show that the ATP-dependent and IP3-sensitive Ca store of HIT cells is almost exclusively recovered in a cell surface-derived vesicle fraction most likely composed of microvillar fragments.

2. MATERIALS AND METHODS

2.1. *Materials*

The polyclonal antiserum against a C-terminal peptide of the GluT1 isoform of the glucose transporter was kindly provided by Dr. M. Mueckler (St Louis), the monoclonal antibody against actin was purchased from Amersham. Thapsigargin, bombesin, and A23187 were obtained from Sigma, and IP3 from Boehringer.

2.2. *Cell culture*

HIT T15 cells were purchased from the American Type Culture Collection (Rockville, MD) Cells were cultured in plastic bottles (Costar) for 1 week in Ham's F12 with 10% horse serum and 2 5% fetal calf serum supplemented with 2 mM glutamine. They were used between the 64th and the 72nd passage. Prior to the experiments, the cells were incubated for 3 h without serum.

2.3. *Preparation of cell samples for scanning electron microscopy*

Cells grown on plastic coverslips were rinsed with HBSS, fixed for

30 min with Karnowsky's solution and washed 3 times with the same buffer. The cells were then dehydrated in aqueous ethanol at concentrations of 30, 50, 75, 95 and 100% for 5 min each. After exchange of ethanol with hexamethyldisilazane (Sigma) the samples were air-dried and sputtered with gold.

2.4. *Preparation of subcellular membrane fractions*

Plasma membrane, microsomal and cell surface-derived membrane fractions were prepared as recently described [3]. Shortly, these fractions were prepared by softly detaching the cells from the growth substrate in ice-cold 1 mM HEPES (pH 7.4)/0.25 M saccharose/1 mM EDTA. Subsequently, the cells, suspended in the same buffer, were gently pressed through hypodermic needles of the following size: No. 1 (0.9 × 40 mm, 0.6 mm internal diameter) and then No. 2 (0.8 mm × 38 mm, 0 5 mm internal diameter). Each step was repeated once The resulting suspension was centrifuged at 2,600 × *g* for 10 min to remove the intact cells (80–90% by Trypan blue exclusion). The 2,600 × *g* supernatant was centrifuged at 16,000 × *g* for 20 min and the supernatant of this step was centrifuged at 180,000 × *g* for 80 min yielding the pellets P2 and P3, respectively. The pellet of the 2,600 × *g* sedimentation (intact cells) was homogenized with a teflon/glass homogenizer for preparation of the plasma membrane (N-PM) and microsomal fraction (N-MIK). Sometimes, homogenization was performed without the shearing step prior to homogenization. The resulting plasma membrane and microsomal fractions were then named PM and MIK, respectively. All operations were performed at 4°C or on ice. The membrane fractions were rapidly frozen in liquid nitrogen and stored at −80°C.

2.5. *Determination of 45-Ca uptake and release from membrane vesicles*

Cell surface-derived vesicles were washed twice with buffer A (140 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 49 μM EGTA, pH 7.2), sedimented at 180,000 × *g* in a Beckman TL 100 centrifuge (TLA 100.3) at 4°C, resuspended in buffer I to give a protein concentration of 300 μg/ml. Ca^{2+} uptake was initiated by addition of $^{45}Ca^{2+}$ (10–5 μCi/ml; final Ca^{2+} concentration = 10 μM; final free Ca^{2+} concentration = 100–200 nM) and 2 mM ATP at room temperature. Effectors were added to each sample (100 μl) in volumes of maximal 1 μl. The uptake reaction was terminated by dilution of the reaction mixture with 3 ml of washing buffer (buffer A plus 10 μM $CaCl_2$; at room temperature), subsequent rapid filtration through Schleicher und Schuell cellulose nitrate filters (type BA 85) and two additional washings with the same buffer. Radioactivity remaining on the filters was determined by liquid scintillation counting. All experiments were carried out at room temperature. Vesicle suspensions were kept on ice until the beginning of the experiment. The basal Ca^{2+} concentration of the used buffers and solutions were controlled by the Fura-2 fluorescence technique. The actual Ca^{2+} concentration of the uptake solutions was computer-calculated according to Fabiato [11].

2 6. *Determination of the ouabain-sensitive ATPase*

The ouabain-sensitive Na^+/K^+-ATPase also exhibits a K^+-dependent phosphatase activity [12]. This activity was measured as described by Colas and Maroux [13] using *p*-nitrophenylphosphate (Sigma 104) as substrate and 100 μg membrane protein/ml. Two enzyme samples were incubated in 1 ml of 50 mM Tris (pH 7.6), 10 mM $MgSO_4$, 5 mM EDTA, 90 mM KCl for 30 min at 37°C, either in the presence or in the absence of 0.7 mM ouabain. The reaction was stopped by addition of 0.2 ml of trichloroacetic acid (30%). The amount of *p*-nitrophenol released was then measured at 410 nm after alkalinization with 2 ml of 1 M Tris solution. The ouabain-sensitive phosphatase activity was computed by subtracting the values found in the absence and in the presence of ouabain.

2.7. *Gel electrophoresis and immunoblots*

SDS-gel electrophoresis of actin and the GluT-1 glucose transporter protein in the subcellular fractions of HIT cells were carried out on 8% (actin) and 10% (GluT-1) SDS-polyacrylamide gels. The gels were blotted onto nitrocellullose. Actin was probed with a monoclonal anti-actin antibody followed by treatment with anti-mouse IgG peroxidase conjugate. GluT-1 was probed with an antibody against a C-terminal peptide of the HepG2/erythrocyte transporter, GluT-1, followed by treatment with ^{125}I-labeled protein A.

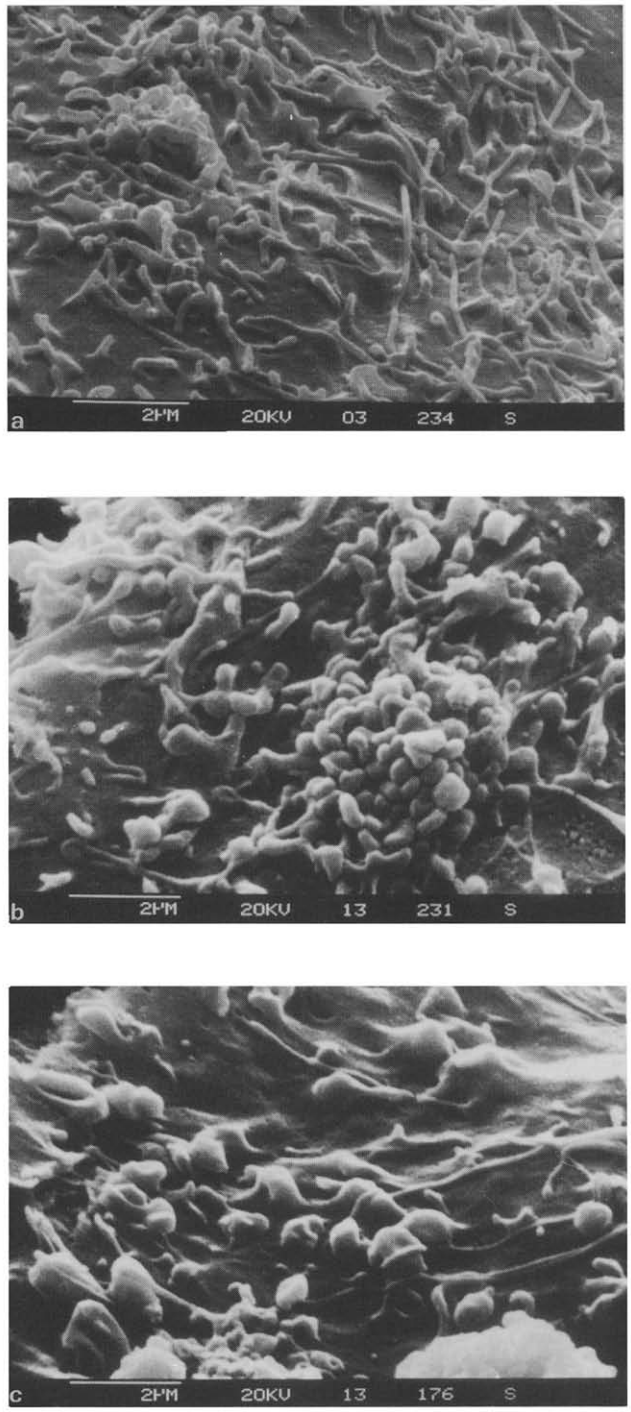

Fig. 1. Scanning electron micrographs of the surface of HIT T15 cells in monolayer culture: microvillar shape changes induced by bombesin and thapsigargin (a) Control. (b) Cell surface of a HIT cell treated for 2 min with 1 μM bombesin, and (c) treated for 1 min with 150 nM thapsigargin.

3. RESULTS

3.1. *Surface morphology of HIT T15 cells*

HIT T15 cells display numerous microvilli covering the surfaces of the islet-like cell clusters formed during several days of culturing (Fig. 1a). As shown by scanning electron microscopy, the shape of these surface protrusions rapidly respond to the PI-response agonist bombesin (Fig. 1b) and thapsigargin (Fig. 1c), a tumor promoter that empties IP3-sensitive Ca^{2+} stores and stimulates Ca^{2+} influx by a phospholipase C-independent pathway in a great variety of cell types. These morphological responses occurred at the same time scale and concentrations at which these effectors elicit their known cellular actions, PI-response and intracellular Ca^{2+} release, respectively. In both cases, microvilli were greatly enlarged indicating the loss of their internal cytoskeletal organization.

3.2. *Preparation and properties of the cell surface derived vesicle fractions*

A hydrodynamic shearing technique, previously described for the isolation of microvilli from tumor cells [14] and 3T3-L1 adipocytes [3], was used for the isolation of two cell surface-derived fractions from HIT cells. Subsequent to the shearing step, the still intact cells were separated by low speed centrifugation, homogenized according to [3] by use of a glass–teflon homogenizer and the plasma membrane and microsomal fractions, named N-PM and N-MIK, respectively, were isolated by differential fractionation and saccharose gradient sedimentation. N-MIK is assumed to be a microsomal fraction which is devoid of membrane vesicles derived from cell surface protrusions.

The cell surface-derived fractions P2 and P3 contained the bulk of the Na^+/K^+-ATPase activity of the HIT cells (Fig. 2). This enzyme was not detectable in N-MIK. Furthermore, P2 and P3 comprised 80% of the total ATP-dependent Ca^{2+} storing activity of these cells (Fig. 3). The content of actin and the glucose transporter isoform GluT-1 in the subcellular fractions of HIT cells was determined by immunoblotting (Fig. 4). The protein-based actin content was almost similar in all fractions except the microsomal fraction which displayed only a weak band. Immunoreactivity against the GluT-1 glucose transporter was also confined to P2 and P3. The observed narrow band of an apparent molecular weight of 36 kDa represents an immunoreactive fragment of the transporter which is completely absent in N-MIK. Considering the amounts of protein recovered in each fraction, the bulk of both components, the GluT-1 fragment and actin, was found in the cell surface-derived fractions P2 and P3 which comprised 80% of the total protein content found in the isolated membrane fractions P2 (46.5%; $n = 4$; S.E.M. = 3.4%), P3 (31.7%; $n = 4$; S.E.M. = 1.9%), N-PM (3.68%; $n = 4$; S.E.M. = 0.44%), and N-MIK (18.3%; $n = 4$; S.E.M. = 2.7).

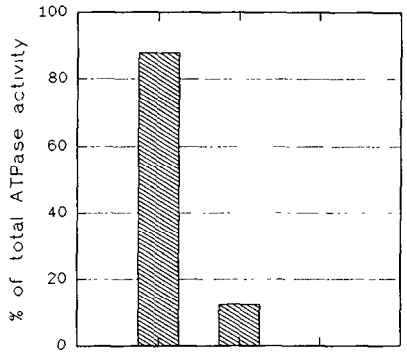

Fig. 2. Subcellular distribution of ouabain-sensitive ATPase activity in HIT cells. Data are expressed as % of the total activity recovered after fractionation. 100% corresponds to an Na^+/K^+-ATPase activity of 1.1 mU/mg of total membrane protein which is about 5% of the ouabain-insensitive ATPase activity. P2 + P3 represents the sum of the activities of the high and low density cell surface-derived fractions, respectively. N-PM and N-Mik are the plasma membrane and the microsomal fractions prepared subsequent to the shearing procedure.

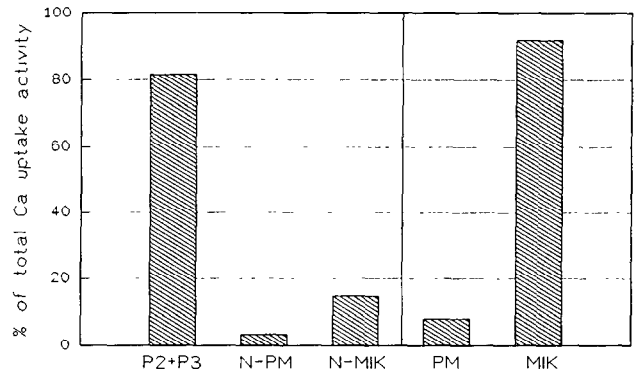

Fig. 3. Subcellular distribution of the Mg^{2+} and ATP-dependent Ca^{2+} storing activity of HIT cells. Data are expressed as % of the total activity recovered after fractionation. Maximal specific uptake activity amounted to 4–5 nmol/mg protein. The fractions are named as in Fig. 2 except those indicated PM and MIK which represent the plasma membrane and the microsomal fraction, respectively, prepared without the shearing step prior to homogenization.

The Ca-storing activity of the subcellular fractions was determined by applying the established filter technique and conditions that have been widely employed for Ca^{2+} storage experiments with microsomal fractions or permeabilized cells of different origin. As shown in Fig. 3, 80–90% of the total cellular ATP-dependent Ca^{2+} storing activity was detected within the two cell surface-derived fractions P2 + P3 and only 15% in the microsomal fraction N-MIK. However, when conventional cell fractionation was carried out without the shearing step prior to homogenization, 90% of the Ca^{2+} storing activity was recovered within the microsomal fraction MIK. This distribution exactly corresponds to the reported distribution of the intracellular non-mitochon-

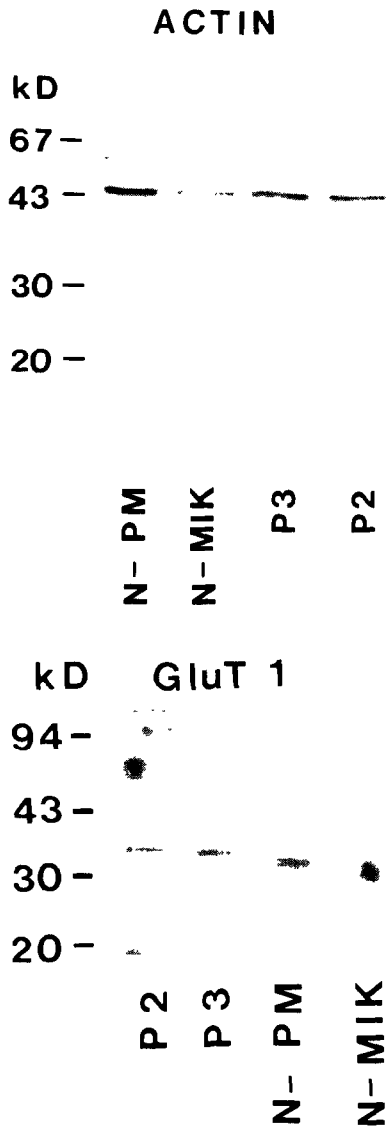

Fig. 4. Western blots of actin and the glucose transporter isoform GluT-1 in subcellular fractions of HIT cells. Each lane of the actin blot and the GluT-1 blot contained 12 μg and 150 μg membrane protein of each fraction, respectively. The fractions are labeled as in Fig. 2. The GluT-1-immunoreactive band of about 36 kDa in represents a fragment of the original transporter protein. The spots on the first and fourth lanes of the GluT-1 blot are artefacts.

drial Ca^{2+} store in subcellular fractions of various cell types using conventional cell fractionation techniques.

The uptake kinetics of $^{45}Ca^{2+}$ of both fractions P2 and P3 are identical with those described for the microsomal Ca^{2+} stores of other cell types. Ca^{2+} loading was essentially complete within 10 to 15 min after addition of ATP (Fig. 5). Ca^{2+} uptake totally depends on the presence of Mg^{2+} and ATP (data not shown). Ca^{2+} uptake experiments were performed at concentrations of free Ca^{2+} between 100 nM and 200 nM. On the other hand, as described for other microsomal Ca^{2+} stores and permeabilized cells, 1 mM vanadate potently inhibited Ca^{2+} uptake in the cell surface-derived vesicles (Fig. 5). In addition, a new effector for the ATP-dependent Ca^{2+} uptake into this pool was detected. Quite similar to vanadate, AlF4- also considerably reduced the rate of $^{45}Ca^{2+}$ uptake (Fig. 5). Rapid liberation ($t_{1/2}$ < 1 min) of 80% of the stored $^{45}Ca^{2+}$ was achieved by addition of the Ca^{2+} ionophore A23187. The tumor promoter, thapsigargin, displayed a much slower ($t_{1/2}$ = 5 min) time course of Ca^{2+} release (Fig. 6). As shown in Fig. 7, IP3 induced a rapid release of 30–40% of the stored Ca^{2+} from surface-derived vesicles. Maximal effects were observed at 1 μM IP3.

All experiments have been repeated for at least 3 times and were found to be highly reproducible.

4. DISCUSSION

Starting point of the present study was the observation that the bulk of the cellular ATP-dependent Ca^{2+}-storing activity is localized within a vesicle fraction derived from cell surface protrusions such as microvilli and membrane lamellae. This vesicle fraction is formed by the action of relatively small shearing forces exerted on the cell surface of suspended cells. The same shearing technique was used in earlier studies for the isolation of microvilli from a mammary tumor cell line [14] and a microvillar vesicle fraction containing the insulin-sensitive glucose transporter species, GluT4, of 3T3-L1 adipocytes [3].

The presented experimental data concerning the Ca^{2+}-storing properties of the cell surface-derived vesicle fraction are generally identical with those described for conventionally prepared microsomal vesicle fractions of various cell types. These data include the ATP- and Mg^{2+}-dependent Ca^{2+} uptake kinetics, the inhibi-

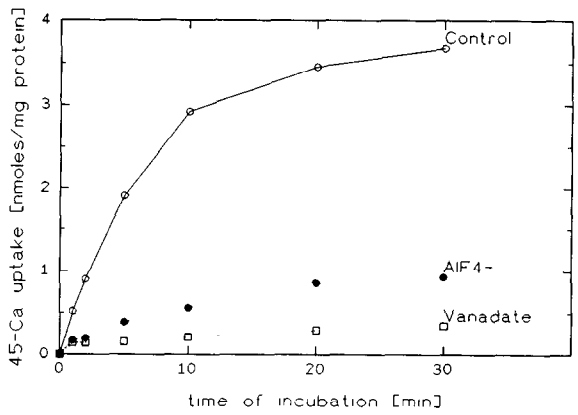

Fig. 5. Time course of ATP-dependent Ca^{2+} uptake into cell surface-derived vesicles (P3) prepared from HIT cells. Uptake experiments were performed at room temperature without additions (open circles), in the presence of 50 μM AlF4– (filled circles), and 1 mM vanadate (open squares). Vesicles were preincubated with vanadate or AlF4– for 5 min prior to initiation of the $^{45}Ca^{2+}$ uptake by addition of 2 mM ATP and $^{45}Ca^{2+}$ as described in section 2. Each point represents the mean of two determinations.

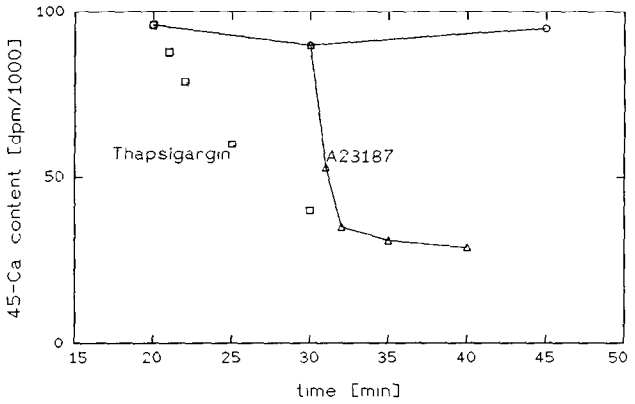

Fig. 6. Time course of thapsigargin- and A23187-induced $^{45}Ca^{2+}$ release from preloaded surface-derived vesicles (P3) from HIT cells. Thapsigargin (150 nM) (squares) and A23187 (2 μM) (triangles) was added to $^{45}Ca^{2+}$-preloaded vesicles. The time scale indicates the time elapsed after initiating $^{45}Ca^{2+}$ uptake by addition of 2 mM ATP and $^{45}Ca^{2+}$ to the vesicle suspension. The upper line (circles) represents the $^{45}Ca^{2+}$ content of the vesicles at the indicated time points in the absence of effectors. Each point represents the mean of two determinations.

tion of Ca^{2+} uptake with vanadate and A23187, as well as the Ca^{2+} release caused by A23187, thapsigargin and IP3. Thus, there seems to be no doubt about the identity of the Ca^{2+}-storing system of the cell surface-derived vesicle fraction with that found in microsomal fractions prepared by conventional techniques.

On the other hand, the non-microsomal character of the surface-derived vesicle fractions was indicated by the finding that these fractions contained a high level of ouabain-sensitive ATPase activity and an immunoreactive GluT-1 fragment which are both absent from the microsomal fraction isolated after the shearing procedure. In addition the surface-derived fractions significantly differ from the microsomal fraction in their higher actin content. A microvillar localization of the Na^+/K^+-ATPase activity has also been demonstrated in rat parotid gland cells [15].

Furthermore, we have shown that the bulk of the cellular Ca^{2+}-storing activity was recovered within the microsomal fraction when the cells were homogenized by conventional techniques. This finding indicates that normal homogenization with teflon–glass homogenizers, which also depends on shearing forces generated within the cleft between the pistil and the wall of the glass tube, gives rise to the same light surface-derived vesicle fraction. Obviously, the sedimentation properties of this fraction are indistinguishable from those of the true microsomal membrane fraction.

In contrast to true microsomal vesicles, cell surface-derived vesicles produced by the low-force shear technique are outside-out oriented [14]. This orientation most likely results from the combined shape-stabilizing effects of their internal cytoskeletal components and the proteoglycan surface coat. Especially the highly charged and hydrated proteoglycan coat of these structures may prevent a reorientation of vesicle membranes. Outside-out orientation of vesicles of microvillar origin has been repeatedly demonstrated by functional and morphological criteria in brush-border preparations from intestine or kidney epithelium [16,17].

The involvement of cell surface protrusions in the Ca^{2+} signalling pathway was further indicated by distinct bombesin- and thapsigargin-induced shape changes of the microvilli which closely resemble those accompanying insulin-induced activation of hexose transport in 3T3-L1 adipocytes [2]. On the other hand, bombesin was recently reported to stimulate hexose transport in Swiss 3T3 cells as well [18]. These morphological observations are in accord with that of Skopicheva et al. [19] who found a reduced number of microvilli and formation of membrane ruffles after EGF treatment of A431 cells.

As a consequence following from this surface localization of the IP3-sensitive Ca^{2+} storage pool one has to assume that Ca^{2+} uptake is mediated by other mechanisms rather than by a Ca^{2+}-pumping membrane ATPase. On the other hand, the cellular localization of the microvillar Ca^{2+} store is in accordance with the well established fact that the internal IP3-sensitive Ca^{2+} pool can be filled directly from the extracellular space. The present findings are compatible with the assumption of a passive Ca^{2+} influx pathway into the microvillar tip compartment and an ATP-dependent storage mechanism acting within this structure. Further studies supporting this concept are in progress.

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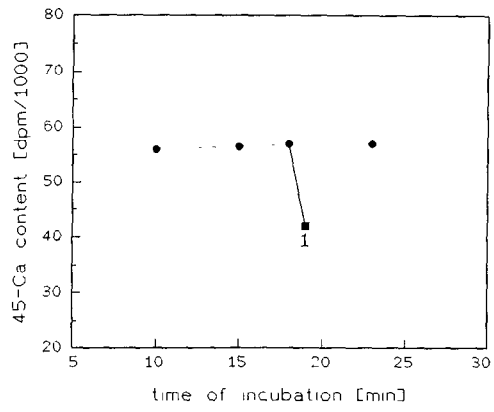

Fig. 7. IP3-mediated Ca^{2+} release from preloaded cell surface-derived vesicles (P3). The upper line (filled circles) represents the $^{45}Ca^{2+}$ content of vesicles preloaded with ^{45}Ca. The square indicates $^{45}Ca^{2+}$ release from preloaded vesicles induced by addition of 1 μM IP3. The general experimental conditions are identical with those of Fig. 6. Each point represents the mean of two determinations. The time scale indicates the time elapsed after starting the $^{45}Ca^{2+}$ uptake by addition of $^{45}Ca^{2+}$ and ATP to the vesicle suspension

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