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Reconstitution of a Passive Ca^{2+}-transport Pathway from the Basolateral Plasma Membrane of Rat Parotid Gland Acinar Cells

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Abstract. We have previously reported that rat parotid gland basolateral plasma membrane vesicles (BLMV) have a relatively high affinity Ca^{2+} transport pathway and an unsaturable Ca^{2+} flux component (Lockwich et al., 1994. J. Membrane Biol. 141:289–296). In this study, we have solubilized BLMV with octylglucoside (1.5%) and have reconstituted the solubilized proteins into proteoliposomes (PrL) composed of *E. coli* bulk phospholipids, by using a detergent dilution method. PrL exhibited 3-5-fold higher $^{45}Ca^{2+}$ influx than control liposomes (without protein). Ca^{2+} uptake into PrL was dependent on the [protein] in PrL and steady state $[Ca^{2+}]$ in PrL was in equilibrium with external $[Ca^{2+}]$. These data demonstrate that a passive, protein-mediated Ca^{2+} transport has been reconstituted from BLMV into PrL. $^{45}Ca^{2+}$ influx into liposomes did not saturate with increasing $[Ca^{2+}]$ in the assay medium. In contrast, PrL displayed saturable $^{45}Ca^{2+}$ influx and exhibited a single Ca^{2+} flux component with an apparent $K_{Ca} = 242 \pm 50.9$ μM and $V_{max} = 13.5 \pm 1.14$ nmoles Ca^{2+}/mg protein/minute. The K_{Ca} of Ca^{2+}-transport in PrL was similar to that of the high affinity Ca^{2+} influx component in BLMV while the V_{max} was about 4-fold higher. The unsaturable Ca^{2+} flux component was not detected in PrL. $^{45}Ca^{2+}$ influx in PrL was inhibited by divalent cations in the order of efficacy, $Zn^{2+} > Mn^{2+} > Co^{2+} = Ni^{2+}$, and appeared to be more sensitive to lower concentrations of Zn^{2+} than in BLMV. Consistent with our observations with BLMV, the carboxyl group reagent N,N′-dicyclohexylcarbodiimide (DCCD) inhibited the reconstituted Ca^{2+} transport in PrL. Importantly, in both BLMV and PrL, DCCD induced a 40–50% decrease in V_{max} of Ca^{2+} transport without an alteration in K_{Ca}. These data strongly suggest that the high affinity, passive Ca^{2+} transport pathway present in BLMV has been functionally reconstituted into PrL. We suggest that this approach provides a useful experimental system towards isolation of the protein(s) involved in mediating Ca^{2+} influx in the rat parotid gland basolateral plasma membrane.

Key words: Reconstitution — Calcium influx — Basolateral membrane vesicles — Proteoliposomes — Parotid gland — Octyl glucoside

Introduction

Ca^{2+} influx pathways are present in the plasma membrane of both excitable and nonexcitable cells and are involved in regulating a wide range of cellular functions, including secretion, contraction, and excitability [3, 9, 30]. In excitable cells, such as neuronal and muscle cells, Ca^{2+} influx is mediated via the well characterized, voltage dependent Ca^{2+} channels [9, 35]. In nonexcitable cells, such as rat parotid and other exocrine gland cells, there is considerable evidence to suggest that stimulation of Ca^{2+} entry is achieved as a result of the depletion of Ca^{2+} from an internal Ca^{2+} pool(s) [3, 27, 33, 34]. However, the molecular events by which the status of $[Ca^{2+}]$ in the internal Ca^{2+} pool is conveyed to the plasma membrane is not yet known [31]. Furthermore, no information is available regarding the molecular nature of the Ca^{2+} influx system and of the components mediating Ca^{2+} influx across the plasma membrane. In contrast to the voltage-gated Ca^{2+} channels which have

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been distinguished based on their differential sensitivities to a number of drugs, toxins, and divalent cations, there are no specific ligands which interact with and modify the Ca^{2+} influx pathway in nonexcitable cells. The lack of such ligands has hindered progress in the identification of the molecular components mediating Ca^{2+} entry into nonexcitable cells. Recently, electrophysiological studies in mast cells [12, 17] demonstrate the presence of a number of different cation channels in cells under stimulated and unstimulated conditions. However, until now, it is not clear which of these channels is the Ca^{2+} entry pathway activated in response to internal Ca^{2+} depletion or whether the Ca^{2+} entry mechanism is, in fact, a channel. Clearly, isolation of the protein(s) involved in Ca^{2+} entry will be essential for complete understanding of its regulation and function.

Studies in our laboratory have been directed towards understanding the biochemical and molecular characteristics of the Ca^{2+} entry pathway in salivary gland cells [16, 27, 28]. More recently, we have examined the passive Ca^{2+} permeability of isolated rat parotid gland basolateral plasma membrane vesicles (BLMV) [19–21]. Membrane vesicles provide an extremely useful experimental system for studying membrane transport functions and have been extensively used for characterizing active and passive Ca^{2+} transport mechanisms, including the Ca^{2+} channels described above and those located in intracellular organelle membranes [13, 14, 24–26]. We hypothesised that the Ca^{2+} transport pathways present in the intact cell basolateral plasma membrane, should be present in isolated BLMV and that during cell disruption, the Ca^{2+} influx pathway might be partially, or fully, activated. We have recently reported the presence of a high-affinity, trypsin-sensitive, passive Ca^{2+} transport component in BLMV, which exhibits characteristics (such as inhibition by low temperature and DCCD) similar to internal Ca^{2+} pool depletion-activated divalent cation entry in rat parotid acinar cells [19, 20]. However, clear interpretation and further characterization of this Ca^{2+} influx component is complicated by our observation, described in a previous report [19], that there are at least two different passive Ca^{2+} entry components in BLMV. In addition to the temperature-sensitive, high-affinity, component described above, there is also a temperature-insensitive, unsaturable, Ca^{2+} transport component which maybe due to Ca^{2+} influx via a nonspecific "leak pathway" or a protein mediating diffusional transport.

To characterize the protein(s) mediating Ca^{2+} influx in rat parotid gland basolateral membranes, it is necessary to purify the molecule in a functional state and determine its transport properties in an artificial membrane system. Towards that goal, we have used a reconstitution approach to characterize the high affinity Ca^{2+} influx pathway in a relatively ion-impermeant membrane system. The characteristics of Ca^{2+} transport in PrL demonstrate the functional reconstitution of the high-affinity passive Ca^{2+} transport component from BLMV.

Materials and Methods

Male Wistar rats were from Harlan Sprague-Dawley. $^{45}CaCl_2$ (2mCi/ml) was obtained from Amersham. Ultragrade mannitol, octyl β D-glucopyranoside (octyl glucoside), and dithiothreitol (DTT) were purchased from Calbiochem (San Diego, CA). N,N′-Dicyclohexylcarbodiimide (DCCD), bovine serum albumin, and phenyl methanesulfonyl fluoride (PMSF) were from Sigma Chemicals (St. Louis, MO). 4-(2-aminoethyl)-benzene-sulfonylfluoride hydrochloride (AEBSF), pepstatin A, and leupeptin were purchased from ICN. Crude *Escherichia coli* (*E. coli*) lipid extract was obtained from Avanti Polar Lipids, and washed with acetone/ether as described [7]. All other reagents used were of the highest available grade.

PREPARATION OF BLMV

BLMV were prepared as described previously [19–21]. Briefly, parotid glands from 16–24 male Wistar rats were excised, cleaned and homogenized (by Polytron) in a medium containing (in mM): 250 sucrose, 10 Tris-HCl (pH 7.5), 1 DTT, and 0.1 PMSF. The homogenate was centrifuged at 3,000 × *g* for 15 min to remove cell debris. The resulting supernatant was centrifuged at 23,500 × *g*. The pellet was resuspended in the homogenization buffer, mixed with Percol (12% v/v) and centrifuged at 49,000 × *g* for 30 min. The BLMV fraction was collected and washed three times with (in mM): 100 mannitol, 1 DTT, 0.1 PMSF, and 10 Tris-HCl (pH 7.5). The final pellet was suspended in 300 mannitol, 1 DTT, and 10 Tris-HCl (pH 7.5) at a concentration of 2–4 mg/ml, quick frozen in liquid N_2, and stored at −70°C until use (maximum two weeks). Protein concentration was determined using the Bio-Rad protein assay (microassay procedure).

This membrane preparation has been well characterized and extensively used in our laboratory. Typically, a 25–30-fold enrichement of basolateral plasma membrane marker enzymes is detected, with no enrichment of mitochondrial or endoplasmic reticulum markers. Further, we do not detect components from other cell types in our cell preparation (cells are prepared by enzymatic digestion of glands dissected and cleaned as described above, i.e., similar procedure till the homogenization step). For example, PLCβ_1 and m_1-muscarinic receptor, proteins found in neuronal cells are not detected in parotid gland membranes (by Western blotting with 100 μg of protein on the gel). Ductal cell contamination is <5% of the cell preparation. Thus, the present BLMV preparation is >95% from parotid gland acinar cells.

SOLUBILIZATION OF BLMV WITH OCTYL GLUCOSIDE AND RECONSTITUTION INTO PROTEOLIPOSOMES

Unless otherwise noted all steps were performed at 4°C. 5 mg of BLMV were diluted into 5–6 ml of (mM): 200 KCl, 50 K-MOPS (pH 7.5), 2.5 $MgCl_2$, and 1 AEBSF, then centrifuged for 15 min at 60,000 × *g*. The supernatant was discarded and the pellet was resuspended in 0.5 ml buffer. Washed membranes were solubilized with 1.5% octylglucoside (w/v) in 2.4 ml of a medium consisting of (in mM): 50 K-MOPS (pH 7.4), 20% glycerol (v/v), 0.37% *E. coli* lipids (w/v), 1.5 $MgCl_2$, 1 DTT, 0.75 AEBSF, 0.167 pepstatin, and 0.167 leupeptin [7]. This mixture was kept for 20 min on ice then centrifuged for 1 hr at 145,000 × *g*. The supernatant (octyl glucoside extract, containing the solubilized BLMV) was used for subsequent reconstitution. Liposomes (vesicles without protein) were prepared by treating the lipid +

glycerol mixture with octyl glucoside under the same conditions (incubation times, centrifugation etc.) described above. This control extract was then treated as given below.

Reconstitution was carried out in a final volume of 1 ml containing 80% (v/v) of the octyl glucoside extract (control or from BLMV) described above, 12.5 mg of bath-sonicated *E. coli* phospholipid, 1.25% octylglucoside, 200 (in mM) KCl, 50 (in mM) K-MOPS (pH 7.5), 1 (in mM) DTT, and 0.5 (in mM) AEBSF. The mixture was briefly vortexed and incubated for 20 min on ice. PrL and liposomes, were then formed by rapidly injecting the mixture into 24 ml of a dilution buffer, which was continuously stirred at room temperature, containing (in mM): 200 KCl, 50 K-MOPS (pH 7.5), 1 DTT, and 1 AEBSF. The stirring was stopped after the addition was completed and the resultant suspension was incubated for 20 min at room temperature. PrL or liposomes were then collected by centrifugation for 1 hr at 105,000 × *g* in a Beckman 40.2 rotor (4°C). The pellet was resuspended in 8 ml of ice-cold dilution buffer (as described above but with 2.5 mM $MgCl_2$). This suspension was centrifuged for 15 min at 7,500 × *g* and the resultant supernatant centrifuged for 1 hr at 145,000 × *g* to obtain PrL (or liposomes). This pellet was resuspended in 300–600 µl of ice-cold dilution buffer containing the protease inhibitors by using a 23-gauge needle. These liposomes and PrL were immediately used for $^{45}Ca^{2+}$ transport experiments. Protein concentrations in BLMV, detergent extract, and PrL were determined using the dye-binding method of Schaffner and Weissmann [32].

^{45}Ca FLUX INTO LIPOSOMES AND PROTEOLIPOSOMES

$^{45}Ca^{2+}$ flux into the PrL (or liposomes) was initiated at 37°C by diluting an aliquot of proteoliposomes 12.5× into an assay medium containing (in mM): 200 KCl, 2.5 $MgCl_2$, 50 K-MOPS (pH 7.5), and $^{45}CaCl_2$ (at concentrations as specified in the figure legends). After various times, as indicated, aliquots (5–20 µg) were removed and filtered under vacuum through Millipore filters (0.22 µ, type GSTF), then washed three times (3 ml each) with ice-cold assay medium (without $^{45}CaCl_2$) and subsequently dried and counted for radioactivity. For determining the kinetic parameters, uptake was performed for 20 sec. Typically, $^{45}Ca^{2+}$ uptake was similarly measured in PrL and an equal volume of liposomes. The radioactivity accumulated into the control liposomes was subtracted from the radioactivity accumulated in PrL to yield a corrected value of $^{45}Ca^{2+}$ uptake into PrL.

^{45}Ca FLUX INTO BASOLATERAL PLASMA MEMBRANE VESICLES (BLMV)

$^{45}Ca^{2+}$ influx into BLMV was measured as described earlier [19]. Briefly, the uptake assay was started by adding 100 µg of BLMV to a assay medium containing 10 mM Tris-HEPES (pH 7.4), 1 mM $MgCl_2$, and 100 µM $^{45}CaCl_2$ at 37°C (final volume 1 ml). After various times aliquots of the assay medium (9 µg BLMV) were removed and filtered through Millipore filters (0.45 µ, type HAWP) using a Millipore filtration system and washed three times (3 ml each) with ice-cold 10 mM Tris-HEPES (pH 7.4), 1 mM $MgCl_2$, and 200 µM $LaCl_3$. The filters were air dried and counted for radioactivity in a scintillation counter.

DCCD TREATMENT OF BLMV AND OCTYLGLUCOSIDE EXTRACT OF BLMV

Octylglucoside extract of BLMV was incubated with 2 mM DCCD, or an equal concentration of the vehicle dimethylsulfoxide (DMSO), at a concentration of 0.5 mg protein/ml at 25°C for 20 min. Following this, the DCCD-treated and control extracts were reconstituted into PrL. PrL were assayed for Ca^{2+} uptake in the first 20 sec after $^{45}Ca^{2+}$addition, was determined, as described above. Liposomes were also prepared under the same conditions.

BLMV, 100 µg protein/ml, were treated with 2 mM DCCD at 25°C for 20 min in medium containing 10 mM Tris-HEPES (pH 7.4) and 1 mM $MgCl_2$. BLMV were then centrifuged at 106,000 × *g* for 40 min, washed, and resuspended in the same medium. Control BLMV were similarly treated but incubated with an equal volume of DMSO. Ca^{2+} uptake was assayed in control and DCCD-treated BLMV. Uptake was initiated by addition of $^{45}CaCl_2$ ($[Ca^{2+}]$ = 10 µM to 7.5 mM) to 10 µg of BLMV in 100 µl of assay medium. After incubation for 5 sec, ice-cold stop buffer containing 10 mM Tris-HEPES (pH 7.4), 2.5 mM, $MgCl_2$, and 0.35 mM $LaCl_2$ was added. The sample was vortexed, filtered through Millipore filters (0.45 µ, HAWP), washed three times with 3 ml of stop buffer. Filters were then air-dried, dissolved in Aquasol (DuPont) and the radioactivity was determined using a scintillation counter. To determine background $^{45}Ca^{2+}$ uptake, i.e., at 0 seconds, stop buffer was added to BLMV prior to addition of $^{45}Ca^{2+}$. Initial rates of Ca^{2+} uptake (nmoles Ca^{2+}/mg protein/minute) at the various $[Ca^{2+}]$ were calculated from the uptake in the first five seconds.

The data in the manuscript have been presented as mean ± SEM for the number of experiments indicated in the figure legends. Where indicated, the Student's *t*-Test was used to statistically evaluate the data.

ABBREVIATIONS

PrL, proteoliposomes; BLMV, basolateral membrane vesicles; DCCD, N,N′-Dicyclo-hexylcarbodiimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethylsulfoxide; BAPTA, 1,2-bis(2-amino-5,5′-difluorophenoxy)-ethane-n,n,n′,n′-tetraacetic acid.

Results and Discussion

$^{45}Ca^{2+}$ UPTAKE INTO LIPOSOMES AND PROTEOLIPSOMES

The basolateral plasma membrane of rat parotid acinar cells contains a number of Ca^{2+} transporters which play a critical role in the regulation of $[Ca^{2+}]_i$ in these cells; e.g., the $(Ca^{2+}+ Mg^{2+})$ – ATPase and the Ca^{2+} influx pathway [4, 8, 30]. We have previously used a well characterized isolated basolateral plasma membrane vesicle (BLMV) preparation to study the $(Ca^{2+}+ Mg^{2+})$ – ATPase [1–3]. More recently, we have examined passive Ca^{2+} transport in these vesicles [19–21]. We have reported that BLMV display a high affinity Ca^{2+} influx component which has certain characteristics similar to those of divalent cation influx in intact acinar cells [19]. To further examine this BLMV Ca^{2+} influx pathway we have reconstituted octyl glucoside-solubilized proteins from BLMV into PrL and examined the characteristics of $^{45}Ca^{2+}$ influx into PrL. The reconstitution protocol involves solubilization of membranes with the detergent, octyl glucoside, in the presence of glycerol, and reconstitution into liposomes by detergent dilution. This method has been used to reconstitute a number of

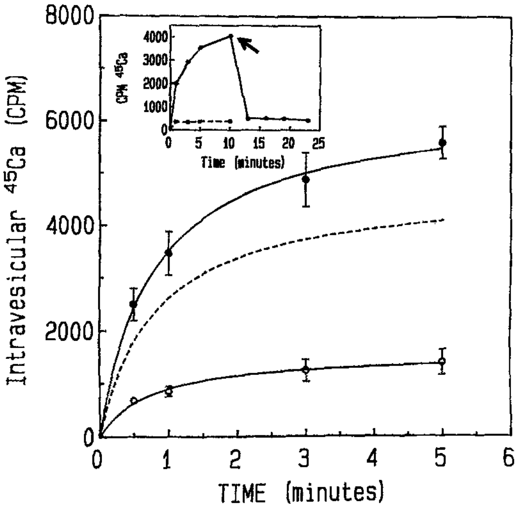

Fig. 1. Ca^{2+} influx into liposomes and proteoliposomes. 20 μl of liposomes (open circles, continuous line) or 20 μl of PrL (closed circles, continuous line) were diluted 12.5× into an uptake assay medium containing (in mM): 200 KCl, 2.5 MgCl$_2$, 50 MOPS (pH 7.5), and 25 μM ^{45}CaCl$_2$ at 37°C. After the times shown, the vesicles were filtered as described in Materials and Methods then counted. The corrected ^{45}Ca^{2+} uptake into PrL (broken line) is obtained by subtracting the ^{45}Ca (CPM) accumulated in PrL from that accumulated in liposomes. The average corrected Ca^{2+} uptake in PrL is about 2.5 nmoles/mg protein at 5 min. The values given are mean ± SEM from 4–6 experiments performed with different BLMV preparations. Inset. After 10 min of uptake in both liposomes (*not shown*) and PrL, 5 μM alamethicin was added, shown by the arrow (continuous line). In a parallel assay alamethicin was included in the assay buffer before the addition of ^{45}Ca^{2+} to both liposomes (*not shown*) and PrL (broken line). Addition of alamethicin did not alter liposomal ^{45}Ca^{2+} accumulation. The data in the inset are representative of two similar experiments.

transporters such as the plasma membrane Ca^{2+} pump from rat parotid BLMV [22], the aquaporin AQP1 [36], P-glycoprotein [6], and the relatively complex multicomponent histidine permease system [10].

The ability of proteoliposomes (PrL) to accumulate ^{45}Ca^{2+} is shown in Fig. 1 (closed circles, continuous line). Following addition of ^{45}Ca^{2+} (25 μM), PrL show a rapid accumulation of ^{45}Ca^{2+}, which reaches a steady state within 3–5 minutes. Typically, PrL accumulate approximately 5-fold more ^{45}Ca^{2+} than an equal volume of liposomes (Fig. 1, open circles, continuous lines) which are prepared using the same procedure (i.e., similar detergent and lipids) as PrL but without any protein (*see* Materials and Methods). Further, ^{45}Ca^{2+} uptake into PrL is increased when the amount of protein reconstituted into PrL is increased (i.e., by using more solubilized protein during reconstitution). It should be noted that <10% of BLMV lipid is extracted with protein and reconstituted into PrL (*data not shown*). Further, since the protein:lipid ratio is very low in PrL, equal volumes of PrL and liposomes have similar lipid contents. The corrected Ca^{2+} uptake in PrL (i.e., due to incorporated protein) can be calculated by subtracting the radioactivity obtained in liposomes (i.e., due to lipid only) from the radioactivity obtained in PrL and then normalized to the amount of protein present in the PrL (broken line, Fig. 1). This corrected Ca^{2+} transport value in PrL, expressed as nmoles Ca^{2+} per mg protein, is shown in all subsequent figures. Typically, PrL reach a intravesicular steady state [Ca^{2+}] of 2–2.5 nmoles/mg protein (scale not shown in Fig. 1). The Ca^{2+} accumulated in PrL is released by the addition of alamethicin (a pore forming antibiotic) to the Ca^{2+}-loaded PrL (Fig. 1 inset, arrow at 10 min reflects addition of alamethicin). Additionally, alamethicin prevents the accumulation of Ca^{2+} into PrL when the PrL are incubated with alamethicin before the addition of ^{45}Ca^{2+} (broken line, Fig. 1 inset). These results are consistent with accumulation of Ca^{2+} in the lumen of the PrL and cannot be explained by an increase in Ca^{2+} binding due to the proteins associated with the PrL.

To determine the nature of Ca^{2+} influx into PrL, we have calculated the intravesicular [Ca^{2+}] at steady state levels of Ca^{2+} influx (i.e., level reached after 3–5 minutes of uptake, *see* Fig. 1). Typically, these PrL have an internal volume of 1 μl/mg of phospholipid [7] and using this value, the calculated steady state intravesicular [Ca^{2+}] is 31 μM and 110 μM in two experiments performed with extravesicular [Ca^{2+}] at 25 μM and 100 μM, respectively. These results demonstrate that the steady state intravesicular [Ca^{2+}] is in equilibrium with the extravesicular [Ca^{2+}]. Notably, similar values of [Ca^{2+}] were obtained by the addition of ionomycin to PrL and BLMV. In aggregate, the data described above suggest that Ca^{2+} influx in PrL occurs via a passive, protein-mediated pathway.

Effect of Octylglucoside Concentration on Protein Yield and Ca^{2+} Transport Activity in Proteoliposomes

To optimize the conditions for reconstitution of the Ca^{2+} transporter(s) from BLMV, the concentration of octylglucoside was varied during solubilization. Figure 2 reveals that the highest Ca^{2+} transport activity (normalized to concentration of protein in PrL, as described above) is obtained when 1.5% octylglucoside is used to solubilize BLMV (continuous line). When the concentration of octylglucoside is increased the amount of protein solubilized is increased until approximately 30% of the protein is extracted (broken line, Fig. 2). At every detergent concentration the percentage recovery of solubilized protein into PrL is constant (50–70%). Since the highest activity of Ca^{2+} transport is obtained using 1.5% octylglucoside, this detergent concentration was used for preparation of PrL in all further experiments described here. The decrease in Ca^{2+} influx activity at higher octyl glucoside concentrations maybe due to inactivation of

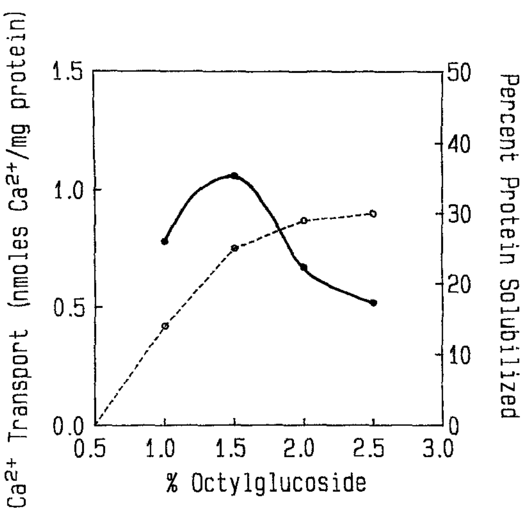

Fig. 2. Effect of octyl glucoside concentration on solubilization of BLMV proteins and recovery of Ca^{2+} transport in proteoliposomes. PrL, prepared from octyl glucoside extracts of BLMV solubilized at various concentrations of octylglucoside (1.0%–2.5%), were assayed for Ca^{2+} transport activity as described in the legend to Fig. 1 (closed circles, continuous line). The total protein extracted as a result of the different octylglucoside concentration was also determined (open circles, broken lines). The final recovery of protein in the PrL was similar in each case (about 23% of initial BLMV protein and about 68% of octyl glucoside extract). The figure shows results from a representative experiment, two experiments, each assayed in duplicate were performed with different BLMV preparations.

the protein(s) involved in Ca^{2+} flux. In addition, in some experiments (*data not shown*), when starting activity in BLMV was low, PrL also demonstrated low Ca^{2+} uptake. In aggregate, these data are also consistent with the suggestion that Ca^{2+} influx into PrL is associated with a specific protein(s).

DIVALENT CATION SENSITIVITY OF $^{45}Ca^{2+}$ UPTAKE IN PROTEOLIPOSOMES AND BLMV

The sensitivity of $^{45}Ca^{2+}$ uptake into PrL and BLMV to various divalent cations was determined by measuring the ability of PrL to accumulate $^{45}Ca^{2+}$ in the presence of increasing concentrations of various divalent cations; $[Ca^{2+}]$ in the assay medium was 100 μM. This experimental approach has been used to characterize Ca^{2+} permeabilities in other membranes [9, 15]. The observed pattern of inhibition of Ca^{2+} uptake in PrL by the various divalent cations tested suggests the following order of effectiveness; $Zn^{2+} > Mn^{2+} > Ni^{2+} > Co^{2+}$ (Fig. 3*A*). In BLMV, under the same experimental condition, a slightly different pattern is observed in the sensitivity of $^{45}Ca^{2+}$ uptake to inhibition by various divalent cations (Fig. 3B). In these membranes, there appears to be less difference in the abilities of the various divalent cations to inhibition Ca^{2+} accumulation, as compared to that in PrL (Fig. 3*A*). At 100 μM of ions, $^{45}Ca^{2+}$ uptake in BLMV is inhibited in the order $Zn^{2+} = Mn^{2+} > Co^{2+} = Ni^{2+}$ (50% loss of activity obtained with 1.1, 1.08, 0.76, and 0.77 mM for Ni^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+}, respectively). While additional experiments are required to determine the IC_{50} values of the various divalent cations on Ca^{2+} influx in BLMV and PrL, the present data indicate that Zn^{2+} exerts maximal inhibitory effects on passive $^{45}Ca^{2+}$ transport in both PrL and BLMV and that it appears to be more effective at lower concentrations in PrL than in BLMV. For example in PrL, Ca^{2+} influx in the presence of 100 μM Zn^{2+} is lower than that obtained with 100 μM Ni^{2+} or Co^{2+}, while in BLMV, < 250 μM is required to observe decreases in Ca^{2+} influx. Further, Co^{2+} and Ni^{2+} exert less inhibition of Ca^{2+} uptake in PrL as compared to BLMV. The somewhat nonspecific effects of the divalent cations in BLMV are likely due to the additional Ca^{2+} permeabilities (e.g., unsaturable, temperature-insensitive, component) present in these vesicles, which we have described previously [20, 21]. Consistently, the increased effectiveness of Zn^{2+} and decreased effectiveness of Co^{2+} in PrL suggest a decrease in the contribution of additional Ca^{2+} permeable pathways.

KINETICS OF Ca^{2+} UPTAKE BY PROTEOLIPOSOMES AND BLMV

We have previously reported that passive Ca^{2+} influx in BLMV is due to at least two influx components; a saturable, relatively high affinity Ca^{2+} transport component (K_{Ca} = 251 ± 54 μM, V_{max} = 3.12 nmoles/mg protein/minute) and an unsaturable Ca^{2+} influx component [19]. To determine the pathways reconstituted in PrL, we measured the kinetics of $^{45}Ca^{2+}$ uptake into PrL. The effects of varying external $[Ca^{2+}]$ on the initial rate (linear phase) of Ca^{2+} influx into PrL is shown in Fig. 4. The rate of Ca^{2+} uptake increases with increasing extravesicular $[Ca^{2+}]$ and approaches saturation when the $[Ca^{2+}]$ is >500 μM. The data shown in Fig. 4 have been corrected, at each $[Ca^{2+}]$, for the corresponding liposomal $^{45}Ca^{2+}$ uptake. Notably, $^{45}Ca^{2+}$ influx into control liposomes does not show saturation (*results not shown*). Figure 4 (inset) shows an Eadie-Hofstee plot of the data shown in Fig. 4. The Eadie-Hofstee plot reveals a linear slope which is reflective of a single transport component. The calculated K_{Ca} is 242 ± 50.9 μM and V_{max} is 13.5 ± 1.14 nmoles Ca^{2+}/mg protein/minute. The K_{Ca} of PrL is not significantly different from the values obtained for the high-affinity Ca^{2+} transport component in BLMV, while the values for V_{max} are significantly higher ($P < 0.025$). The increase in V_{max} indicates a 3-4-fold enrichment of the relatively high affinity Ca^{2+} transport component in PrL, which is consistent with the recovery of protein during reconstitution (i.e., about 80% loss of protein during solubilization). It is important to note that the unsat-

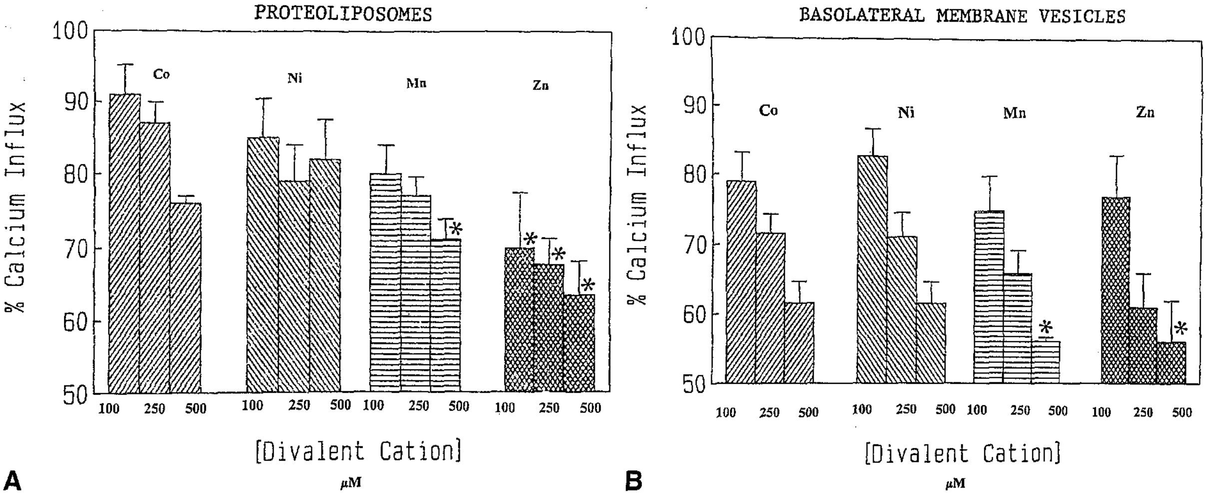

Fig. 3. Divalent cation sensitivity of $^{45}Ca^{2+}$ uptake into PrL and BLMV. Figure 3*A*. 20 µl of PrL or 20 µl of liposomes were diluted 12.5× into similar Ca^{2+} uptake assay medium described for Fig. 1 (but with 100 µM $^{45}CaCl_2$) and several divalent cations, i.e., Mn^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} (concentrations are shown in the figure). After a 1-min incubation at 37°C, the PrL and liposomes were filtered and counted. The corrected $^{45}Ca^{2+}$ in PrL in each condition is expressed relative to $^{45}Ca^{2+}$ uptake in the absence of any added divalent cation. Similarily, BLMV (Fig. 3*B*) were diluted 12.5× into a Ca^{2+}-uptake medium consisting of 10 mM Tris-Hepes (pH 7.4), 100 µM $^{45}CaCl_2$, and 1 mM $MgCl_2$ containing the same concentrations of divalent cations used for PrL. After 1 min the BLMV were filtered and the intravesicular Ca^{2+} was determined. The Ca^{2+} accumulation in the presence of the divalent cations is expressed relative to Ca^{2+} uptake in the absence of cations (Fig. 3*B*). The data show mean ± SEM for 6–7 experiments with different BLMV preparations. In Fig. 3*A* all values, except for the value obtained with 100 µM Co^{2+}, are significantly different from control ($P < 0.05$) and the values marked * (for Zn^{2+} and Mn^{2+}) are significantly different from values obtained with equivalent concentrations of Mn^{2+}, Co^{2+} and Ni^{2+} ($P < 0.05$). In Fig. 3*B*, all values are significantly different from control ($P < 0.025$), and the values marked * (with Zn^{2+} and Mn^{2+}) are different ($P < 0.05$) from the values obtained with equivalent concentrations of Ni^{2+} and Co^{2+}.

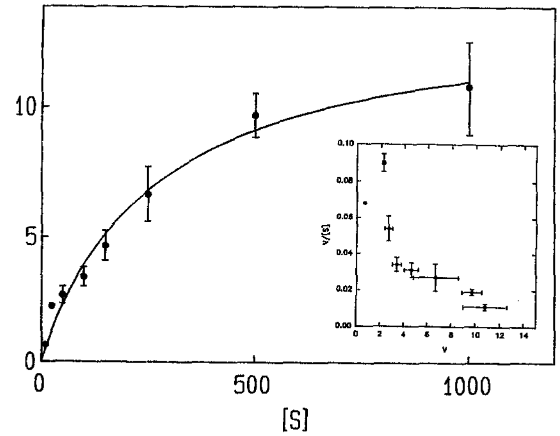

Fig. 4. Kinetic analysis of Ca^{2+} influx into proteoliposomes. 20 µl of liposomes and PrL were diluted 12.5× into the uptake assay medium described above at 37°C for 20 sec then filtered and $^{45}Ca^{2+}$ incorporation was determined. The corrected initial rate of intravesicular [$^{45}Ca^{2+}$] accumulation in PrL was determined for each extravesicular [Ca^{2+}]. Data shown are mean ± SEM for 4–6 experiments with different BLMV preparations. Inset: Eadie-Hoffstee plot of the data.

urable component seen in BLMV is not detected in PrL, at least within the range of [Ca^{2+}] we have used, 10–1000 µM. Analysis of data using either nonlinear regression or Eadie Hoffstee plots demonstrate the presence of a single flux component in PrL, whereas in the case of BLMV, two statistically significant flux components were detected [19]. It is possible that higher [Ca^{2+}] may be required to observe flux via the unsaturable component in PrL. However, such high [Ca^{2+}] are difficult to use with PrL since [Ca^{2+}] > 1 mM induces aggregation/fusion effects on liposomes.

Effect of DCCD on High Affinity Ca^{2+} Flux in BLMV and Proteoliposomes

Since passive Ca^{2+} uptake into BLMV is inhibited by the carboxyl group reagent, DCCD [19, 20], it was of interest to see whether DCCD also inhibited Ca^{2+} influx into PrL. As direct treatment of liposomes with DCCD resulted in large increases in Ca^{2+} permeability, to determine the effects of DCCD on Ca^{2+} influx in PrL, the octyl glucoside-extract from BLMV was treated with 2 mM DCCD for 20 min at 25°C prior to reconstitution into PrL (DCCD-treated PrL). It is important to note that DCCD did not affect the Ca^{2+} transport in control liposomes prepared under these conditions (*data not shown*). Additionally, control PrL were also prepared with the same incubations etc., but without DCCD. Figure 5 reveals that DCCD-treated PrL accumulate 40–50% less Ca^{2+} after 3 min of incubation than PrL treated with DMSO alone. This observation is consistent with our

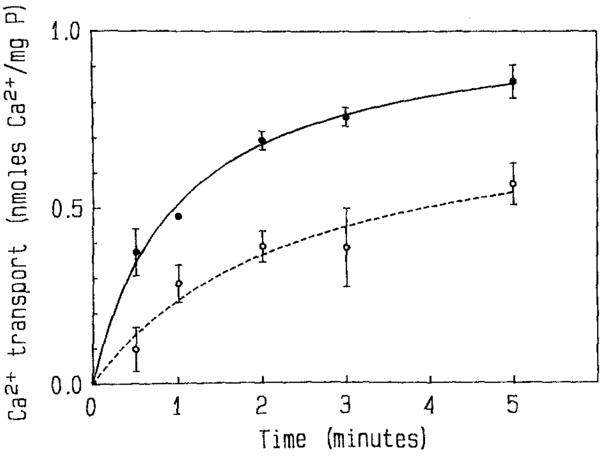

Fig. 5. Effect of DCCD on Ca^{2+} accumulation in proteoliposomes. Octyl glucoside extract of the BLMV was treated with 2 mM DCCD, or an equal volume of DMSO, at 25°C for 20 min before the subsequent preparation of PrL. DCCD-treated PrL, control DMSO-treated PrL, DMSO-treated liposomes, and DCCD-treated liposomes were then assayed for $^{45}Ca^{2+}$ uptake. Corrected uptake into control PrL (continuous line) and DCCD-treated PrL (broken line) is shown. Data represent mean ± SEM obtained from three experiments with different BLMV preparations.

previous results obtained with DCCD-treated BLMV. It has been previously observed that octyl glucoside-extracted membrane proteins are relatively unstable at room temperature than when kept on ice [5]. This may explain the somewhat lower levels of Ca^{2+} transport obtained in the control PrL in this experiment. In fact, incubation at 37°C induces further reduction in activity (*data not shown*). Therefore, DCCD treatments of octyl glucoside extracts were done at 25°C instead of 37°C, a condition used previously for BLMV [10, 12]. Further, (i) the protein yields in PrL prepared from DCCD-treated and control octyl glucoside extract are similar, and (ii) SDS gel electrophoresis of these PrLs revealed similar protein composition (*data not shown*).

We have examined the effect of DCCD on the kinetics of Ca^{2+} influx in PrL and BLMV and the data are summarized in the Table. The decrease in uptake in PrL induced by incubating the octyl glucoside extract of BLMV at 25°C for 20 min prior to reconstitution (as discussed above) is due to a decrease in the V_{max} of the high affinity site from 13.5 ± 1.14 nmoles Ca^{2+}/mg protein/minute to 6.2 ± 1.6 nmoles Ca^{2+}/mg protein/minute, without a significant change in K_{Ca} (242 ± 50.9 μM and 194 ± 62 μM, respectively). DCCD induces a further decrease in the V_{max} to 1.48 ± 0.9 nmoles/mg protein/minute, without significantly affecting the K_{Ca} (194 ± 62 μM and 110 ± 42 μM in control and DCCD-treated PrL, respectively). Thus, it appears that DCCD exerts its effects on PrL mainly by decreasing the V_{max} of Ca^{2+} influx via the high affinity site and not by altering the affinity of the protein(s) for Ca^{2+}.

We had previously shown that DCCD inhibits Ca^{2+} influx into BLMV [19] and maximum inhibition obtained was 40%. To assess whether DCCD affects the high affinity Ca^{2+} transport site in BLMV, or has a more general effect on the membrane permeability, we have examined the kinetics of Ca^{2+} influx into DCCD-treated BLMV using the procedure described earlier [19] and the data are given in the Table. There is decreased Ca^{2+} uptake into DCCD-treated (2 mM for 20 min) at every $[Ca^{2+}]$ tested. Consistent with our earlier report, Eadie-Hofstee plots (*data not shown*) of these data show the presence of a saturable, relatively high affinity, Ca^{2+} influx component and a nonsaturable Ca^{2+} influx component. These plots also indicate that the V_{max} of the high affinity is decreased by about 40% in DCCD-treated BLMV, without any change in the nonsaturable component. This was further confirmed by nonlinear regression analysis. While in DCCD-treated BLMV there is a consistent decrease in V_{max} (2.72 ± 0.46 nmoles Ca^{2+}/mg protein/minute in control BLMV to 1.72 ± 0.31 nmoles Ca^{2+}/mg protein/minute in DCCD-treated BLMV), the difference is not statistically significant (n = 5). There is no significant change in K_{Ca} (157.6 ± 49 μM and 151.8 ± 34 μM in control and DCCD treated BLMV, respectively). The K_{Ca} of the high affinity Ca^{2+} influx component is slightly lower that that previously reported by us [19]. This is most likely explained by the fact that in the experiments described here BLMV were incubated for 20 min at 37°C followed by centrifugation. In addition, the wash medium in the assay was also slightly modified (*see* Materials and Methods). In aggregate the data in Table 1 clearly demonstrate that the high affinity Ca^{2+} influx site in BLMV and PrL are similarly affected by DCCD.

The data described above suggest that a relatively high affinity Ca^{2+} influx component has been reconstituted into PrL. We have shown that Ca^{2+} influx in PrL and high affinity Ca^{2+} influx in BLMV demonstrate an impressive similarity in K_{Ca} and sensitivity to DCCD. Previously, we have reported that the characteristics of the high affinity Ca^{2+} influx site in BLMV and divalent cation (Mn^{2+} and Ca^{2+}) influx in intact parotid acini are similar, i.e., inhibition by pH < 7.0, low temperature, and DCCD. In addition, Mn^{2+} entry into internal Ca^{2+} pool-depleted acini is inhibited by divalent cations in the order $Zn^{2+} > Ni^{2+} > La^{2+}$, but not by Co^{2+} (I.S. Ambudkar, *unpublished observations*). This again is consistent with the present observations with BLMV and PrL. One of the main characteristics we have used to compare the Ca^{2+} influx pathway in PrL and BLMV is the similarity in the kinetic parameters. Presently, it is difficult to extend this comparison to intact cells since there are no studies which clearly describe the kinetics of Ca^{2+} (or Mn^{2+}) influx into intact parotid or any exocrine gland cells.

Previous reports show that $^{45}Ca^{2+}$ uptake into pa-

Table. Effect of DCCD on kinetic parameters of Ca^{2+} influx into BLMV and proteoliposomes

	–DCCD		+DCCD	
	K_{Ca} (μM)	V_{max} (nmoles/min/mg protein)	K_{Ca} (μM)	V_{max} (nmoles/min/mg protein)
BLMV	157 ± 49	2.72 ± 0.46	151 ± 34	1.72 ± 0.31
PrL	194 ± 62	6.2 ± 1.6	110 ± 42	*1.48 ± 0.9

BLMV, or octyl glucoside extract, was treated for 20 min at 25°C either with or without 2 mM DCCD, as described in Materials and Methods. Following the incubation, BLMV were centrifuged at 106,000 × *g*, washed, resuspended in 10 mM HEPES (pH 7.4) containing 1 mM $MgCl_2$, and assayed for $^{45}Ca^{2+}$ uptake. DCCD-treated octyl glucoside extract of BLMV was reconstituted into PrL as described for Fig. 5. $^{45}Ca^{2+}$ uptake into BLMV and PrL was measured at various [Ca^{2+}]; 10 μM to 10 mM for BLMV and 10 μM to 1 mM for PrL (as described for Fig. 4). Initial rates of Ca^{2+} uptake were determined from $^{45}Ca^{2+}$ uptake during first 10 sec for BLMV and 20 sec for PrL. Kinetic parameters were determined either by nonlinear regression or from Eadie-Hoffstee plots. The data shown are the mean ± SEM obtained from 5 experiments. The value marked * is significantly different from the corresponding value in the –DCCD condition.

rotid, and other nonexcitable, cells saturates at external [Ca^{2+}] > 1 mM, suggesting the presence of a relatively low Ca^{2+} affinity for this Ca^{2+} transporter [23, 28, 29]. However, any Ca^{2+} entering the cell is rapidly accumulated into intracellular Ca^{2+} stores via the activity of a high capacity Ca^{2+}-ATPase in the internal Ca^{2+} store membrane. Thus, the apparent Ca^{2+} influx rate, determined in previous studies by assessing internal store refill, also reflects the uptake of Ca^{2+} into internal Ca^{2+} stores. Recently, a K_d of 3.3 mM has been reported by Hoth and Penner for the I_{CRAC} current in mast cells [18] and a $K_d = 3.3$ mM has been reported by Donnadieu et al. [11] for Ca^{2+} influx into thapsigargin-treated Jurkat cells. We have recently determined the kinetics of Ca^{2+} entry in parotid acinar cells treated with thapsigargin to deplete internal Ca^{2+} stores and to prevent reuptake of Ca^{2+} into these stores (J. Chauthaiwale, S.E. Taylor, and I.S. Ambudkar, *in preparation*). We have observed a low affinity Ca^{2+} influx component ($K_{Ca} = 4.2$ mM) which is consistent with the previous reports. In addition, we also detect, a novel, relatively high-affinity, Ca^{2+} influx component with K_{Ca} similar to that in BLMV. In the former study [18], to measure an inward Ca^{2+} current using a patch clamp technique, a high concentration of the Ca^{2+} buffer, BAPTA, was used intracellularly and a high [Ca^{2+}] (>500 μM) was used extracellularly. Similarly, Donnadieu et al. [11] have also used [Ca^{2+}] above 200 μM. It is likely that under these experimental conditions, a high affinity Ca^{2+} influx component, similar to the one we have observed, would not be detectable. Alternatively, the high affinity Ca^{2+} influx component would likely have a poor ion conductivity [35] and therefore, not be easily detected.

In summary, we have described herein the functional reconstitution of a high affinity, passive Ca^{2+} influx pathway from rat parotid gland BLMV into PrL. Further, since the unsaturable Ca^{2+} flux component present in BLMV is not detected in PrL, the high affinity Ca^{2+} influx pathway can be studied independent of other Ca^{2+} transport activities. Our data demonstrate that the characteristics of Ca^{2+} influx in PrL are similar to those in BLMV and consistent with those of divalent cation entry into intact parotid acinar cells. To our knowledge this study is the first attempt to reconstitute a passive Ca^{2+} transport pathway from the plasma membrane of parotid acini (or any other nonexcitable cell type). Future studies should address the role of this putative Ca^{2+} influx pathway in the regulation of Ca^{2+} influx into parotid acinar cells. Such studies will require isolation and purification of the Ca^{2+} transport protein(s). Due to the lack of a specific ligand or gating agent for the Ca^{2+} influx pathway in nonexcitable cells, a more classical biochemical approach will likely have to be used to isolate and purify protein(s) that mediate Ca^{2+} transport in rat parotid gland acinar cells. We believe that the present study represents the first step towards this goal.

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