Volume 325, number 3, 205–209 FEBS 12621 July 1993

Rapid uptake of calcium, ATP, and inositol 1,4,5-trisphosphate via cation and anion channels into surface-derived vesicles from HIT cells containing the inositol 1,4,5-trisphosphate-sensitive calcium store

Klaus Lange and Ursula Brandt

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, Germany

Received 8 April 1993; revised version received 26 April 1993

In a previous study [K. Lange and U. Brandt (1993) FEBS Lett. 320, 183–188], we showed that the bulk of the ATP-dependent IP_3-sensitive Ca^{2+} store of the hamster insulinoma cell line, HIT-T15, resides in cell surface-derived vesicles most likely of microvillar origin. The origin and orientation of these vesicles suggested that Ca^{2+} storage is not due to a membrane-located Ca^{2+} pumping ATPase but rather to ATP-dependent Ca^{2+}-binding within the vesicles. In this case, Ca^{2+}, ATP and IP_3 should have free access to the vesicle lumen. This hypothesis was tested. ATP-independent Ca^{2+} uptake occurred with biphasic kinetics. An initial rapid uptake, which was complete within 30 s, was followed by a slow linear uptake lasting about 10 min. The rapid component was shown by efflux experiments to have an equilibration half-time of about 4 s. This rapid Ca^{2+} efflux pathway was inhibited by externally applied La^{3+} (0.1 mM). A similar rapidly equilibrating La^{3+}-sensitive Ca^{2+} pool was also present in vesicles which had been actively loaded with Ca^{2+} in the presence of ATP. The intravesicular distribution space of this labile Ca^{2+} pool was identical with that of the non-metabolizable hexose analogue 3-*O*-methyl-D-glucose, demonstrating that rapid Ca^{2+} uptake occurs into a true vesicular water space and is not due to binding. ATP and IP_3 were also shown to enter the vesicles by an energy-independent pathway which is inhibited by the anion channel inhibitor, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS; 0.5 mM). Both ATP-dependent Ca^{2+} uptake and IP_3-induced Ca^{2+} release from preloaded vesicles were inhibited by DIDS. These findings clearly demonstrate that (1) the vesicle membrane is permeable to ATP and IP_3 via anion channels, and (2) Ca^{2+} uptake into as well as IP_3-induced Ca^{2+} release from the vesicles occur by passive diffusion through a cation channel which is not regulated by IP_3. Consequently, the mechanisms for Ca^{2+} storage and IP_3-induced Ca^{2+} release must be located in the vesicle lumen. Moreover, the microvillar diffusion-barrier concept, originally proposed for the regulation of hexose transport may also be valid for the receptor-operated regulation of cation and anion influx pathways. Functional coupling of the microvillar Ca^{2+} stores with the associated cation influx pathway is also strongly supported by the previously demonstrated microvillar shape changes accompanying depletion of the Ca^{2+} stores by bombesin or thapsigargin in HIT cells [K. Lange and U. Brandt (1992) FEBS Lett. 320, 183–188].

Calcium ion store (HIT cells); Microvilli; Cation channel, Anion channel, Inositol trisphosphate; ATP

1. INTRODUCTION

In a preceding study [1], we isolated from HIT insulinoma cells a surface-derived vesicle fraction which contained 80% of the total cellular Ca^{2+} stores. Up to 40% of the Ca^{2+} stored in this pool was found to be IP_3-sensitive. This vesicle fraction was prepared by applying low shear forces to the surface of suspended cells, a procedure which leaves the bulk of the cells intact but pulls off small surface protrusions. Morphological and biochemical data indicated that this fraction is derived from microvilli abundantly occurring on the surface of HIT cells. Furthermore, scanning electron microscopy revealed that the microvilli on the HIT cell surface conspicuously responded by shape changes to agents such as bombesin and thapsigargin known to release the cellular IP_3-sensitive Ca^{2+} pool [1]. The biochemical properties of the Ca^{2+} storage process of these vesicles were identical with those found in the microsomal fractions of other cells. Furthermore we demonstrated that this cell surface-derived vesicle fraction was quantitatively recovered within the microsomal fraction when cell fractionation was performed according to the classical preparation protocol by cell homogenization with a glass-teflon homogenizer.

The microvillar origin of the surface-derived vesicles strongly suggests that their membrane orientation is right-side-out as shown by other authors [2–4,7]. Consequently, the ATP-dependent Ca^{2+} storage process is unlikely to proceed via a membrane ATPase, pumping Ca^{2+} against a concentration gradient into the vesicle lumen. Another possible storage mechanism may be the existence of a passive Ca^{2+} influx pathway through the vesicle membrane combined with an ATP-dependent Ca^{2+}-binding process located in the vesicle lumen. This hypothesis is tested in the present work by studying the uptake properties of the cell surface-derived vesicles for Ca^{2+}, ATP, and IP_3.

Correspondence address K. Lange, Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, Germany. Fax: (49) (30) 831 5954.

Abbreviations: DIDS, 4,4′-diisocyanostilbene-2,2′-disulfonic acid; IP_3, inositol 1,4,5-trisphosphate; 3-OMG, 3-*O*-methyl-D-glucose.

2. MATERIALS AND METHODS

2.1. *Cell culture*

HIT T15 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultivated in plastic bottles (Costar) for one 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

2.2. *Preparation of the cell surface-derived vesicles*

Cell surface-derived membrane fractions were prepared as recently described by a hydrodynamic shearing procedure [7]. Shortly, these fractions were prepared by softly detaching the cells from the growth substrate in ice-cold 1 mM HEPES (pH 7.4) with 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 internal diameter). Each shearing 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* yielding the cell surface-derived vesicle fractions P_2 and P_3, respectively, which have been further characterized in a previous report [1]. P_2 and P_3 have been shown to be different from the true microsomal fraction by their high content of Na^+/K^+-ATPase and an immunoreactive species of the GluT1 isoform of the glucose transporter. The fraction P_2 has been used for all experiments of this study. All preparation steps were performed at 4°C or on ice. The membrane fractions were rapidly frozen in liquid nitrogen and stored at −80°C

2.3. *Calcium uptake into and release from cell surface-derived vesicles*

Cell surface-derived vesicles were washed twice with buffer A (140 mM KCl, 2 mM Mg^{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 A 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 0.5 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), rapid filtration through Schleicher und Schuell cellulose nitrate filters (type BA 85) and two additional washings with the same buffer. Each of the filtration procedures was completed within 4 s. Radioactivity remaining on the filters was determined by liquid scintillation counting

The same technique was also used for the passive $^{45}Ca^{2+}$ uptake and release experiments which were carried out in the absence of ATP and at room temperature Different times of elution were obtained by repeated filtrations with washing buffer A 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 fura-2 fluorescence (K_d = 220 nM).

2.4. *Uptake of tritium-labeled 3-OMG, ATP, and IP_3 into cell surface-derived vesicles*

Preparation and determination of 3-OMG, ATP, and IP_3 uptake into the vesicles was carried out following the protocol described in Section 2.3 Uptake was terminated by the addition of cold buffer A and two additional rapid washings with the same buffer. The filtration and washing procedures were performed within 12-15 s. 3-OMG uptake was terminated by ice-cold 0.1 mM phloretin-containing buffer A and 2 washings with the same cold buffer.

3. RESULTS

The passive Ca^{2+} influx and efflux kinetics of the cell surface-derived vesicles are shown in Fig. 1. Uptake of Ca^{2+} into the vesicles in the absence of ATP occurred in a biphasic manner (Fig. 1A). A rapid initial uptake which occurred within the first 30 s, the earliest possible time point for uptake measurements, was followed by a much slower linear uptake process. Interpolation yielded a Ca^{2+} distribution space (clearance) for the fast influx component of 4.93 μl/mg membrane protein. The efflux kinetics shown in Fig. 1B revealed that the rapidly accumulating Ca^{2+} pool is also rapidly equilibrating with the external medium with a half-time of 4 s. This rapid efflux pathway is effectively inhibited by 0.1 mM La^{3+} externally applied in the washing buffer. The difference between the vesicular $^{45}Ca^{2+}$ content after 3 washings determined in the presence and absence of La^{3+} corresponded to a Ca^{2+} distribution space of about

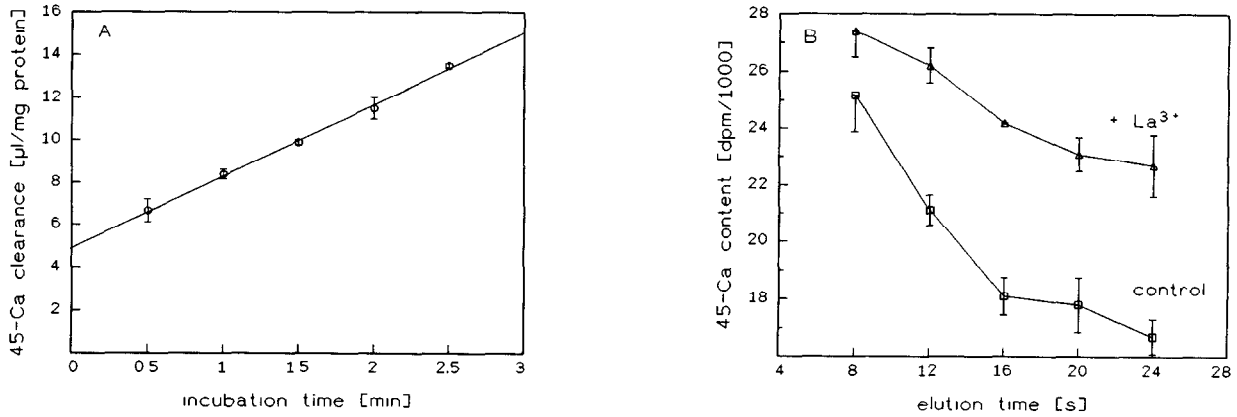

Fig. 1. Time course of the passive $^{45}Ca^{2+}$ uptake into (A) and release from (B) HIT cell surface-derived vesicles (fraction P_2). $^{45}Ca^{2+}$ uptake was performed as described in Section 2, without ATP in the incubation medium. The samples in A (open circles) and B (open triangles) were stopped and washed with a buffer A containing 0.1 mM La^{3+}. The control samples (open squares in A) were stopped and washed with buffer A without La^{3+}. In B, a higher concentration of free Ca^{2+} (15 μM) was used because EGTA was omitted from the preincubation medium. Each point of A and B represents the mean ± S.D. of 3 determinations. The data of A are presented as μl of incubation medium cleared from $^{45}Ca^{2+}$ per mg membrane protein.

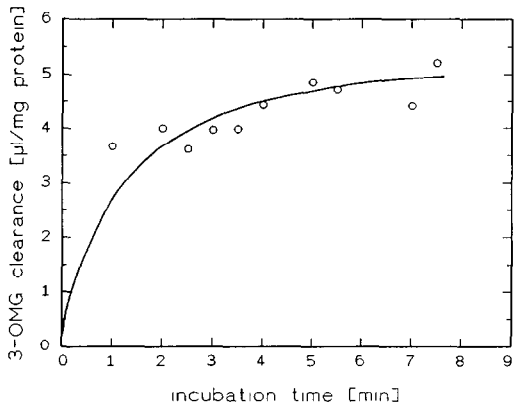

Fig. 2. Determination of the 3-OMG distribution space of cell surface-derived vesicles (fraction P_2). 3-OMG (20 μCi/ml; 2.4 Ci/mmol) uptake was achieved as described in Section 2. Data are presented as μl of incubation medium cleared from [^{3}H]3-OMG per mg membrane protein

7 μl/mg protein. This higher value may be due to the unavoidable partial elution of vesicular Ca^{2+} during the washings in the control group.

In order to exclude the possibility that the rapid Ca^{2+} uptake is merely due to binding, the vesicular distribution space of the rapid component was compared with that of the non-metabolizable glucose analogue 3-OMG (Fig. 2). 3-OMG can enter the vesicles via glucose transporters shown to be present in the cell surface derived vesicles of HIT cells [1] as well as in the microvilli of islet B cells [9]. The sizes of the distribution spaces for Ca^{2+} (4.93 μl/mg membrane protein) and 3-OMG (4.80 μl/mg membrane protein) were found to be identical. It is, therefore, highly probable that the rapidly accumulated amount of Ca^{2+} is located within the vesicular water space and is in diffusional equilibrium with the extravesicular fluid.

A similar but about 3-fold greater La^{3+}-sensitive Ca^{2+} pool (14 μl/mg protein) was also found in vesicles in which the Ca^{2+} store has previously been loaded in the presence of ATP (Fig. 3). The higher amount of freely diffusible Ca^{2+} in the presence of ATP compared to that under passive loading conditions is assumed to be due to the Ca^{2+}-complexing properties of ATP (app. $K_d = 1.6 \times 10^{-4}$ M) which is also present within the vesicles as shown below. On the other side, this ATP effect indicates that ATP and free Ca^{2+} coexist in a common vesicular water space.

Evidence for a rapid influx pathway for ATP and IP_3 into cell surface-derived vesicles of HIT cells is presented in Figs. 4 and 5, respectively. Uptake of both agents was inhibited by preincubation (10 min, 37°C) of the vesicles with the impermeant anion channel inhibitor, DIDS (0.5 mM). Uptake of external ATP greatly exceeds the value expected for equilibration with the internal water space alone. These high uptake values are most likely due to intravesicular binding and metabolism of ATP. DIDS-sensitive uptake of IP_3 is partially obscured by a high amount of IP_3 bound to the vesicles by a DIDS-insensitive process.

DIDS-sensitive entry of ATP and IP_3 into the vesicles is also shown by inhibition of both the ATP-dependent Ca^{2+} uptake into and IP_3-mediated Ca^{2+} release from DIDS-pretreated vesicles (Fig. 6). IP_3 entry into the vesicles via a DIDS-sensitive pathway was demonstrated by the fact that DIDS treatment of preloaded vesicles almost completely abolished subsequent IP_3 action on the Ca^{2+} store. This experiment further indicates that DIDS does not affect the function of the loaded Ca^{2+} store. DIDS alone did not impair the loading of the Ca^{2+} store as seen in an experiment carried out in the presence of DIDS but without preincubation with DIDS which is assumed to be necessary for covalent modification of the anion channel protein. Under these conditions Ca^{2+} uptake occurred in the presence of DIDS at room temperature for at least 5–10 min and then ceased rapidly (data not shown).

Each of the shown experiments have been repeated at least 3 times yielding highly reproducible results.

4. DISCUSSION

In a series of previous studies, a new concept of hexose transport regulation has been depicted [5–8] based on the assumption of a regulated entrance compartment for cellular hexose uptake formed by the microvillar tip compartment. This entrance compartment was shown by kinetic data to be separated from the cytoplasmic main compartment by a diffusion barrier [5,6] which is

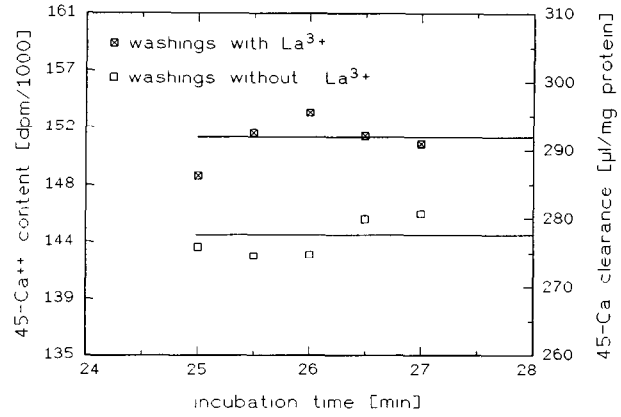

Fig. 3. Determination of the rapidly equilibrating pool of free Ca^{2+} in Ca^{2+}-preloaded cell surface-derived vesicles (fraction P_2). $^{45}Ca^{2+}$ uptake into the vesicle suspension was carried out as described in Section 2 in the presence of 0.5 mM ATP. After an incubation period of 25 min, at which time a filling steady-state has been achieved, two sets of $^{45}Ca^{2+}$ determinations were carried out, each consisting of 5 measurements performed within the shortest possible time period (2 min). The washings were carried out either with (squares with cross) or without 0.1 mM La^{3+} (open squares) in buffer A at room temperature. The washings consisted of 3 filtration procedures lasting 4 s each

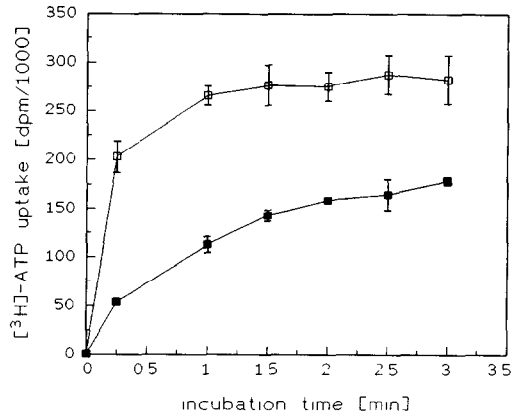

Fig. 4. Inhibition by DIDS of ATP uptake into cell surface-derived vesicles (fraction P_2). [^{3}H]ATP (25 μCi/ml; 57 Ci/mmol) uptake was carried out as described in Section 2 with (filled squares) and without preincubation (open squares) of the vesicle suspension with 0.5 mM DIDS for 10 min at 37°C. The values are expressed as mean ± S.D. of 3 determinations

thought to be formed by the internal microfilament structure of the microvilli. Insulin was shown to break down this diffusion barrier, as visualized by the microvillar shape changes, and to open a hexose influx pathway from the tip compartment into the cytoplasm [5,6]. In analogy to the previously shown surface localization of the ATP-dependent Ca^{2+} store, the insulin-sensitive pool of glucose transporters in adipocytes and other cell types generally was found in the microsomal fraction when using classical homogenization techniques. Following the shearing protocol, however, we found this transporter pool in the cell surface-derived vesicle fractions of 3T3-L1 adipocytes [7]. The detection of the IP_3-sensitive Ca^{2+} pool in the surface-derived vesicle fraction once more shows that shear forces which are much lower than those exerted on the cells during homogenization with a teflon–glass homogenizer are sufficient to cause vesiculation of cell surface protrusions such as microvilli, ruffles, veils and other structures. After subcellular fractionation these small membrane components were then recovered in the microsomal fraction which is generally assumed to contain only intracellular membrane components. The apparent cell surface localization of the IP_3-sensitive Ca^{2+} pool of HIT cells opens the question whether the diffusion barrier model, originally designed for glucose transport regulation, may also have relevance for the Ca^{2+} signaling pathway of the cell, i.e., the internal IP_3-sensitive Ca^{2+} store and the associated Ca^{2+} influx pathway.

In the present study, we have tested some predictions following from the microvillar localization of the bulk (80%) of the IP_3-sensitive Ca^{2+} store of HIT cells [1]. The Ca^{2+} storing properties of the HIT cell surface-derived vesicle fraction have been shown to be identical with those of the microsomal fraction of various cell types including the extent of the IP_3-sensitive Ca^{2+} pool which amounts to about 40% of the total ATP-dependent Ca^{2+} pool [1].

These findings have suggested a mechanistic concept for Ca^{2+} storage and release quite different from that of the current hypothesis which postulates a membrane-located ATP-driven Ca^{2+} pump combined with an IP_3-sensitive Ca^{2+} channel. Here we show that Ca^{2+} uptake into the cell surface-derived vesicles proceeds via a passive La^{3+}-sensitive cation pathway which allows rapid equilibration of the internal vesicular water space with the external medium. La^{3+} sensitivity of microsomal Ca^{2+} release has been repeatedly described by other authors [10–14]. Moreover, we present evidence that IP_3 and ATP rapidly enter the vesicles via a DIDS-sensitive anion pathway, and we demonstrate that the entry of both compounds into the vesicular lumen via the DIDS-sensitive anion pathway is essential for ATP-dependent loading and IP_3-induced emptying of the Ca^{2+} store. The latter finding further indicates that the vesicle population containing the DIDS-inhibitable anion pathway is identical with that containing the IP_3-sensitive Ca^{2+} pool. On the other hand, we could also present evidence indicating that the vesicle population containing the La^{3+}-sensitive Ca^{2+} influx pathway is identical with that containing the DIDS-sensitive anion pathway and, hence, also the IP_3-sensitive Ca^{2+} pool. Such evidence comes from the observation that the rapidly equilibrating Ca^{2+} pool was considerably increased by the presence of ATP. The amount of this increase corresponds to the value that can be calculated on the basis of the apparent dissociation constant of ATP for Ca^{2+} binding (0.16 mM): in a Ca^{2+}-buffered system, 0.5 mM ATP always binds 3.1 times the amount of free Ca^{2+}. Thus, Ca^{2+} binding to ATP would cause a 4-fold enhancement of the amount intravesicular Ca^{2+} compared to the ATP-free condition: we observed a 3-fold enhancement.

In addition, the 3-OMG equilibration experiments yielded two important items of information. First, the

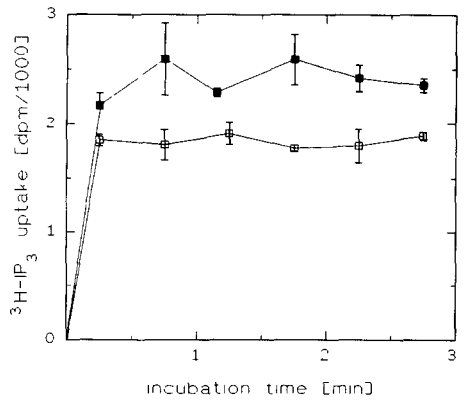

Fig. 5. Inhibition by DIDS of IP_3 uptake into cell surface-derived vesicles (fraction P_2). [^{3}H]IP_3 (0.6 μCi/ml; 50 Ci/mmol) uptake was performed as described for Fig. 4 with (filled squares) and with preincubation with 0 5 mM DIDS for 10 min at 37°C (open squares). The values are expressed as mean ± S.D. of 3 determinations.

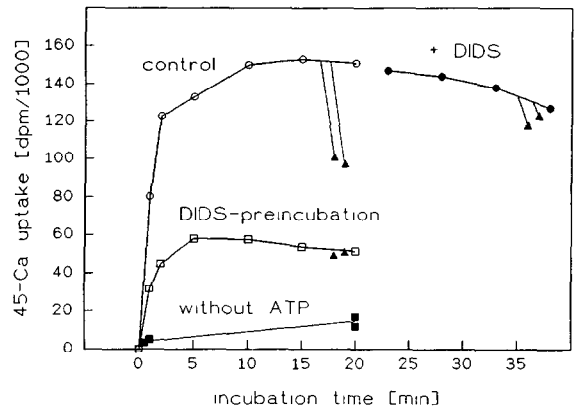

Fig. 6. Inhibition by DIDS of ATP-dependent Ca^{2+} uptake into and IP_3-induced Ca^{2+} release from surface-derived vesicles (fraction P_2). $^{45}Ca^{2+}$ uptake in the presence of ATP (open circles) was carried out as described in Section 2. After 20 min of incubation, 0.5 mM DIDS was added to the preloaded vesicles (filled circles). In a second experiment, uptake of $^{45}Ca^{2+}$ was performed after preincubation (10 min; 37°C) of the vesicles with 0.5 mM DIDS (open squares). In each of the 3 experiments, IP_3-induced Ca^{2+} release (10 μM; 1 min) was initiated at the time points indicated by straight lines connected with the filled triangles. The lower line (filled squares) represents passive Ca^{2+} uptake into the vesicles in the absence of ATP. Passive uptake values at 20 min represent steady-state levels (upper point: without DIDS; lower point: preincubated with DIDS). The open and filled circles and the open squares represent the means of 2 determinations.

presence of a rapid uptake pathway for hexoses indicates that the cell surface-derived vesicles are provided with glucose transport proteins. Since the presence of the bulk of the cell surface glucose transporters in the microvilli of islet β-cells has recently been demonstrated [9], this finding is compatible with the assumed microvillar origin of the used surface-derived vesicle fraction. Second, the vesicular 3-OMG distribution space was found to be identical with that of the La^{3+}-sensitive vesicular Ca^{2+} pool. This indicates that the Ca^{2+} pool is indeed localized in the intravesicular water space and is not due to some sort of reversible binding, for instance.

To summarize, the presented findings provide strong evidence that the mechanisms for both processes, the ATP-consuming Ca^{2+} storage and the IP_3-dependent Ca^{2+} release, are located in the lumen of the vesicles and that free Ca^{2+}, ATP, and IP_3 can rapidly enter and leave the vesicle lumen via cation and anion channels. Vesicular influx and efflux of free Ca^{2+} occurs through La^{3+}-sensitive cation channels in an IP_3-independent manner. Consequently, the IP_3 sensing mechanism of the Ca^{2+} store must also be located in the vesicle lumen.

Furthermore, the demonstration of microvillar shape changes induced by Ca^{2+} store-emptying agents such as the IP_3-agonist, bombesin, and the Ca^{2+}-ATPase inhibitor, thapsigargin, point to another interesting implication emerging from the concept of a microvillar Ca^{2+} store [1]. In intact cells, the Ca^{2+} influx pathway, detected in the surface-derived vesicle membranes, is located in the microvillar membranes. Under basal conditions, Ca^{2+} uptake into the cytoplasm through this cation channel may be restricted by the diffusion barrier consisting of the internal cytoskeletal structure of the microvilli. However, after receptor-mediated reorganization of the microvillar shaft structure, as visualized by shape changes of the microvilli on depletion of the Ca^{2+} stores, this internal cytoskeletal diffusion barrier is impaired. The diminished diffusional resistance of the microvillar pathway allows Ca^{2+} to enter the cytoplasm via the La^{3+}-sensitive cation influx pathway.

In the case of the insulin-induced activation of glucose transport in adipocytes, morphological and kinetic evidence indicated that the normal microvillar shaft organization represents an effective diffusion barrier for low molecular weight solutes such as glucose and other hexoses [6]; the same cytoskeletal barrier system may also regulate the microvillar Ca^{2+} influx pathway. Such structural connection between the Ca^{2+} store and the associated cation channel may be the basis for the well known inverse relationship between the filling state of the IP_3-sensitive Ca^{2+} store and the activation of a Ca^{2+} influx pathway as first described by Putney [15].

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

[1] Lange, K. and Brandt, U. (1993) FEBS Lett. 320, 183–188.
[2] Carothers Carraway, C.A., Cerra, R.F., Bell, P.B. and Carraway, K.L. (1982) Biochim. Biophys. Acta 719, 126–139.
[3] Bretscher, A. and Weber, K. (1978) Exp. Cell Res. 116, 397.
[4] Mooseker, M.S. and Howe, C.L. (1982) Methods Cell Biol. 25, 143–174.
[5] Lange, K., Brandt, U., Keller, K. and Zimmermann, B. (1989) J. Cell. Physiol. 141, 29–43.
[6] Lange, K., Brandt, U. and Zimmermann, B. (1990) J. Cell. Physiol 142, 1–14.
[7] Lange, K. and Brandt, U. (1990) FEBS Lett. 261, 459–463.
[8] Lange, K. and Brandt, U. (1990) FEBS Lett 276, 39–41.
[9] Orci, L., Thorens, B., Ravazzola, M. and Lodish, H.F (1989) Science 245, 295–297.
[10] Dargemont, C., Hilly, M. and Mauger, J.-P. (1988) Biochem. J. 256, 117–124.
[11] Palade, P., Dettbarn, C and Volpe, P. (1989) Mol. Pharmacol. 36, 673–680.
[12] Kwan, C.Y., Takemura, H., Obie, J.F., Thastrup, O. and Putney, J.W. (1990) Am. J. Physiol. 258, C1006–C1015.
[13] Hartl, C., Fischer, S. and Hasselbach, W. (1990) Z. Naturforsch. 45, 121–124.
[14] Mason, M.J., Mahaud-Smith, M.P. and Grinstein, S. (1991) J. Biol. Chem. 266, 10872–10879.
[15] Putney Jr., J.W. (1986) Cell Calcium 7, 1–12.