J. Membrane Biol. 126, 183–193 (1992)

The Journal of
Membrane Biology

Membrane Potential Modulates Divalent Cation Entry in Rat Parotid Acini

Lawrence M. Mertz*, Bruce J. Baum, and Indu S. Ambudkar
Clinical Investigations and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

Summary. This study examines the effect of membrane potential on divalent cation entry in dispersed parotid acini following stimulation by the muscarinic agonist, carbachol, and during refill of the agonist-sensitive internal Ca^{2+} pool. Depolarizing conditions (addition of gramicidin to cells in Na^+-containing medium or incubation of cells in medium with elevated $[K^+]$) prevent carbachol-stimulated hyperpolarization of acini and also inhibit carbachol activation of Ca^{2+} and Mn^{2+} entry into these cells. Conditions promoting hyperpolarization (cells in medium with Na^+ or with N-methyl-D-glucamine instead of Na^+) enhance carbachol stimulation of divalent cation entry. Intracellular Ca^{2+} release (initial increase in $[Ca^{2+}]_i$) does not appear to be affected by these manipulations. Mn^{2+} entry into resting and internal Ca^{2+} pool-depleted cells (10-min carbachol stimulation in a Ca^{2+}-free medium) is similarly affected by membrane potential modulations, and refill of the internal pool by Ca^{2+} is inhibited by depolarization. The inhibitory effects of depolarization on divalent cation entry can be overcome by increasing extracellular $[Ca^{2+}]$ or $[Mn^{2+}]$. These data demonstrate that the modulation of Ca^{2+} entry into parotid acini by membrane potential is most likely due to effects on the electrochemical gradient ($E_m - E_{Ca}$) for Ca^{2+} entry.

Key Words Ca^{2+} entry · membrane potential · intracellular calcium mobilization · fluid secretion · exocrine gland

Introduction

The regulation of fluid and electrolyte secretion in the rat parotid gland by calcium-mobilizing agonists is mediated via changes in the cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$). Such agonists stimulate a biphasic increase in $[Ca^{2+}]_i$; an initial rapid, transient increase due to intracellular Ca^{2+} release, followed by a lower, sustained elevation primarily due to Ca^{2+} entry [4, 22, 24]. There is considerable evidence to suggest that intracellular release of Ca^{2+} is mediated by inositol 1,4,5, trisphosphate. However, in the parotid and other exocrine gland cells, as well as in a number of nonexcitable cells, the events involved in the activation of Ca^{2+} entry are not yet fully understood. Putney [27, 28] has proposed that Ca^{2+} entry is a "capacitative process," which is regulated by the depletion of the internal Ca^{2+} pool. This theory has been supported by studies reported from several laboratories [9, 16, 17], including ours [14, 15], which together show that depletion of the internal Ca^{2+} pool stimulates divalent cation influx while, reciprocally, its refill inactivates divalent cation entry. The mechanism(s) mediating Ca^{2+} influx at the plasma membrane and the factors involved in this apparent capacitative regulation have not yet been resolved.

In addition to the $[Ca^{2+}]_i$ mobilization response, neurohormonal stimulation of exocrine cells such as the parotid acinar cell, induces a number of other responses (e.g., changes in plasma membrane potential (hyperpolarization) [6, 18, 22], cytosolic pH (mild alkalinization) [12], and cell volume (shrinkage) [7]). These responses are coincident with agonist-stimulated changes in $[Ca^{2+}]_i$. Based on data obtained with other cell types [8, 26, 29], it is conceivable that such responses could affect Ca^{2+} mobilization events in the parotid acinar cell. For example, several recent studies have shown that the plasma membrane potential in nonexcitable cells may have a role in $[Ca^{2+}]_i$ homeostasis [1, 20, 23, 29]. Also, it has been suggested that in endothelial cells Ca^{2+} influx is related to the Ca^{2+} electrochemical gradient across the plasma membrane [11]. While it is widely accepted that Ca^{2+} entry in the parotid and other exocrine cells is not mediated via voltage (depolarization)-gated channels, the available data do not rule out the possibility that Ca^{2+} entry in these cells could be voltage regulated. Alternatively, Ca^{2+} entry could be dependent on the electrochemical gradient, in which case changes in either the

* *Present address:* Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892.

membrane potential or the inwardly directed Ca^{2+} gradient could modulate Ca^{2+} entry. Previous to this report, there are no data which directly demonstrate the effects of membrane potential modulations on divalent cation entry in parotid acini. Although, as noted above, such effects have been proposed in other cell types, the mechanism by which alterations in the membrane potential affect Ca^{2+} entry in these cells has not yet been established.

We have examined the influence of membrane potential changes on divalent cation entry into parotid acini under various conditions: resting, upon carbachol stimulation, after internal Ca^{2+} pool depletion, and during refill of the internal Ca^{2+} pool. The data demonstrate that carbachol activation of Ca^{2+} and Mn^{2+} entry into parotid acinar cells cannot be detected when the cells are in a depolarized state. Further, the depolarizing conditions also decrease divalent cation entry into Ca^{2+}-depleted cells, and consequently, refill of the internal agonist-sensitive Ca^{2+} pool. Importantly, we show that the decrease in Ca^{2+} entry by depolarization can be overcome by increasing the inwardly directed gradient of Ca^{2+}. Earlier studies from our laboratory [15] have shown that an increase in the inwardly directed Ca^{2+} gradient augments Ca^{2+} entry in parotid acini. Together these data are consistent with a transport mechanism where Ca^{2+} influx is driven by the electrochemical gradient for Ca^{2+} entry.

Materials and Methods

The animals used in this study were male Wistar rats (Harlan Sprague-Dawley) with an average weight of 200–300 g. The rats were caged in temperature-controlled, independently ventilated enclosures and allowed chow (NIH Purina) and water *ad libitum*. Collagenase (CLSPA; specific activity 300 U/mg) was purchased from Cooper Biochemical. Carbamylcholine chloride (carbachol), atropine sulphate, hyaluronidase (Type 11), bovine serum albumin (BSA), and lima bean trypsin inhibitor (type 11-L) were from Sigma Chemical. Fura-2/AM was obtained from Calbiochem and stored at −70°C as a 5-mM solution in dimethylsulfoxide. Bis-1,3-dibutylbarbituric acid trimethineoxonol, DiBaC4(3), was obtained from Molecular Probes. Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution (HBSS) was purchased from GIBCO. All other reagents were of the highest chemical grade available.

Rats were killed between 0900–1200 hr by cardiac puncture following ether anesthesia. Enzymatically dispersed acini were prepared as described [3] using collagenase and hyaluronidase in the HBSS buffered with HEPES (33 mM, pH 7.4) containing 1.28 mM $CaCl_2$, 0.81 mM $MgSO_4$, and 0.01% BSA (HBSS medium). This medium was used in all subsequent procedures. After dispersion the cells were washed and resuspended in the same medium with lima bean trypsin inhibitor (2 mg/10 ml). Fura-2/AM (2 μM) was added and the cells were incubated for 45 min at 30°C, following which they were washed and kept at 30°C until use with gassing every 20 min (95% O_2/5% CO_2).

Fura-2 fluorescence was measured in an SLM 8000 spectrofluorimeter with the excitation and emission band passes adjusted to 4 nm. Before each assay the cells were gently pelleted at 400 × *g* and resuspended in fresh medium (specified for each experiment, *see* the text and figure legends). For each assay 1.5 ml of cells were gently stirred in a cuvette maintained at 37°C. Excitation and emission wavelengths were 340 and 510 nm or 363 and 510 nm, as specified. The Ca^{2+}-insensitive excitation wavelength was 363 nm [14]. In each figure representative fluorescence traces are shown without data manipulation. As indicated in the legends, either F_{max} or resting Fura-2 fluorescence was set as 100%. The data are representative of results obtained with at least five different cell preparations. $[Ca^{2+}]_i$ was calculated as described earlier [3]. Mn^{2+} entry has been expressed in terms of relative rate of Fura-2 quenching during the first 60 sec, determined as described previously [14]. Where indicated, data were analyzed for statistical significance using the Student's *t* test.

Membrane potential in acini was assessed by measuring DiBAC4(3) fluorescence using the SLM 8000 spectrofluorimeter with excitation and emission at 495 and 525 nm, respectively. Two-μM dye was added to 2 ml of cells in a cuvette maintained at 37°C. About 10 min were required for the dye to equilibrate. Membrane potential has been expressed as arbitrary units with an increase in signal representing depolarization and a decrease in signal representing hyperpolarization. To determine the relative membrane potential in the various media used (as indicated), cells were preincubated with 2-μM dye in HBSS medium for 10 min at 37°C. Thereafter, the cells were pelleted at 400 × *g*. Two ml of the required medium with 2-μM dye was then added to the pellet, and the cells were resuspended in the cuvette immediately before fluorescence measurements were started.

Results

Figure 1 shows representative $[Ca^{2+}]_i$ changes induced by carbachol (10 μM) stimulation of acini suspended in three different media which either contain 1.28 mM Ca^{2+} or are nominally Ca^{2+} free (no added Ca^{2+}). We have used Na^+-containing HBSS medium (*HBSS*, Fig. 1*A*), HBSS medium with 50 mM KCl added 10 sec before the agonist (*HBSS + KCl* or K medium, Fig. 1*B*), or HBSS medium with Na^+ replaced by the impermeant cation N-methyl-D-glucamine (*NMDG*, Fig. 1*C*). Resting and peak $[Ca^{2+}]_i$ following stimulation with carbachol in the various media are shown in the Table. Resting $[Ca^{2+}]_i$ of parotid acini in either Ca^{2+}-containing or Ca^{2+}-free K medium, or in Ca^{2+}-free HBSS medium are not significantly different from that in cells in Ca^{2+}-containing HBSS medium (178 ± 17 nM). However, resting $[Ca^{2+}]_i$ in parotid acini suspended in Ca^{2+}-containing NMDG medium (231 ± 18 nM) is significantly different ($P < 0.05$) from that in Ca^{2+}-containing HBSS medium, while in Ca^{2+}-free NMDG medium it is not. Peak $[Ca^{2+}]_i$ in all the media are similar to that in Ca^{2+}-containing HBSS (420 ± 44 nM), except for that in Ca^{2+}-containing NMDG medium (535 ± 19 nM), which is ~27% higher ($P < 0.05$). However, the change in $[Ca^{2+}]_i$ upon stimulation (i.e., peak minus resting) in the Ca^{2+}-containing

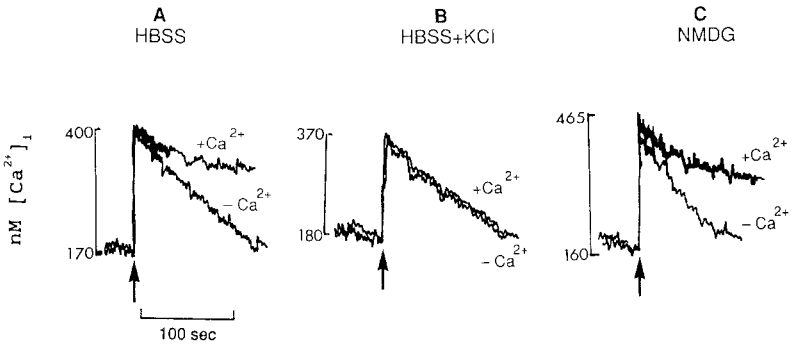

Fig. 1. Effects of ionic composition of the extracellular medium on carbachol-induced $[Ca^{2+}]_i$ mobilization in dispersed rat parotid acini. Enzymatically dispersed rat parotid acini were loaded with Fura-2 as described in the experimental procedures section. Fura-2 fluorescence was measured (with excitation at 340 nm) in cells suspended in HBSS medium (*A*), HBSS medium with 50 mM KCl added 30 sec prior to carbachol (*HBSS + KCl*) (*B*), and in NMDG medium (*C*) (details of these media are given in the text). Representative changes in fura-2 fluorescence in Ca^{2+}-containing and Ca^{2+}-free media are shown. Additions of 10 μM carbachol are indicated by arrows. *See* the Table for additional data.

Table. Effect of the ionic composition of the extracellular medium on carbachol-stimulated $[Ca^{2-}]_i$ increase in rat parotid acini

	$[Ca^{2+}]_i$ (nM)					
Medium	HBSS		K		NMDG	
Ca^{2+}	+	−	+	−	+	−
(*n*)	(7)	(7)	(9)	(8)	(6)	(4)
Resting	178 ± 17	161 ± 18	189 ± 12	170 ± 12	231 ± 19[a]	197 ± 80
Stimulated	420 ± 44	362 ± 22	381 ± 13	416 ± 34	535 ± 19[b]	483 ± 28

These data have been calculated from experiments similar to those shown in Fig. 1. In each case resting $[Ca^{2+}]_i$ and peak $[Ca^{2+}]_i$ (6 sec after agonist addition) were calculated.

[a] Value significantly different ($P < 0.05$) from resting $[Ca^{2+}]$ in HBSS + Ca^{2+} medium.

[b] Value significantly different ($P < 0.05$) from peak $[Ca^{2+}]_i$ in HBSS − Ca^{2+} medium.

All other resting $[Ca^{2+}]_i$ values are not significantly different from that in HBSS + Ca^{2+}, and all other peak $[Ca^{2+}]_i$ values are not different from that in HBSS + Ca^{2+} medium.

The numbers in parentheses indicate the number of experiments in each condition.

NMDG medium (304 ± 30 nM), though on average higher than that in the Ca^{2+}-containing HBSS medium (242 ± 36 nM), is not statistically different. In all cases $[Ca^{2+}]_i$ increases are 2.3-fold over resting. These data indicate that intracellular Ca^{2+} release, which is associated with agonist-stimulated inositol trisphosphate generation, is apparently not affected by introducing parotid acini to different media.

The sustained elevation in $[Ca^{2+}]_i$, which follows the initial peak elevation in $[Ca^{2+}]_i$ and is dependent on extracellular Ca^{2+} [13, 15] is, however, affected by the manipulations in the extracellular medium described above. As shown in Fig. 1, the sustained elevation of $[Ca^{2+}]_i$ in cells suspended in Ca^{2+}-containing HBSS medium is attenuated in Ca^{2+}-containing K medium (Fig. 1*B*), but not in Ca^{2+}-containing NMDG medium (Fig. 1*C*). In fact, the pattern of $[Ca^{2+}]_i$ changes in Ca^{2+}-containing K medium cannot be distinguished from that in nominally Ca^{2+}-free K medium. In the Ca^{2+}-free HBSS medium and NMDG medium the duration of the $[Ca^{2+}]_i$ increase is markedly decreased (Figs. 1*A* and *C*). These data suggest that Ca^{2+} entry is inhibited in the K medium and are consistent with an earlier report by Merritt and Rink [13] which showed that substitution of Na^+ with K^+ in the extracellular medium attenuated the extracellular Ca^{2+}-dependent phase of the $[Ca^{2+}]_i$ response but did not appear to change the peak $[Ca^{2+}]_i$ increase due to intracellular release. However, that study did not clarify whether the attenuation of the sustained $[Ca^{2+}]_i$ increase was a result of a change in membrane potential or, as suggested by the authors, a more direct effect on the plasmalemmal influx mechanism ex-

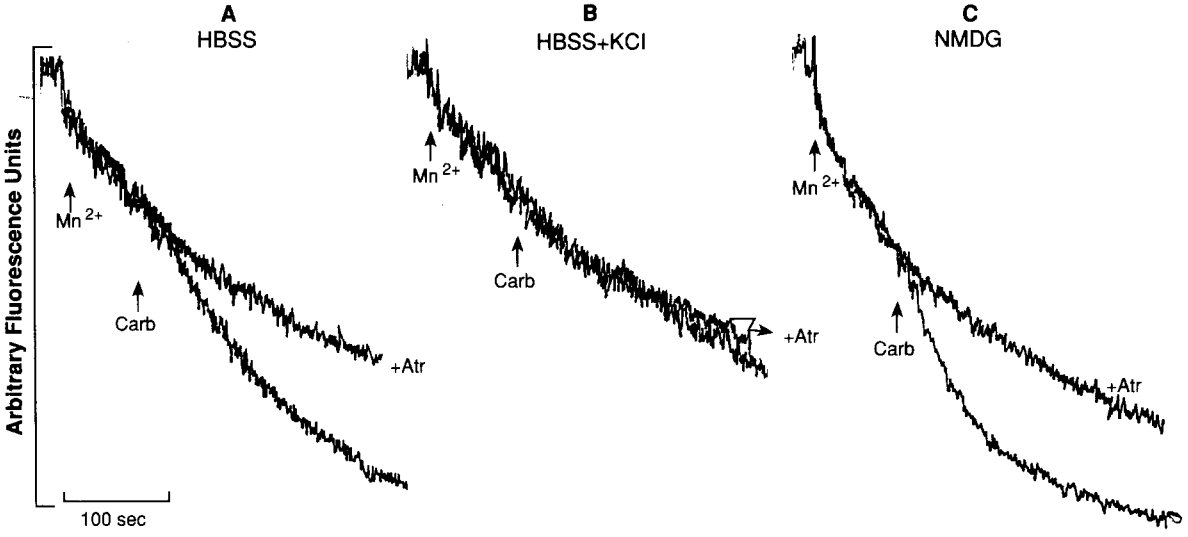

Fig. 2. Effect of the ionic composition of the extracellular medium on carbachol stimulation of Mn^{2+} entry in parotid acini. All experimental conditions were similar to those described for Fig. 1: HBSS medium (*A*); K medium (*HBSS + KCl*) (*B*); NMDG medium (*C*). Fura-2 fluorescence was measured with Ca^{2+}-insensitive excitation wavelength. Mn^{2+} (50 μM) and carbachol (*Carb*) (10 μM) were added at the times indicated by arrows to cells in Ca^{2+}-free media. Where indicated 100 nM atropine (*Atr*) was added 30 sec before carbachol. Fluorescence is expressed as arbitrary units relative to the initial fluorescence before Mn^{2+} addition. Rates of Fura-2 quenching in the various conditions were calculated based on the initial decrease in Fura-2 fluorescence after carbachol addition. The values (mean ± SEM, in U/sec) obtained from three experiments are as follows: Basal (HBSS) −0.091 ± 0.001, Carb (HBSS) −0.168 ± 0.01, Basal (NMDG) −0.131 ± 0.001, Carb (NMDG) −0.24 ± 0.005, Basal (KCl) −0.073 ± 0.005, and Carb (KCl) −0.088 ± 0.02.

erted by the high external $[K^+]$. Additionally, such changes could also be induced by membrane potential effects on other parotid cell Ca^{2+}-transport systems, e.g., Ca-ATPases in the basolateral plasma membrane and in the endoplasmic reticulum which appear to be sensitive to changes in membrane potential [2, 5]. The experiments described below address these possibilities.

To assess directly whether the effects (described above) of different media on $[Ca^{2+}]_i$ changes are due to effects on Ca^{2+} entry, we have examined the entry of Mn^{2+} into parotid acini under conditions similar to those described in Fig. 1. We have previously demonstrated that Mn^{2+} can enter parotid acini in response to stimulation by Ca^{2+}-mobilizing agonists and that, like Ca^{2+}, its entry is regulated by the refill status of the internal Ca^{2+} pool [14]. Therefore, as in a number of other cell types, Mn^{2+} can be used as a Ca^{2+} surrogate to investigate mechanisms of Ca^{2+} influx in parotid acinar cells. The data in Fig. 2 show that with cells in nominally Ca^{2+}-free HBSS medium (Fig. 2*A*), carbachol stimulates Mn^{2+} entry, which can be seen as a 1.8-fold enhancement in the Fura-2 quench rate (*see* legend). We have used the relative rates of Fura-2 quenching as a basis for comparing Mn^{2+} entry into cells in the different media, and the values obtained from the various experiments are given in the figure legend. With cells suspended in nominally Ca^{2+}-free K medium (Fig. 2*B*), carbachol enhancement in Fura-2 quench rate is not observed, while in cells in nominally Ca^{2+}-free NMDG medium (Fig. 2*C*) the quench rate is higher (~42%) than that in HBSS medium. The Fura-2 quench rate due to Mn^{2+} entry into unstimulated cells (100 nM atropine added 30 sec before carbachol) is also higher in the NMDG (~40%) and lower in the K medium (~20%) as compared to that in HBSS medium. However, the difference between Fura-2 quenching in carbachol-stimulated cells suspended in NMDG medium and HBSS medium is greater than the difference between Fura-2 quenching in unstimulated cells in the same media. Thus the increased Mn^{2+} entry into carbachol-stimulated cells in the NMDG medium cannot be solely accounted for by the increase in Mn^{2+} entry into unstimulated cells due to the hyperpolarizing medium.

Membrane potential was assessed in cells under conditions similar to those described above using the fluorescent dye DiBAC4(3), and the data are shown in Fig. 3. Following carbachol stimulation in normal HBSS medium (Fig. 3*A*, solid line), cells hyperpolarize relative to their resting potential (fluorescence decrease). This hyperpolarization can be prevented by the prior addition of atropine (dotted line). When 50 mM KCl is added to the medium, the cells depolarize (Fig. 3*B*), i.e., fluorescence in-

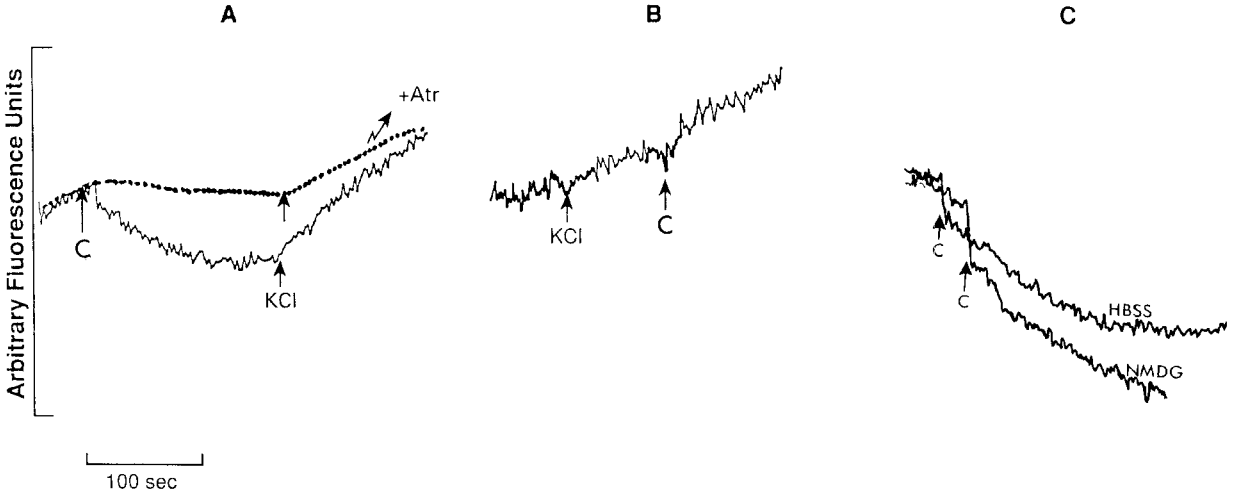

Fig. 3. Effect of ionic composition of extracellular medium on carbachol-stimulated membrane potential changes in dispersed parotid acini. Membrane potential was assessed by measuring the fluorescence of DiBAC4(3) as described in the Materials and Methods section. Dye (2 μM) was added to 2 ml of cells gently stirred in a cuvette. The fluorescence increases gradually as the dye equilibrates in the cell (about 10 min); this is not shown. Additions: 100 nM atropine (*Atr*), 10 μM carbachol (*C*), and 50 mM KCl (*KCl*) are indicated by arrows. Cells were suspended in Ca^{2+}-containing HBSS medium (*A* and *B*). In the traces shown in *C* the cells were first incubated with 2-μM dye in Ca^{2+}-containing HBSS medium for 10 min for dye equilibration, after which they were washed and resuspended in the indicated medium (NMDG or HBSS) containing 2-μM dye immediately prior to fluorescence measurements.

creases relative to the resting potential. No fluorescence change is seen if the same concentration of NaCl, NMDG or Tris are added instead of KCl (*not shown*). When carbachol is added after the KCl-induced depolarization, the hyperpolarization typically induced in HBSS medium is not observed, and the rate of depolarization is further increased. When cells are resuspended in the NMDG medium (Fig. 3*C*) there is an initial decrease in the fluorescence, indicating a hyperpolarization without carbachol stimulation. The quenching rate of the dye is further increased when carbachol is added to cells in this medium as compared to cells in the HBSS medium. It should be noted that addition of 50 μM Mn^{2+} to the medium does not directly alter the fluorescence of the dye or change the pattern of carbachol stimulation of membrane hyperpolarization (*not shown*).

Our earlier studies have shown that Mn^{2+} entry into Ca^{2+} pool-depleted cells is not altered by the addition of atropine [14], and a similar observation has been reported earlier by Takemura and Putney [28] for Ca^{2+} entry. Therefore, divalent cation entry into Ca^{2+} pool-depleted acini appears to be independent of receptor stimulation and the relatively short-lived known products of phosphatidylinositol 4,5-bisphosphate hydrolysis. We have examined the effects of membrane potential on Mn^{2+} entry into internal Ca^{2+} pool-depleted cells and these studies (*see below*) rule out effects of membrane potential on receptor occupation. Also, since intracellular Ca^{2+} pools are empty in this case, direct effects of membrane potential on the intracellular Ca^{2+} release process, which would secondarily affect Ca^{2+} entry (by capacitative regulation) can be excluded.

The effects of membrane potential modulations on Mn^{2+} entry into Ca^{2+} pool-depleted cells are similar to those seen upon carbachol stimulation. Cells were depleted of internal Ca^{2+} by stimulation with 10 μM carbachol for 10 min in nominally Ca^{2+}-free medium. The rate of Fura-2 quenching, due to Mn^{2+} entry, in such pool-depleted cells suspended in Ca^{2+}-free HBSS medium (shown as a dashed line in Fig. 4*A* and solid trace in Fig. 4*B*), is significantly enhanced (~1.8-fold over basal, $P < 0.005$ by Student's *t* test, *see* figure legend for the relative rates of Fura-2 quenching) compared to that in unstimulated cells. When pool-depleted cells are suspended in K medium (in this case all the Na^+ was replaced by K^+) Mn^{2+} entry is not significantly different from that in unstimulated cells (Fig. 4*A*). On the other hand, when cells are suspended in NMDG medium, Mn^{2+} entry is significantly greater (23%, $P < 0.025$, Student's *t* test) than in HBSS medium (Fig. 4*B*). To further assess the effects of increased extracellular [K^+] on divalent cation influx into parotid acini, we have examined Mn^{2+} entry at different concentrations of extracellular Na^+ (Na^+ in the HBSS medium was replaced by K^+), and the data are shown in Fig. 5. Fura-2 quench rate, due to Mn^{2+} entry into internal Ca^{2+} pool-depleted cells is linearly altered

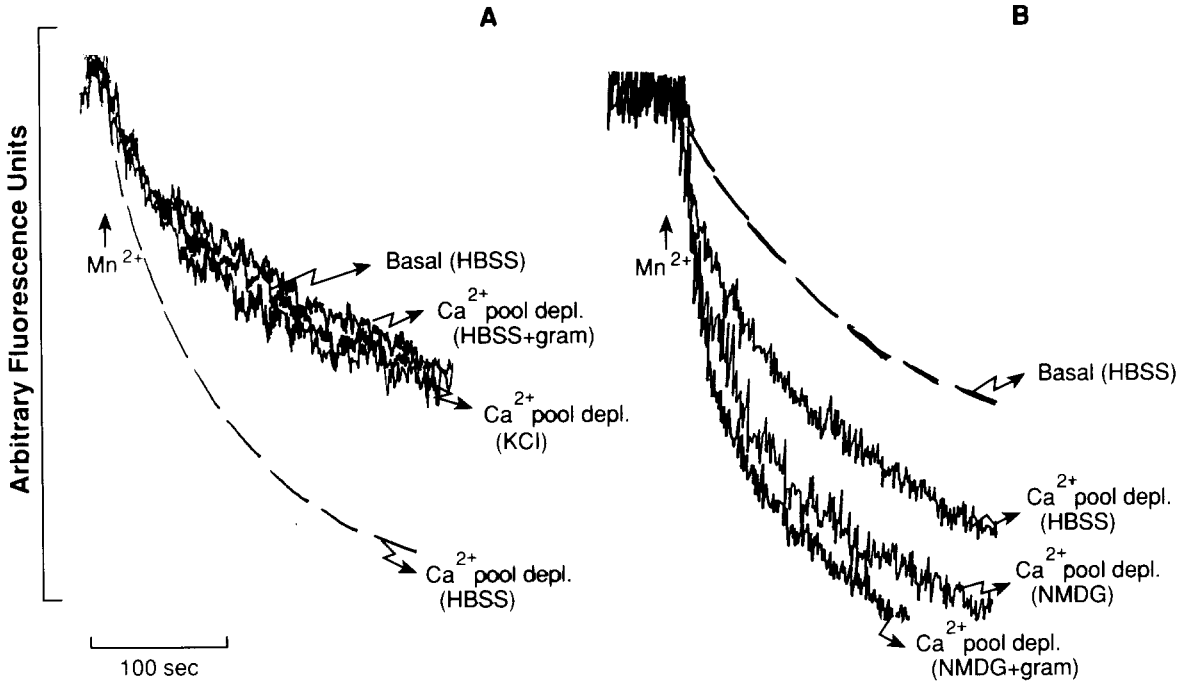

Fig. 4. Effect of membrane potential on Mn^{2+} entry in Ca^{2+} pool-depleted acini. Dispersed rat parotid acini were washed and resuspended in Ca^{2+}-free HBSS medium and stimulated with 10 μM carbachol for 10 min after which the Ca^{2+} pool-depleted cells (*Ca^{2+} pool depl.*) were washed and resuspended in the Ca^{2+}-free medium indicated, HBSS (HBSS medium), KCl (HBSS with NaCl replaced by KCl), NMDG (HBSS with NaCl replaced by NMDGCl) and 100 nM atropine was added. Mn^{2+} (50 μM) was added where indicated in all the traces. Gramicidin (10 μM) was added to the cells before Mn^{2+} in the traces indicated. Fura-2 fluorescence was measured with 363-nm excitation. Rates of Fura-2 quenching were calculated based on the initial decrease in Fura-2 fluorescence after Mn^{2+} addition. The values obtained (mean ± SEM, the number of experiments in each case is indicated in parentheses) in U/sec are as follows: *Basal (HBSS)* −0.158 ± 0.001 (6), *Ca^{2+} pool depl. (HBSS)* −0.286 ± 0.017 (7), *Ca^{2+} pool depl. (KCl)* −0.157 ± 0.001 (4), *Ca^{2+} pool depl. (NMDG)* −0.354 ± 0.002 (3), *Ca^{2+} pool depl. (NMDG + gram)* −0.402 ± 0.001 (4), and *Ca^{2+} pool depl. (HBSS + gram)* −0.164 ± 0.015 (5). The difference in the rates of basal Fura-2 quench in the basal condition (HBSS) calculated here and in Fig. 2 should be noted. This is likely due to the nonlinearity of Fura-2 fluorescence as intracellular [Mn^{2+}] increases with time due to passive Mn^{2+} influx. The rates given here agree well with our previously reported values (*see* reference [14]).

as a function of the log of extracellular [K^+]. These data are consistent with an earlier report showing a linear relationship between membrane potential and extracellular [K^+] [19]. We have observed that, conversely, when Na^+ in the medium is substituted with increasing concentrations of $NMDG^+$, there is a corresponding increase in Mn^{2+} entry (*data not shown*), with the highest rates obtained in the medium where all the Na^+ is substituted by $NMDG^+$ (Fig. 4*B*). As shown in Fig. 3*C*, parotid acinar cells are hyperpolarized to a greater degree in this medium than in the HBSS medium. When gramicidin is added to Ca^{2+} pool-depleted cells in the NMDG medium the cells can be expected to hyperpolarize further. Under these conditions (Fig. 4*B*), Mn^{2+} entry is greater than that in the NMDG medium without gramicidin. In contrast, addition of gramicidin to cells in HBSS medium results in the inhibition of Mn^{2+} entry to levels not greater than that observed in Ca^{2+}-depleted cells in K medium or in basal cells in HBSS medium. In the HBSS medium gramicidin will facilitate the entry of Na^+ into the cells, resulting in depolarization. This latter observation further supports our suggestion that depolarization due to high external [K^+], and not direct effects of external [K^+], accounts for the inhibition of Mn^{2+}, or Ca^{2+}, entry into parotid acini which is observed in a high K^+ medium as described above and by Merritt and Rink [13].

It is also important to consider the effects on Ca^{2+} (Mn^{2+}) entry of changes in intracellular pH (pH_i), which may be induced by inhibition of the Na^+/H^+ exchanger as a result of varying extracellular [Na^+] in the different media used to manipulate the membrane potential. Both the NMDG and K media would be expected to induce acidification of the cytosol. Measurements of pH_i showed that the pattern of pH_i in cells incubated in either media are similar (*data not shown*) and very slight acidification (<0.1 units compared to control cells in HBSS me-

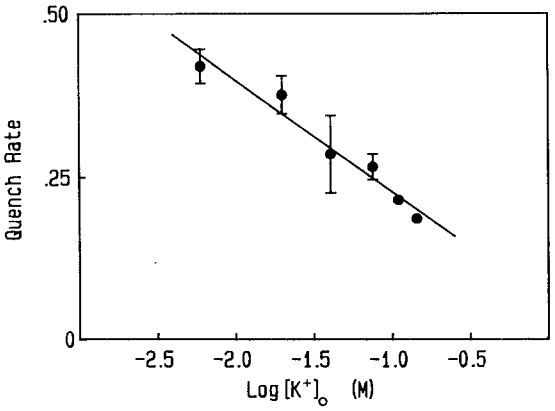

Fig. 5. Effect of substituting extracellular Na^+ with varying concentrations of K^+ on Mn^{2+} entry into Ca^{2+}-depleted parotid acini. Experimental conditions were similar to those described in Fig. 4. The Na^+ in HBSS medium (147 mM) was isosmotically substituted with K^+. Relative rates of initial Fura-2 quenching after the addition of 50 μM Mn^{2+} were determined as described for Fig. 4. The data shown were obtained from 3–5 experiments. Linear regression of these values gave a correlation coefficient of 0.984.

dium) is seen in either case. However, these two conditions induce opposite effects on divalent cation entry; K medium inhibits, while NMDG medium stimulates entry. Thus we can suggest that alterations in pH_i do not account for the observed changes in $[Ca^{2+}]_i$ mobilization. The inhibition of divalent cation entry observed in the presence of gramicidin and by KCl addition in the presence of extracellular Na^+ also rules out possible effects of pH_i.

Several studies demonstrate that Ca^{2+} entry into Ca^{2+} pool-depleted cells, following termination of receptor activation by the addition of the antagonist, leads to the refill of the intracellular Ca^{2+} pools [10, 13, 14, 16, 24]. Since $[Ca^{2+}]_i$ increase due to Ca^{2+} entry during refill of the internal pool is very transient, Ca^{2+} entry under these conditions can be monitored by assessing the extent of refill of the internal agonist-sensitive Ca^{2+} pool. In Fig. 6 we show the effect of depolarizing conditions on the extent of refill of the internal Ca^{2+} pool. Cells were stimulated with carbachol for 10 min in Ca^{2+}-free HBSS medium and then resuspended in either Ca^{2+}-free K medium (Fig. 6*B*) or in Ca^{2+}-free HBSS medium (Fig. 6*A*) with atropine for 5 min. The cells depolarized during this incubation in the Ca^{2+}-free K medium (fluorescence trace not shown). Following this, 1.28 mM Ca^{2+} was added to both media and 5 min later epinephrine was added. The $[Ca^{2+}]_i$ response to epinephrine (which indicates the extent of refill) was greatly blunted in cells allowed to refill in the K medium as compared to that in cells incubated in HBSS. Thus, conditions which retard Ca^{2+} and Mn^{2+} entry into the cytosol, consequently, also retard the refill of the internal Ca^{2+} pool. It should also be noted that the transient $[Ca^{2+}]_i$ increase, seen when Ca^{2+} is reintroduced into Ca^{2+}-depleted cells [28], is much slower in cells suspended in the K medium. This agrees well with the inhibitory effect of depolarizing conditions on Mn^{2+} entry in Ca^{2+}-depleted cells (shown in Fig. 4). These data also show that depolarization decreases but does not inhibit Ca^{2+} entry.

In the experiments shown in Figs. 7 and 8 we have assessed whether the decrease in divalent cation influx induced by depolarization is due to a direct effect on the transport mechanism or due to changes in the electrochemical gradient for Ca^{2+} entry. In an earlier report [15] we had demonstrated that Ca^{2+} entry into rat parotid acini is a Ca^{2+} gradient-responsive process, i.e., greater entry at higher extracellular $[Ca^{2+}]$. In Fig. 7*A*, the cells were subjected to K^+ depolarization (as in the experiments described above) but in the presence of elevated extracellular Ca^{2+} (10 mM). Carbachol stimulation of acini suspended in this medium leads to a sustained elevation of $[Ca^{2+}]_i$ in contrast to the attenuated response observed in normal Ca^{2+} (1.28 mM) containing K medium (shown in Fig. 1*B*). We have also examined whether the increase in Ca^{2+} entry seen in Fig. 7*A* can result in refill of the internal Ca^{2+} pool which, otherwise, is attenuated by these depolarization conditions. As shown in Fig. 7*B*, this elevation in extracellular $[Ca^{2+}]$ also reverses the attenuation of refill of the internal Ca^{2+} pools seen in the 1.28-mM Ca^{2+}-containing K medium. Figure 7*B* shows the effect of extracellular $[Ca^{2+}]$ during refill of the internal Ca^{2+} pool in HBSS and K medium. The data clearly demonstrate that with increasing concentrations of extracellular $[Ca^+]$ the efficiency of refill in the K medium is increased and at 10 mM Ca^{2+} it is not significantly different from that in cells in the HBSS medium. We suggest that the apparent inhibition of internal Ca^{2+} pool refill by depolarizing conditions is not due to direct effects on the refill process but as a result of retardation of Ca^{2+} entry from the extracellular medium into the cytosol.

In Fig. 8*A* we have shown that a high K medium fully retards the entry of Mn^{2+} into Ca^{2+}-depleted parotid acini, when the extracellular concentration of Mn^{2+} is 50 μM. When extracellular $[Mn^{2+}]$ is increased fourfold to 200 μM, the same K^+ depolarization medium only partially retards Mn^{2+} entry (Fig. 8*B*). With 200 μM Mn^{2+} in the extracellular medium the basal rate of entry is also higher than that seen with 50 μM Mn^{2+}. The results from these experiments demonstrate that the extent of inhibition of divalent cation entry by depolarization de-

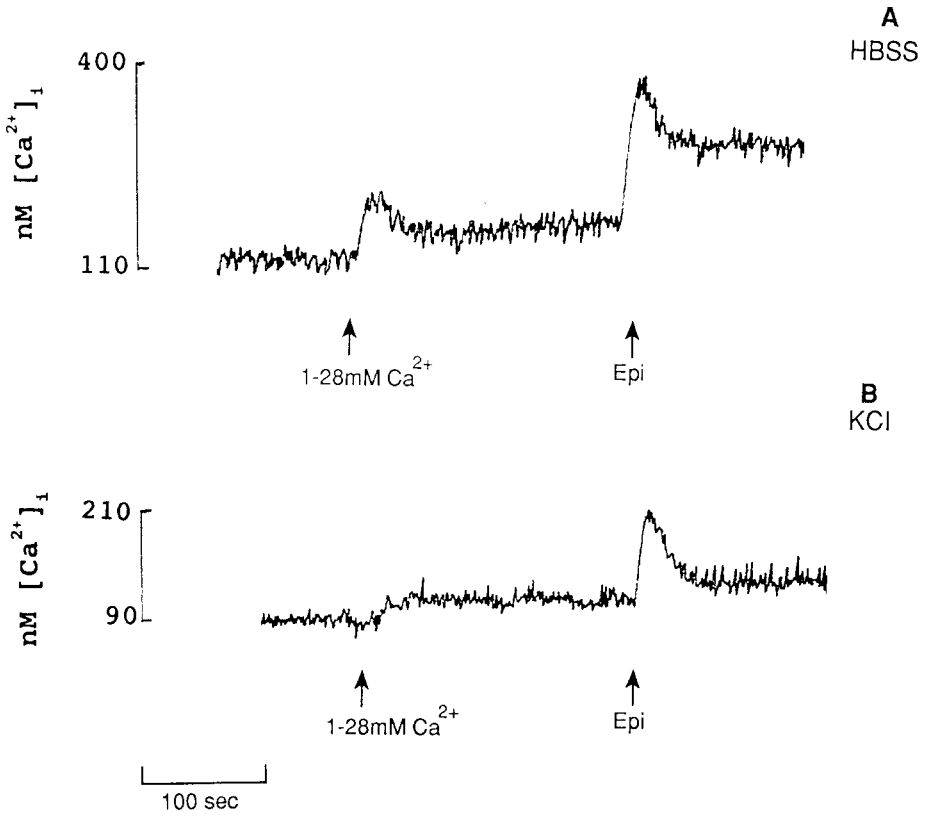

Fig. 6. Effect of K^+ depolarization on Ca^{2-} entry during refill of the internal Ca^{2+} pool. Cells were depleted of internal Ca^{2+} pool as described for Fig. 4. Following this, cells were washed and resuspended in either Ca^{2+}-free HBSS medium (*A*) or Ca^{2+}-free K medium (*B*, HBSS with all Na^+ replaced with K^+) for 5 min with 10 μM atropine. Ca^{2+} (1.28 mM) was subsequently added (as indicated), and the cells were further incubated for 5 min as shown. Epinephrine, *Epi*, (10 μM) was then added as indicated. Fura-2 fluorescence was measured with excitation at 340 nm.

pends upon the extracellular concentration of divalent cation. This suggests that the chemical gradient of the divalent cation can influence the inhibitory effects of depolarization on divalent cation entry in parotid acinar cells.

Discussion

The results presented above show that the rate of Ca^{2+} and Mn^{2+} entry into unstimulated and agonist-stimulated parotid acini can be altered by conditions which change the membrane potential (i.e., alteration of monovalent cation composition of the extracellular medium or addition of the monovalent cation ionophore, gramicidin). These data demonstrate that divalent cation entry into rat parotid acinar cells is modulated by membrane potential. Such changes in the membrane potential could modulate Ca^{2+} entry either by altering the electrochemical gradient for Ca^{2+} entry or by voltage regulation of the Ca^{2+} influx mechanism *per se*. We have shown that the inhibitory effects of depolarization on divalent cation entry can be overcome by increasing the extracellular concentration of the divalent cation. This observation strongly indicates that depolarization does not inactivate the Ca^{2+} influx mechanism. Thus, although we cannot presently rule out direct voltage regulation of the Ca^{2+} influx mechanism, we can suggest that Ca^{2+} influx in parotid acini involves a transfer of charge and is related to the electrochemical gradient ($E_m - E_{Ca}$) for Ca^{2+} entry.

Several earlier reports support the proposal that following agonist stimulation of parotid acini the depletion of the internal Ca^{2+} pool stimulates Ca^{2+} entry (by capacitative regulation) [9, 14, 27, 28]. The data reported by us earlier agree well with this proposal [14], while our present data suggest that in addition, the activation of Ca^{2+} entry by carbachol can be modulated by changes in the membrane potential. Importantly, we have also demonstrated that membrane potential modulates divalent cation entry in the absence of any accompanying changes in the intracellular Ca^{2+} pool, i.e., in Ca^{2+} pool-depleted

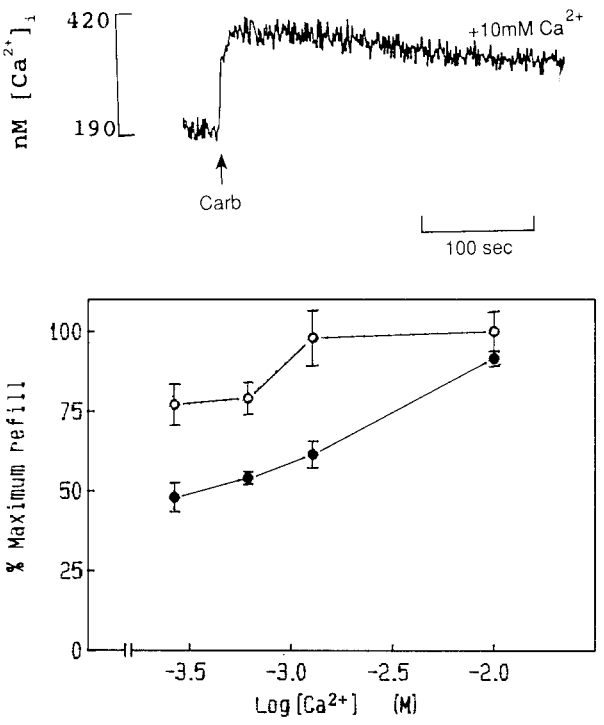

Fig. 7. Effect of extracellular $[Ca^{2+}]$ on K depolarization-induced inhibition of Ca^{2+} entry into parotid acini. Conditions for the experiment shown in *A* were similar to those described for Fig. 1*B*. Cells were suspended in HBSS medium with 50 mM KCl, containing 10 mM Ca^{2+} and stimulated with 10 μM carbachol added where shown by arrow. Conditions for the experiments shown in *B* were similar to those described for Fig. 6. Ca^{2+} pool-depleted cells were incubated in K medium with 10 μM atropine for 5 min after which the indicated concentration of Ca^{2+} was added and cells further incubated for 5 min. Epinephrine (10 μM) was then added to restimulate the cells. All measurements were done with Fura-2 excitation of 340 nm. The data in *B* were obtained with four experiments with different cell preparations. The $[Ca^{2+}]_i$ reached after epinephrine addition was used to represent refill. Refill obtained with 10 mM Ca^{2+} in HBSS medium (open circles) was set as 100% (395 ± 25 nM), and this is not significantly different from the refill with 10 mM Ca^{2+} in the K medium (filled circles, 359 ± 15 nM). At all other $[Ca^{2+}]$ refill in HBSS is significantly higher ($P < 0.05$) than that in K medium.

cells. As mentioned above, in such Ca^{2+} pool-depleted cells the entry of Ca^{2+} or Mn^{2+} does not appear to be dependent on receptor-mediated intracellular signals since neither is altered by the addition of the antagonist, atropine [14, 28]. These observations rule out the possibility that the observed effects of membrane potential on divalent cation entry are secondarily induced due to alterations, either in agonist-receptor interactions or in the intracellular Ca^{2+} pool via the inositol-phosphate signaling system. This is further supported by the data in Fig. 1 and the Table which show that intracellular Ca^{2+} release is not affected by changes in the membrane potential. Additionally, Ca^{2+}-free conditions were used for the Mn^{2+} entry experiments and $[Ca^{2+}]_i$ values in carbachol-stimulated cells in Ca^{2+}-free HBSS, KCl, and NMDG media are comparable (*see* the Table). Based on these observations we propose that the effects of membrane potential are directly exerted on divalent cation influx.

The membrane potential of parotid acini in the unstimulated state ranges from about −20 to −70 mV [6, 18, 19, 30], and upon stimulation there is a hyperpolarization of 10 to 50 mV [6, 19, 30]. Together with the existing, large, inwardly directed Ca^{2+} gradient ($[Ca^{2+}]_o$ is approximately 10^4-fold higher than resting $[Ca^{2+}]_i$); a large driving force for Ca^{2+} entry is present in resting cells. The plasma membrane, under these conditions, provides an extremely effective barrier against Ca^{2+} entry, although a low level of divalent cation influx can be detected in unstimulated cells (*see* Fig. 2) with the use of Mn^{2+}, likely due to the high sensitivity of the Fura-2-Mn^{2+} interaction. Upon agonist stimulation, the "gating" mechanism, presumably triggered as a result of intracellular Ca^{2+} mobilization, results in a change in the Ca^{2+} permeability of this membrane, which allows Ca^{2+} entry along its electrochemical gradient. Such a change in the divalent cation permeability of the plasma membranes is evident from the data we have presented above and from earlier studies reported with parotid and pancreatic acinar cells [14–17, 28]. Previous reports from our laboratory and others indicate that following agonist stimulation, Ca^{2+} entry apparently commences at a time when $[Ca^{2+}]_i$ is elevated several-fold above basal [13–15]. The initial elevation in $[Ca^{2+}]_i$ would also alter the inwardly directed Ca^{2+}. Thus, it seems reasonable to suggest that the initial hyperpolarization may augment the driving force for Ca^{2+} entry during this phase. This speculation is supported by our observation (shown in Fig. 2) that further hyperpolarization of parotid acini, observed in acini incubated in the NMDG medium, increases the rate of initial Mn^{2+} entry.

While the activation of the "maxi" K^+ channel has been well characterized in the parotid and other exocrine cells, little is yet known about the molecular mechanisms involved in the activation and regulation of Ca^{2+} entry in exocrine and other "nonexcitable" cells [21, 25]. Earlier studies (which we have discussed above) have clearly shown that the release of Ca^{2+} from the intracellular pool, mediated either by inositol 1,4,5, trisphosphate or by inhibition of the Ca^{2+} pump involved in loading this pool, activate Ca^{2+} influx in a capacitative manner. The data we have described here show that in addition to pool depletion, the magnitude of Ca^{2+} entry into agonist-stimulated rat parotid acinar cells is increased by

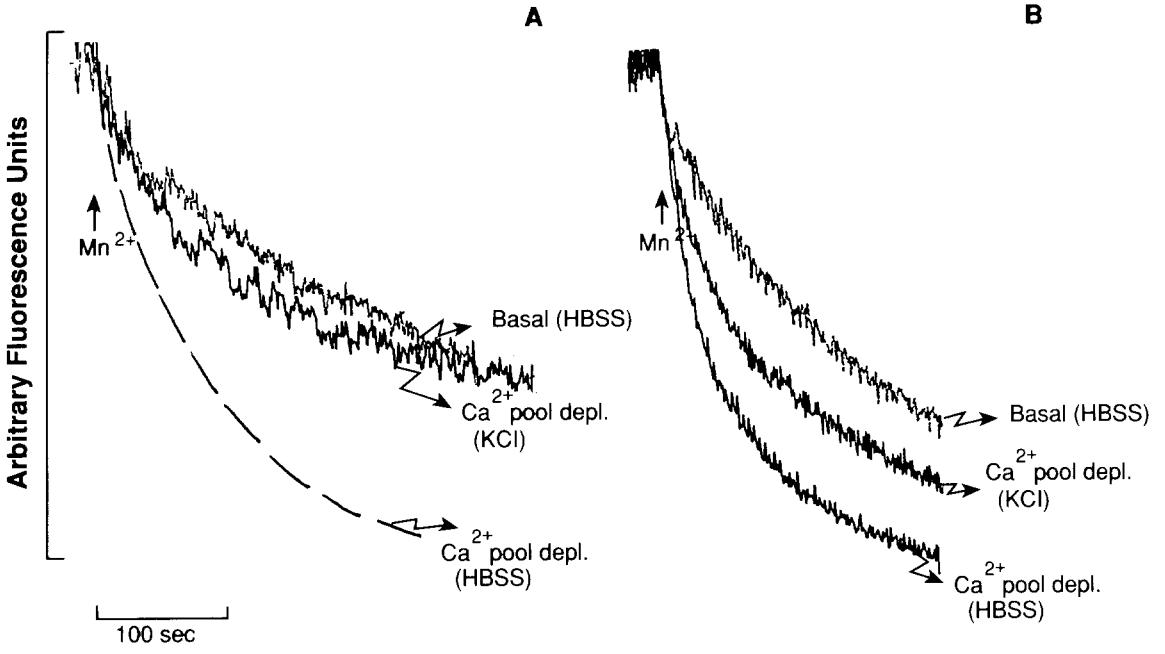

Fig. 8. Effect of extracellular $[Mn^{2+}]$ on the K depolarization-induced inhibition of Mn^{2+} entry in parotid acini. Cells were depleted of internal Ca^{2+} pool as described for Fig. 4. Mn^{2+}-dependent Fura-2 quenching was measured by using Fura-2 excitation at 363 nm. Cells were resuspended in the different media as indicated (similar to those described for Fig. 4) and 50 μM Mn^{2+} (*A*) or 200 μM Mn^{2+} (*B*) was added where shown by arrows. Fluorescence has been expressed relative to the value before addition of Mn^{2+} in each case. Basal cells indicate unstimulated cells with intact internal Ca^{2+} pool.

hyperpolarization of the cell and that depolarization can completely overcome the capacitative activation of Ca^{2+} entry. Based on these observations, it can be suggested that any condition leading to the release of Ca^{2+} from the intracellular pool would increase $[Ca^{2+}]_i$ and activate the K^+ channel, resulting in hyperpolarization of the cell. This change in membrane potential would then increase the driving force for Ca^{2+} influx. Thus, in order to describe completely the activation of divalent cation entry in rat parotid acini following agonist stimulation, it will be necessary to resolve the effects on Ca^{2+} influx due to intracellular Ca^{2+} release (capacitative effects) from those due to changes in the membrane potential and/or the $[Ca^{2+}]_o/[Ca^{2+}]_i$ gradient (electrochemical gradient effects).

We would like to thank Dr. James R. Turner and Dr. Yukiharu Hiramatsu for the helpful discussions during the preparation of this manuscript.

References

1. Adams, D.J., Barakeh, J., Laskey, R., van Breeman, C. 1989. Ion channels and regulation of intracellular Ca^{2+} in vascular endothelial cells. *FASEB J.* **3:**2389–2400
2. Ambudkar, I.S., Baum, B.J. 1988. ATP-dependent Ca^{2+} transport in rat parotid basolateral membranes is modulated by membrane potential. *J. Membrane Biol.* **102:**59–69
3. Baum, B.J., Ambudkar, I.S., Helman, J., Horn, V.J., Melvin, J.E., Mertz, L.M., Turner, R.J. 1990. Dispersed salivary gland acinar cell preparations for use in studies of neuroreceptor coupled secretory events. *Methods Enzymol.* **192:**26–37
4. Baum, B.J., Ambudkar, I.S., Horn, V.J. 1991. Neurotransmitter regulation of calcium mobilization in salivary cells. *In:* The Biology of the Salivary Glands. *(in press)*
5. Baum, B.J., Horn, V.J., Ambudkar, I.S. 1988. ATP-dependent Ca^{2+} transport in rat parotid cell endoplasmic reticulum requires charge compensation. *Biochem. J.* **254:**649–654
6. Foskett, J.K., Gunter-Smith, P.J., Melvin, J.E., Turner, R.J. 1989. Physiological localization of an agonist-sensitive pool of Ca^{2+} in parotid acinar cells. *Proc. Natl. Acad. Sci.* **86:**167–171
7. Foskett, J.K., Melvin, J.E. 1989. Activation of salivary secretion: Coupling of cell volume and $[Ca^{2+}]_i$ in single cells. *Science* **244:**1582–1585
8. Ganz, M.B., Rasmussen, J., Bollag, W.B., Rasmussen, H. 1990. Effect of buffer systems and pH_i on the measurement of $[Ca^{2+}]_i$ with Fura 2. *FASEB J.* **4:**1638–1644
9. Jacob, R. 1990. Agonist-stimulated divalent cation entry into single cultured umbilical vein endothelial cells. *J. Physiol.* **421:**55–57
10. Kwan, C.-Y., Putney, J.W., Jr. 1990. Uptake and sequestration of divalent cations in resting and methacholine-stimulated mouse cells. Dissociation by Sr^{2+} and Ba^{2+} of agonist-stimulated divalent cation entry from the refilling of the agonist-sensitive intracellular pool. *J. Biol. Chem.* **265:** 678–684

11. Laskey, R.E., Adams, D.J., Johns, A., Rubanyi, G.M., van Breeman, C. 1990. Membrane potential and Na^+-K^+-pump activity modulate resting and bradykinin-stimulated changes in cytosolic free Ca^{2+} in cultured endothelial cells from bovine atria. *J. Biol. Chem.* **265:**2613–2619
12. Melvin, J.E., Moran, A., Turner, R.J. 1988. The role of HCO_3^- and Na^+/H^+ exchange in the response of rat parotid acinar cells to muscarinic stimulation. *J. Biol. Chem.* **263:**19564–19569
13. Merritt, J.E., Rink, T.J. 1987. Regulation of cytosolic free Ca^{2+} in Fura-2 loaded rat parotid acinar cells. *J. Biol. Chem.* **262:**17362–17369
14. Mertz, L.M., Baum, B.J., Ambudkar, I.S. 1990. Refill status of the agonist-sensitive Ca^{2+} pool regulates Mn^{2+} influx into parotid acini. *J. Biol. Chem.* **265:**15010–15014
15. Mertz, L.M., Horn, V.J., Baum, B.J., Ambudkar, I.S. 1990. Calcium entry in rat parotid acini: Activation by carbachol and aluminum fluoride. *Am. J. Physiol.* **258:**C654–C661
16. Muallem, S. 1989. Calcium transport pathways of pancreatic acinar cells. *Annu. Rev. Physiol.* **51:**83–105
17. Muallem, S., Khademazad, M., Sachs, G. 1990. The route of Ca^{2+} entry during reloading of the intracellular Ca^{2+} pool in pancreatic acini. *J. Biol. Chem.* **265:**2011–2016
18. Nauntofte, B., Dissing, S. 1988. K^+ transport and membrane potentials in isolated rat parotid acini. *Am. J. Physiol.* **255:**C508–C518
19. Pedersen, G.L., Petersen, O.H. 1973. Membrane potential measurement in parotid acinar cells. *J. Physiol.* **234:** 217–227
20. Penner, R., Matthews, G., Neher, B. 1988. Regulation of calcium influx by second messengers in rat mast cells. *Nature* **334:**499–504
21. Petersen, O.H. 1989. Does inositol tetrakisphosphate play a role in the receptor-mediated control of calcium mobilization? *Cell Calcium* **10:**375–383
22. Petersen, O.H., Gallacher, D.V. 1988. Electrophysiology of pancreatic and salivary acinar cells. *Annu. Rev. Physiol.* **50:**65–80
23. Pittett, D., Virgilio, F.D., Pozzan, T., Monod, A., Lew, D.P. 1990. Correlation between plasma membrane potential and second messenger generation in the promyelocytic cell line HL-60. *J. Biol. Chem.* **265:**14256–14263
24. Putney, J.W., Jr. Identification of cellular activation mechanisms associated with salivary secretion. *Annu. Rev. Physiol.* **48:**75–78
25. Rink, T.J. 1990. Receptor-mediated calcium entry. *FEBS Lett.* **268:**381–385
26. Siffert, W., Akkerman, J.W.N. 1989. Na^+/H^+ exchange and Ca^{2+} influx. *FEBS Lett.* **259:**1–4
27. Takemura, H., Hughes, A.R., Thastrup, O., Putney, J.W., Jr. 1989. Activation of calcium entry by the tumor promotor thapsigargin in rat parotid acinar cells. Evidence that an intracellular calcium pool, and not an inositol phosphate, regulates calcium fluxes at the plasma membrane. *J. Biol. Chem.* **264:**12266–12271
28. Takemura, H., Putney, J.W., Jr. 1989. Capacitative calcium entry in parotid acinar cells. *Biochem. J.* **258:**409–412
29. Yamaguchi, D.T., Green, J., Kleeman, C.R., Muallem, S. 1989. Characterization of volume-sensitive, calcium-permeating pathways in the osteosarcoma cell line, UMR-106-01. *J. Biol. Chem.* **264:**4383–4390
30. Young, J.A., van Lennep, E.W. 1979. Transport in salivary and salt glands. *In:* Membrane Transport in Biology, Vol. 4*b*, pp. 563–624. Springer Verlag, Berlin

Received 13 August 1991; revised 8 November 1991