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Temperature-dependent Modification of Divalent Cation Flux in the Rat Parotid Gland Basolateral Membrane

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Abstract. Divalent cation (Mn^{2+}, Ca^{2+}) entry into rat parotid acinar cells is stimulated by the release of Ca^{2+} from the internal agonist-sensitive Ca^{2+} pool via a mechanism which is not yet defined. This study examines the effect of temperature on Mn^{2+} influx into internal Ca^{2+} pool-depleted acini (depl-acini, as a result of carbachol stimulation of acini in a Ca^{2+}-free medium for 10 min) and passive $^{45}Ca^{2+}$ influx in basolateral membrane vesicles (BLMV). Mn^{2+} entry into depl-acini was decreased when the incubation temperature was lowered from 37 to 4°C. At 4°C, Mn^{2+} entry appeared to be inactivated since it was not increased by raising extracellular [Mn^{2+}] from 50 μM up to 1 mM. The Arrhenius plot of depletion-activated Mn^{2+} entry between 37 and 8°C was nonlinear, with a change in the slope at about 21°C. The activation energy (E_a) increased from 10 kcal/mol (Q_{10} = 1.7) at 21–37°C to 25 kcal/mol (Q_{10} = 3.0) at 21–8°C. Under the same conditions, Mn^{2+} entry into basal (unstimulated) cells and ionomycin (5 μM) permeabilized depl-acini exhibit a linear decrease, with E_a of 7.8 kcal/mol (Q_{10} = 1.5) and 6.2 kcal/mol ($Q_{10} < 1.5$), respectively. These data suggest that depletion-activated Mn^{2+} entry into parotid acini is regulated by a mechanism which is strongly temperature dependent and distinct from Mn^{2+} entry into unstimulated acini.

As in intact acini, Ca^{2+} influx into BLMV was decreased (by 40%) when the temperature of the reaction medium was lowered from 37 to 4°C. Kinetic analysis of the initial rates of Ca^{2+} influx in BLMV at 37°C demonstrated the presence of two Ca^{2+} influx components: a saturable component, with K_{Ca} = 279 ± 43 μM, V_{max} = 3.38 ± 0.4 nmol Ca^{2+}/mg protein/min, and an apparently unsaturable component. At 4°C, there was no significant change in the affinity of the saturable component, but V_{max} decreased by 61% to 1.3 ± 0.4 nmol Ca^{2+}/mg protein/min. There was no detectable change in the unsaturable component. When BLMV were treated with DCCD (5 mM) or trypsin (1:100, enzyme to membrane) for 30 min at 37°C there was a 40% decrease in Ca^{2+} influx. When BLMV were treated with DCCD or trypsin at 4°C and subsequently assayed for Ca^{2+} uptake at 37°C there was no significant loss of Ca^{2+} influx. These data suggest that the temperature-sensitive high affinity Ca^{2+} flux component in BLMV is mediated by a protein which undergoes a modification at low temperatures, resulting in decreased Ca^{2+} transport.

Key words: Divalent cations — Basolateral membrane vesicles — Ca^{2+} flux — Mn^{2+} influx — Rat parotid acinar cells — Carbachol

Introduction

Several studies demonstrate that Ca^{2+} entry into rat parotid acinar cells is activated by the release of Ca^{2+} from the internal agonist-sensitive Ca^{2+} pool and is correlated with the extent of depletion of the internal Ca^{2+} pool [15, 28, 29]. However, the molecular mechanism(s) involved in relaying the status of the internal Ca^{2+} pool to the plasma membrane in parotid and other nonexcitable cells is yet unclear. Among the proposed mechanisms are: diffusion of soluble messengers from the internal Ca^{2+} pool to the plasma membrane, modulation of cytosolic factors as a result of internal Ca^{2+} release, and physical interaction between the internal pool membrane and the plasma membrane

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[4, 11, 30]. Additionally, direct activation by second messengers has been proposed [14, 22]. Although some electrophysiological measurements of the Ca^{2+} release-stimulated Ca^{2+} influx have been recently reported in mast cells and pancreatic acinar cells [2, 10], the molecular nature of the component(s) mediating Ca^{2+} flux across the plasma membrane in these cells has not yet been described.

To elucidate this Ca^{2+} entry mechanism and understand its regulation, we have used Mn^{2+}, as a Ca^{2+} surrogate ion, to study divalent cation permeability in dispersed parotid acini. In addition, we have examined $^{45}Ca^{2+}$ flux across isolated plasma membrane vesicles (BLMV). Our previous studies demonstrate a similarity in the characteristics of divalent cation entry in dispersed cells and $^{45}Ca^{2+}$ flux in BLMV. For example, divalent cation permeability in both cases is affected by such factors as the Ca^{2+} (or Mn^{2+}) gradient across the plasma membrane [16], membrane potential [17], carbodiimides [12], pH [12], $[Ca^{2+}]$ on the cytoplasmic side of the membrane [5, 13], and is inhibited by Ni^{2+} and Zn^{2+} but not by Co^{2+}. In this study, we have assessed the nature of the Ca^{2+} entry mechanism in the rat parotid gland basolateral membrane by studying the effects of temperature on Mn^{2+} entry in dispersed acini and $^{45}Ca^{2+}$ influx in BLMV.

Materials and Methods

Materials

$^{45}CaCl_2$ (2 mCi/ml) was obtained from Amersham. Ultra-grade mannitol and dithiothreitol (DTT) were purchased from Calbiochem (San Diego, CA). *N,N'*-Dicyclo-hexylcarbodiimide (DCCD), TPCK-trypsin (type XIII, bovine pancreas), trypsin inhibitor (lima bean, for cell preparation and Type II-T: turkey egg white, for BLMV experiments), hyaluronidase (Type V), ionomycin, and carbachol were obtained from Sigma Chemical (St. Louis, MO). CLSPA collagenase was purchased from Worthington Biochemical (Freehold, NJ). Fura 2-AM was obtained from Molecular Probes (Eugene, OR), dissolved in DMSO, aliquoted, and kept at −70°C until use (not more than one month). Ca^{2+}-, Mg^{2+}- and indicator-free Hank's balanced salt solutions (HBSS) were purchased from GIBCO BRL (Gaithersburg, MD). Percoll was from Pharmacia (Upsala, Sweden).

Protein was determined by using the Biorad protein assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as standard.

Cell Preparation and Fura 2 Loading

Dispersed parotid cells were prepared by collagenase-hyaluronidase digestion as described previously [9, 15, 17]. Briefly, minced and cleaned rat (Wistar male, from Harlan Sprague-Dawley) parotid glands were incubated in Hank's balanced salt solution buffered with HEPES (HBSS medium) for 80 min at 37°C with CLSPA collagenase, 300–400 U/ml, with gassing and dispersion every 20 min. Cells were washed 4–6 times, resuspended in HBSS containing 2 μM Fura 2-AM and 1 mg/10 ml lima bean trypsin inhibitor and incubated at 30°C for 45 min. The cells were then washed three times with HBSS and maintained with gassing every 20 min at 30°C.

Fluorescence Measurements

Fura 2 fluorescence was measured using an SLM-8000 spectrofluorimeter. It should be noted that only cells with similar levels of Fura 2 loading (monitored by 363 nm fluorescence) were used. Under our experimental conditions, Fura 2 loading was very consistent and stable. For Mn^{2+} entry measurements, cells were washed with Ca^{2+}-free HBSS and incubated at 30°C in the same medium with 10 μM carbachol for 10 min. The cells were then washed two times and resuspended in the same medium, then incubated at the desired temperature. Conditions for the specific experiments are provided in the figure legends. Mn^{2+} (concentrations used in the specific experiments are given in the figure legends) was added and Fura 2 quenching was determined at the isosbestic wavelength, 363 nm. To quantitatively assess Mn^{2+} entry, the slope of Fura 2 quenching in the first 30 sec after Mn^{2+} addition was determined [9, 17]. The very initial, sharp drop in fluorescence observed in some experiments, probably due to external free or bound dye, was not included in the calculations.

Preparation of BLMV and Treatment with Trypsin and DCCD

BLMV were prepared as described previously [13]. Briefly, parotid glands from 10–12 male Wistar rats (Sprague-Dawley, 150–200 g) were excised, cleaned, and homogenized in a medium containing 250 mM sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. 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 medium, mixed with Percoll (12% v/v) and centrifuged at 49,000 × *g* for 30 min. The BLMV fraction was collected and washed three times with 100 mM mannitol, 1 mM DTT, 0.1 mM PMSF, and 10 mM tris-HCl, pH 7.5. The final pellet was suspended in 300 mM mannitol, 1 mM DTT, and 10 mM Tris-HEPES (pH 7.4) at a concentration of 1–3 mg/ml, aliquoted, quick frozen in liquid N_2, and stored at −70°C until use (maximum two weeks). Before use, aliquots of BLMV were thawed out on ice; all preparations were subjected to only one freeze-thaw.

BLMV (100 μg/ml) were pretreated for 20 min with either 2 mM DCCD or TPCK-trypsin (at the concentrations described in the text) in 10 mM Tris-HEPES (pH 7.4) and 1 mM $MgCl_2$. In the case of TPCK treatment, the digestion was stopped after 20 min by adding a fourfold excess of trypsin inhibitor. The DCCD- or trypsin-treated BLMV were then separated from the reaction media by centrifugation (106,000 × *g*, 20 min, 4°C). The resultant pellet was rehomogenized in 10 mM Tris-HEPES (pH 7.4), 1 mM $MgCl_2$ and used for ^{45}Ca flux measurements described below.

^{45}Ca Flux into Basolateral Plasma Membrane Vesicles (BLMV)

$^{45}Ca^{2+}$ influx into BLMV was measured as described earlier [12]. Briefly, 100 μg of BLMV were incubated (100 μg/ml) in 10 mM Tris-HEPES (pH 7.4) and 1 mM $MgCl_2$ (assay medium) at 37°C unless otherwise noted. $^{45}CaCl_2$ (100 μM) was then added and aliquots (9 μg) were removed at the times indicated in the figures. These aliquots were filtered through Millipore filters (0.45 μ, type HA) 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 $LaCl_3$ (wash medium). The filters were then dried, dissolved in Aquasol (DuPont) and the radioactivity determined using a scintillation counter (Hewlett-Packard, Tricarb).

For determining initial rates of Ca^{2+} influx, 100 μg of BLMV were incubated in the assay medium (1 ml) described above. Nine-

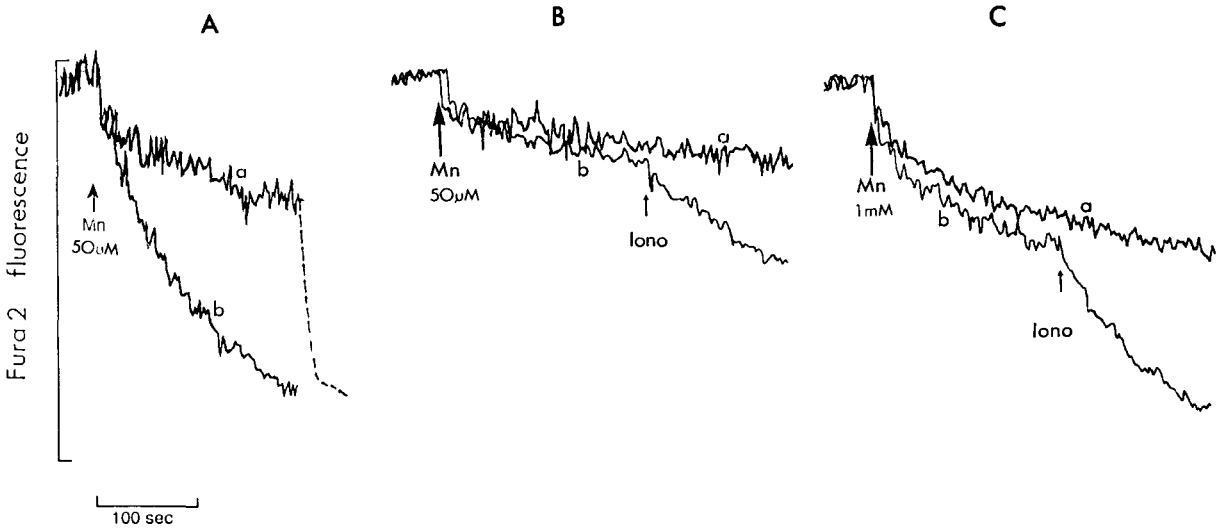

Fig. 1. Effect of temperature on Mn^{2+} entry into internal Ca^{2+}-pool-depleted parotid acinar cells. Dispersed parotid acini were loaded with Fura 2 as described in Materials and Methods. (*A*) Depl-acini (*b*) and unstimulated acini (*a*) were kept at 37°C. Internal Ca^{2+} pool was depleted by stimulation of the cells with 100 μM carbachol for 10 min in a Ca^{2+}-free HBSS medium. Before Mn^{2+} entry measurement, 1.5 ml aliquots of cells were washed and resuspended in the same medium. (*B* and *C*) Depl-acini were washed two times and resuspended in medium at 4°C, and kept, with stirring, in a cuvette maintained at that temperature. 50 μM (*A* and *B*) or 1 mM Mn^{2+} (*C*) and 5 μM ionomycin (*Iono*) were added where indicated by arrows. The data are averaged from three to five experiments with different cell preparations.

ty microliters (9 μg protein) aliquots were withdrawn and placed in a tube either maintained at 37°C or on ice. $^{45}CaCl_2$ (10 μl) at the required concentration (10 μM–10 mM) was then added and vortexed. Five seconds later, ice-cold wash medium was added, and the sample vortexed, filtered and washed as described above. It should be noted that the 5 sec time point was used on the basis of time course experiments performed (*data not shown*) at each $^{45}CaCl_2$ to determine the linearity of the uptake. To obtain a "0" time point, 9 μg BLMV were first added to ice-cold wash medium. The appropriate concentration of $^{45}CaCl_2$ was then added, and the sample vortexed, filtered and washed as described above. Additionally, increasing the concentration of $LaCl_3$ in the wash medium did not affect the counts obtained at the higher [$^{45}CaCl_2$]. Kinetic parameters of Ca^{2+} influx were obtained by nonlinear regression analyses. F test (where indicated) and Student's *t*-test were used to statistically evaluate the data.

Results

Effect of Temperature on Mn^{2+} Influx into Dispersed Parotid Acini

Internal Ca^{2+} pool depletion (i.e., stimulation of acini with 10 μM carbachol for 15 min in a Ca^{2+}-free medium) leads to a substantial increase in Mn^{2+} entry into parotid acinar cells at 37°C (Fig. 1*A*, *see also* refs. 9, 15, 17) as shown by the increase in Fura 2 quenching into internal Ca^{2+} pool-depleted acini (depl-acini) compared to that in unstimulated cells (*compare* traces *a* and *b*). When Mn^{2+} entry is measured in depl-acini incubated at 4°C, Fura 2 quenching in depl-acini is not distinguishable from that in unstimulated cells (*compare* traces *a* and *b*). However, addition of ionomycin (5 μM) further increases the rate of Fura 2 quenching. To determine the extent to which Mn^{2+} entry is decreased in cells incubated at 4°C, we examined Fura 2 quenching with higher [Mn^{2+}] in the external medium. Fura 2 quenching in both depl-acini and unstimulated cells is slightly increased when [Mn^{2+}] is increased from 50 μM to 1 mM (Fig. 1*C*) or 5 mM (*not shown*). In contrast, when ionomycin is added to depl-acini at 4°C, Fura 2 quenching is markedly greater with 1 mM Mn^{2+} than with 50 μM Mn^{2+} in the medium. We have previously shown that Mn^{2+} entry into depl-acini acini at 37°C increases dramatically with increase in external [Mn^{2+}] [9, 17] and that decreases in Mn^{2+} influx due to reduced driving force can be overcome by increasing the external [Mn^{2+}] [17]. Thus, since the decrease in Mn^{2+} permeability induced as a result of cooling the cells to 4°C is not overcome by increasing (by 20- to 100-fold) the extracellular [Mn^{2+}], we suggest that the Mn^{2+} entry mechanism is inactivated at low temperature.

The data in Fig. 2 show the decrease in the rate of Mn^{2+} entry as the temperature is lowered. When the temperature is decreased from 37° to 12°C, there is a 85–90% decrease in the rate of Mn^{2+} entry (shown by the slower rate of Fura 2 quenching). Ionomycin-stimulated Mn^{2+} entry is relatively less affected by the decrease in temperature; when the temperature is lowered to 12°C there is a 45–50% decrease in the quench rate. It should be noted that at all temperatures, the final level of quenching induced by ionomycin is the same, which shows that the availability of intracellular Fura 2 is not altered when temperature is decreased.

To examine the response of Mn^{2+} influx in parotid acini to changes in temperature, we expressed the data

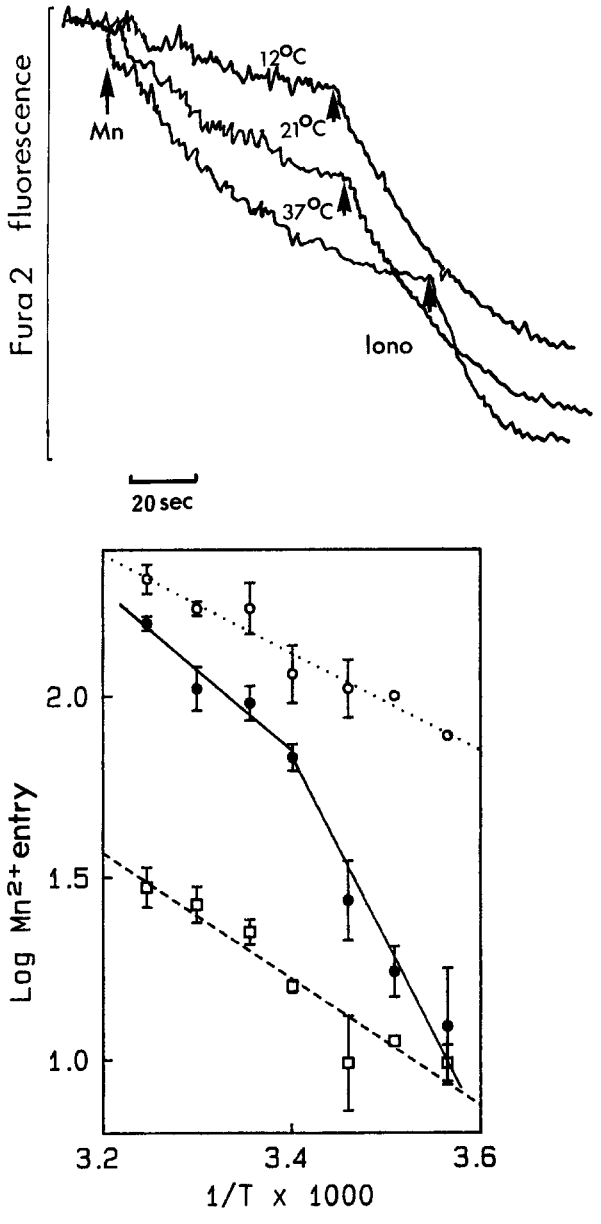

Fig. 2. Response of Mn^{2+} entry into parotid acinar cells to changes in temperature. (Top) Experimental conditions were similar to those described for Fig. 1. Depl-acini were incubated at the various temperatures indicated. Ionomycin (*Iono* 5 μM) was added when shown by arrows. The data represent similar results obtained from five experiments. (Bottom) For Arrhenius plots, data were obtained from experiments similar to those shown in Figs. 1 and 2, Top. Initial Fura 2 quenching due to Mn^{2+} (50 μM) entry at the various temperatures was determined as described in Materials and Methods in unstimulated cells (open squares, dashed line), internal Ca^{2+}-pool-depleted cells and ionomycin-permeabilized depl-acini. (Filled circles, unbroken line)—effect of temperature on the difference between Mn^{2+} entry in unstimulated cells and depl-acini (i.e., the capacitatively stimulated entry component); (Open circles, dotted line)—difference between Mn^{2+} entry into unstimulated cells and ionomycin-permeabilized depl-acini. The data are mean ± SEM obtained from at least four experiments with different cell preparations.

from experiments similar to those shown in Fig. 2, Top, and those obtained with unstimulated (basal) cells (*traces not shown*) in the form of Arrhenius plots (Fig. 2, Bottom). The depletion-activated Mn^{2+} entry component (the difference between Mn^{2+} entry into depl-acini and Mn^{2+} entry into unstimulated, basal, acini) decreases linearly when temperature is lowered from 37 to about 21°C, and, from the slope, an apparent activation energy (E_a) of 10 kcal/mol (Q_{10} = 1.7) can be calculated. At about 21°C, there is an abrupt increase in the sensitivity of Mn^{2+} entry to temperature. Below this temperature, the (E_a) increases by 2.5-fold to 25 kcal/mol (Q_{10} increases to about 3.0), reflecting a much higher energy barrier to translocation of the Mn^{2+} through the plasma membrane. In these experiments the internal Ca^{2+} pool of the acini was depleted by carbachol stimulation prior to the change in temperature. Thus, effects of temperature on receptor-signaling processes can be ruled out. Additionally, since Mn^{2+} is not transported by Ca^{2+} pumps [26], temperature effects on ATP-dependent Ca^{2+} transporting mechanisms cannot account for these observations. Importantly, we have observed that Mn^{2+} entry into unstimulated cells, and ionomycin-permeabilized depl-acini, do not exhibit a nonlinear response to decrease in the incubation temperature. Linear Arrhenius plots are obtained in both cases, with E_a values of 7.8 kcal/mol (Q_{10} = 1.5) and 6.2 kcal/mol (Q_{10} < 1.5), respectively. These data show that Mn^{2+} entry into unstimulated and stimulated cells can be distinguished based on the characteristic temperature dependence observed in each case.

Effect of Temperature on Ca^{2+} Influx into BLMV

To further characterize the effect of temperature on the divalent permeability of rat parotid acinar cells, we examined Ca^{2+} influx into BLMV incubated at 37 and 4°C (Fig. 3). At 37°C, there is rapid uptake of Ca^{2+} until a steady-state level of about 6–7 nmol Ca^{2+}/mg protein is reached after 2 min. At 4°C, Ca^{2+} influx into BLMV decreases by 40%. This decrease in $^{45}Ca^{2+}$ influx into BLMV at low temperature could be due to (i) a reduction in the overall Ca^{2+} permeability, or (ii) a decrease in a specific Ca^{2+} flux pathway. To distinguish between these possibilities, we examined the effect of temperature on the kinetics of Ca^{2+} influx in BLMV. Figure 4, Top, shows the initial rates of Ca^{2+} influx at 37 and 4°C as a function of [Ca^{2+}] in the extravesicular medium. At either temperature, Ca^{2+} influx in BLMV increases dose dependently with increasing [Ca^{2+}], but rates of influx are lower at 4°C than at 37°C at every [Ca^{2+}] tested. Kinetic parameters of Ca^{2+} influx were determined by nonlinear regression analyses of the data shown in Fig. 4, Top. The data provide evidence for the presence of more than one site of Ca^{2+} influx in BLMV (P < 0.025, F test). At 37°C, there is a clearly defined saturable component with K_{Ca} = 279 ± 43 μM and V_{max}

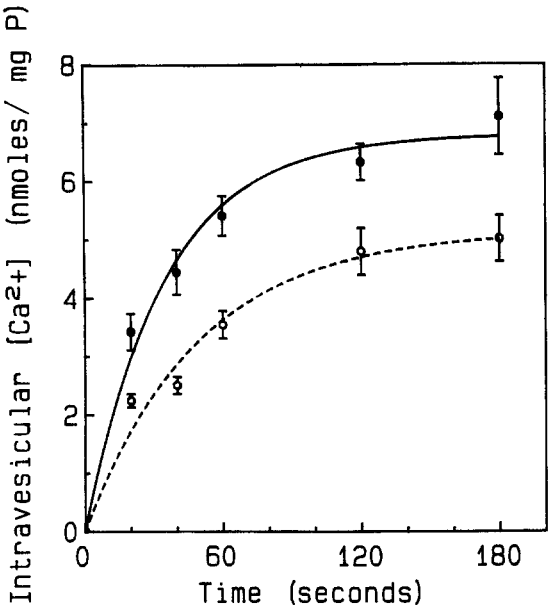

Fig. 3. Effect of temperature on Ca^{2+} influx into BLMV. $^{45}Ca^{2+}$ entry into BLMV incubated at either 37°C (open circles) or 4°C (filled circles) was determined as described in Materials and Methods. $^{45}CaCl_2$ (100 μM) was used. Data are shown as mean ± SEM and obtained from three experiments performed with different BLMV preparations.

= 3.38 ± 0.4 nmol/mg protein/min and a second component, which does not appear to be saturable within the range of $[Ca^{2+}]$ used in this experiment. When BLMV are maintained at 4°C, there is no significant change in the K_{Ca} of the saturable, high affinity, component but there is a 61% decrease in the V_{max} (*see* figure legend for values). Figure 4, Bottom, shows the data from Fig. 4, Top, plotted as Eadie-Hofstee plots. A marked decrease in the V_{max} of the high affinity Ca^{2+} influx component can be clearly seen, but there is no significant decrease in the K_{Ca} (*compare* slopes of the high-affinity component). We did not detect any substantial change in the unsaturable Ca^{2+} influx component. Thus, consistent with our observation with intact acini, these data demonstrate the presence of a specific temperature-sensitive Ca^{2+} flux component in BLMV.

Effect of Temperature on Inhibition of Ca^{2+} Influx in BLMV by DCCD and Trypsin

We have previously reported that treatment of deplacini and BLMV with the hydrophobic carbodiimide, DCCD, induces a marked decrease in Mn^{2+} and Ca^{2+} permeability, respectively [12]. These previous studies suggest that DCCD-induced inhibition of divalent cation flux is due to its interaction with a carboxyl group, likely involved in Ca^{2+} flux, located on the cytosolic side of the plasma membrane. Since it is clear that at least one Ca^{2+} entry component is altered by low temperature, it was of interest to see if low temperature affects the site of DCCD interaction. Consistent with our previous observations, Figure 5*A* shows that when BLMV are treated with DCCD at 37°C, their ability to accumulate Ca^{2+} is decreased. However, when BLMV are treated with DCCD at 4°C (Fig. 5*B*-dashed line), washed, and then assayed for Ca^{2+} influx at 37°C, there

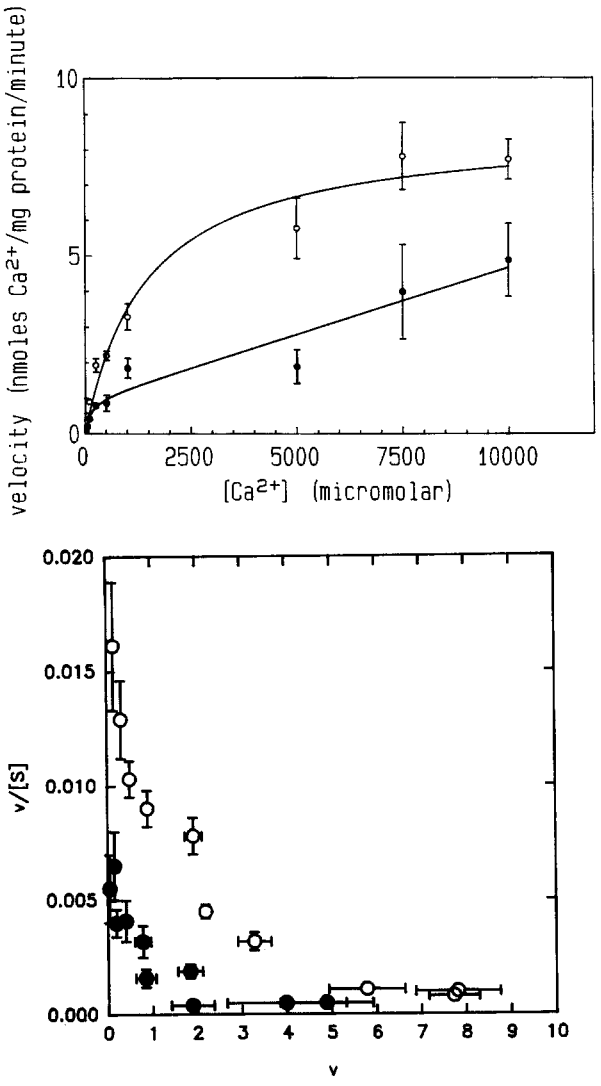

Fig. 4. Kinetics of Ca^{2+} influx into BLMV. Initial rate of $^{45}Ca^{2+}$ influx into BLMV was measured as described in Materials and Methods. (Top) Velocity (v, nmol Ca^{2+}/mg protein/min) as a function of extravesicular $[Ca^{2+}]$ at 37°C (filled circles) and 4°C (open circles). Data are shown as mean ± SEM from at least five experiments in each case, using different BLMV preparations. Nonlinear regression analysis was used to calculate the kinetic parameters of Ca^{2+} influx. The data significantly indicate the presence of more than one site of Ca^{2+} influx ($P < 0.025$, by F test); a saturable (high affinity) site and an apparently nonsaturable site. The high affinity site has K_{Ca} = 279 ± 43 μM at 37°C and 208 ± 86 μM at 4°C (no significant change with decrease in temperature) and V_{max} 3.38 ± 0.4 nmol/mg protein/min at 37°C and 1.3 ± 0.4 nmol/mg protein/min at 4°C (significantly decreased with decrease in temperature, $P < 0.02$ by F test). (Bottom) Data from Fig. 4, Top (filled circles-4°C and open circles-37°C) presented in the form of Eadie-Hofstee plots.

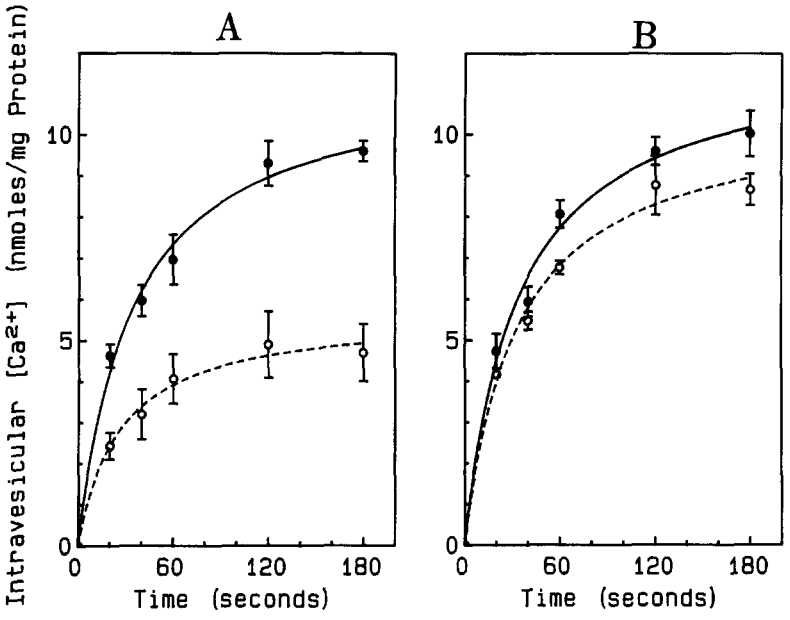

Fig. 5. Effect of temperature on DCCD-induced decrease in Ca^{2+} influx in BLMV. BLMV were incubated for 20 min with DCCD (5 mM) at either 37°C (*A*) or 4°C (*B*), washed, and resuspended in assay medium. Ca^{2+} influx was measured at 37°C as described in Materials and Methods, in control BLMV (filled circles, unbroken line) and DCCD-treated BLMV (open circles, dashed line). Data are shown as mean ± SEM obtained from at least three experiments with different membrane preparations.

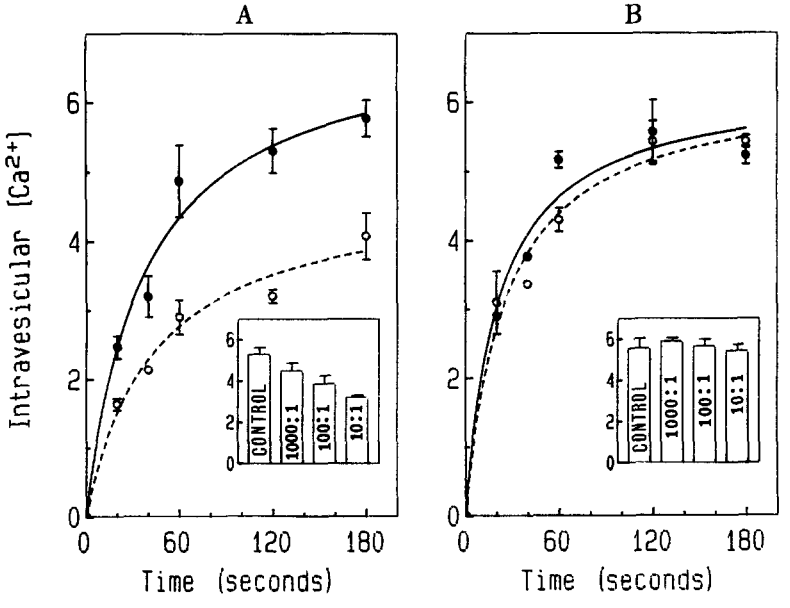

Fig. 6. Effect of temperature on trypsin-induced decrease in Ca^{2+} influx in BLMV. BLMV were incubated with TPCK-trypsin (100:1 protein:trypsin) for 20 min at 37°C (*A*) or 4°C (*B*), washed and resuspended in assay medium. Ca^{2+} uptake was assayed at 37°C as described in Materials and Methods in control BLMV (filled circles, unbroken line) and trypsin-treated BLMV (open circles, dashed line). The effect of various concentrations of TPCK-trypsin, at 37 and 4°C, are shown in the insets (values represent Ca^{2+} uptake after two minutes of incubation). All data are shown as mean ± SEM and were obtained from three experiments with different BLMV preparations.

is no significant inhibition of Ca^{2+} influx. Thus, the site in BLMV which interacts with DCCD at 37°C is also affected by low temperature.

Further, to confirm the involvement of a protein in the Ca^{2+} flux process and to assess whether it is modified by low temperature, we tested the effect of TPCK-trypsin on Ca^{2+} influx in BLMV. The data in Fig. 6, show that like DCCD, trypsin treatment of BLMV for 30 min at 37°C but not at 4°C, decreases Ca^{2+} flux in a dose-dependent manner. It is important to note that the decrease in the inhibitory effect of trypsin at the lower temperature is not due to a reduction in the activity of the enzyme; (i) we have detected <10% decrease in the hydrolysis of the trypsin substrate, BAEE, at 4°C compared to 37°C and (ii) we have not detected any effect on Ca^{2+} flux even when BLMV are treated at 4°C with tenfold higher concentration of trypsin (1:10 ratio of trypsin to protein). Thus, the observed lack of effect of trypsin at low temperature is due to its substrate, i.e., a change in the sensitivity of a protein(s) in BLMV to trypsin. Additionally, when BLMV are treated with DCCD (5 mM for 20 min at 37°C), washed, and subsequently digested with trypsin (1:100:: trypsin:protein for 20 min at 37°C), the level of Ca^{2+} flux obtained is similar to that obtained when BLMV are treated with either agent alone.

Discussion

The data presented above demonstrate that Mn^{2+} entry into rat parotid acinar cells is strongly dependent on temperature and is apparently inactivated at low temperatures, e.g., 4°C. These results are consistent with two previous reports in nonexcitable cells; avian nasal salt gland cells [27] and thymocytes [18], which demonstrate that Ca^{2+} (and Mn^{2+}) entry is decreased at temperatures below 37°C, i.e., 23 and 17°C, respectively. In addition, we have shown that a nonlinear Arrhenius plot (with a dramatic change in Mn^{2+} influx at about 21°C) is obtained for Ca^{2+}-release-stimulated Mn^{2+} entry. Conversely, linear Arrhenius plots are obtained for Mn^{2+} entry into ionomycin-permeabilized acini and unstimulated acini. These data also exclude the possibility that the transition observed in the case of capacitative Mn^{2+} entry is due to effects of temperature on the binding properties of Fura 2. Moreover, a linear change in the binding characteristics of Fura 2 with change in temperature has been reported previously [27]. Our data clearly demonstrate that the temperature dependence of Mn^{2+} entry, which is stimulated in response to internal Ca^{2+} pool depletion of rat parotid acini, is distinct from that in unstimulated acini. This difference in the effects of temperature on Mn^{2+} entry into unstimulated acini and depl-acini may indicate that a different flux pathway, activated as a result of internal Ca^{2+} pool depletion, is present in depl-acini. Alternatively, different modes of regulation of the same divalent cation influx pathway, in unstimulated and depl-acini, can also account for these observations.

Typically, the activity of a membrane transport protein is sensitive to changes in temperature, as a result of a change either in the structure of the protein itself or in its membrane environment, i.e., associated membrane lipids. Depending on the response of the protein and its membrane environment to changes in temperature, there is either a linear or nonlinear change in the transport activity. Although the exact mechanisms which account for a nonlinear change in activity are not yet clearly defined, it has been generally suggested to be a result of a change in the fluidity of the membrane lipids. For example, some types of transport proteins such as carriers and pumps, exhibit a nonlinear effect of temperature, with an increase in E_a when the membrane fluidity is decreased at lower temperatures [6, 24]. On the other hand, ion flux mediated by channels are believed to be less affected by changes in membrane fluidity and exhibit a linear change in activity with change in temperature and low E_a (e.g., the water channel CHIP-28 [33] and gramicidin [3]). Ca^{2+} entry into excitable cells, such as ventricular myocytes and chick and cat sensory neurons, which is mediated via the well-characterized depolarization-gated Ca^{2+} channels, has been reported to be a temperature-sensitive process [7, 20, 31]. These studies have indicated that an increase in temperature produces an increase in Ca^{2+} current, mainly as a result of an increased probability of the channels to open. Linear Arrhenius relationships have been generally observed in such excitable cells, except in the case of neuroblastoma cells [19], where the conductance of Ca^{2+} channels, but not the channel open times, shows a nonlinear decrease when the temperature is lowered, with a transition around 20°C. It was suggested that such effects of temperature reveal a membrane environment-dependent regulation of either the gating or the ion permeation of these channels. Thus, although at present we cannot conclusively describe the nature of the mechanism which mediates divalent cation entry into parotid acini, i.e., channel or other, we can suggest that it is regulated by a temperature-dependent mechanism which is distinct from that in unstimulated cells.

Several recent reports describe various factors as possible activators of the Ca^{2+} entry mechanism which is stimulated in response to the release of Ca^{2+} from the internal Ca^{2+} pool. The involvement of GTP, via GTP hydrolysis likely catalyzed by an as yet undefined small molecular weight G protein, has been reported by Bird and Putney in lacrimal acinar cells [5] and Fasolato et al. in mast cells [8]. A role for tyrosine kinase has been suggested in platelets [25, 32]. Studies with *Xenopus* oocytes and neutrophils have provided evidence for a diffusible messenger [21, 23] and studies with pancreatic acinar cells suggest the involvement of cGMP [2]. While it is presently unclear whether more than one mechanism exists in any cell type or whether the mechanisms described above are cell specific, all the proposed mechanisms are most likely to be temperature dependent and therefore consistent with our suggestion that depletion-activated Ca^{2+} entry involves a temperature-dependent regulatory mechanism.

In this study, we have also defined a saturable high affinity Ca^{2+} influx component in BLMV which, like Mn^{2+} entry in intact depl-acini, is decreased by low temperature. Importantly, we have clearly demonstrated that Ca^{2+} influx in BLMV is decreased by trypsinization of the membrane, which provides the first evidence that Ca^{2+} influx in the rat parotid gland basolateral membrane is mediated by a protein(s). In addition, we have observed that trypsin does not alter Mn^{2+} entry into depl-acini. We have previously reported that the hydrophobic carbodiimide DCCD decreases influx both in intact acini and BLMV, while the hydrophilic agents EAC and CMCD are only effective with BLMV. Based on this differential effect, we had suggested that the site of DCCD action is located on the cytosolic side of the membrane, which is exposed in inside-out vesicles. The similarity in the effect of trypsin, i.e., effective in BLMV but not intact acini, suggests that trypsin, like DCCD, most likely acts on the cytosolic side of the basolateral plasma membrane. Additionally, since the ef-

fects of DCCD and trypsin are not additive, it is likely that these agents act on the same component of Ca^{2+} influx and in the same region of the protein. This is further demonstrated by our results that low temperature similarly decreases the effectiveness of both DCCD and trypsin. These data suggest that the DCCD- and trypsin-sensitive region of the protein, likely on the cytoplasmic side of the membrane, is modified by low temperature. It is most likely that this modification also accounts for the decrease in Ca^{2+} influx BLMV at low temperatures and, thus, this region of the protein is critical to the regulation of passive Ca^{2+} transport in this membrane. Consistent with this suggestion, we have demonstrated that the saturable high affinity Ca^{2+} influx component in BLMV is substantially decreased at low temperatures. In future studies it would be important to establish whether the high affinity Ca^{2+} transport component detected in BLMV is involved in mediating temperature-sensitive Mn^{2+} influx in depl-acini.

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