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Inhibitors of the intracellular Ca^{2+} release mechanism prevent muscarinic-induced Ca^{2+} influx in rat sublingual mucous acini

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The effects of inhibitors of the intracellular Ca^{2+} release mechanism on divalent cation fluxes were examined in acinar cells loaded with the Ca^{2+}-sensitive, Mn^{2+}-quenchable dye, fura-2. TMB-8 and dantrolene (DTL) dramatically inhibited the carbachol (CCh)-stimulated increase in $[Ca^{2+}]_i$ and Mn^{2+} influx. These agents do not directly inhibit divalent cation entry since addition of TMB-8 or DTL *after* CCh stimulation did not block Mn^{2+} influx. TMB-8 did not influence the $[Ca^{2+}]_i$ increase or the Mn^{2+} influx produced by thapsigargin. These results indicate that TMB-8 and DTL do not interfere with divalent cation influx by inhibiting a step distal to depletion of the intracellular Ca^{2+} pool. TMB-8 and DTL did not significantly influence the muscarinic-stimulated production of inositol trisphosphate (IP_3) and inositol tetrakisphosphate (IP_4), although TMB-8, but not DTL, did decrease the CCh-stimulated 1,4,5-IP_3 levels approximately 55%. The above results directly demonstrate that the filling state of the intracellular Ca^{2+} store primarily regulates the Ca^{2+} entry mechanism in sublingual mucous acinar cells.

Ca^{2+} release, Ca^{2+} entry; Salivary gland

1. INTRODUCTION

Ca^{2+} influx occurs immediately upon activation of receptors associated with Ca^{2+} mobilization [1]. Early models of Ca^{2+} uptake predicted that Ca^{2+} influx was either directly mediated by receptor activated-channels or was coupled to receptors via G-proteins (see [2,3]). More recently, the underlying mechanism of Ca^{2+} influx has been suggested to be activation of 1,4,5-IP_3 and/or 1,3,4,5-IP_4 receptors [4]. According to this model, 1,4,5-IP_3 receptors interact with a protein, possibly 1,3,4,5-IP_4 receptors located at the plasma membrane, to activate Ca^{2+} entry. When either 1,4,5-IP_3 or 1,3,4,5-IP_4 bind to their respective receptors, dissociation of the two proteins occurs, and Ca^{2+} influx is activated [4]. However, current evidence suggests that Ca^{2+} influx in salivary gland acinar cells is not directly regulated by a receptor-mediated Ca^{2+} entry mechanism. Instead, Ca^{2+} influx is thought to be activated by the emptying of an 1,4,5-IP_3-sensitive, intracellular Ca^{2+} pool. Studies using the Ca^{2+}-sensitive fluorescent probe fura-2 and/or patch-clamp techniques [5–11] support this hypothesis. First, depletion of the intracellular Ca^{2+} store by blocking endoplasmic Ca^{2+}-ATPase with thapsigargin activates Ca^{2+} influx without stimulating receptors and without increasing cellular inositol phosphate production [8–14]. Second, after depleting the agonist-sensitive Ca^{2+} pool, receptor antagonists fail to block the stimulation-induced Ca^{2+} influx [8,9,11–14].

Although previous studies of salivary gland acinar cells clearly demonstrated that depletion of the intracellular Ca^{2+} store activates divalent cation influx [7–11,14], these studies only examined the properties of this pathway *after* activation. The results of these studies thus indicate that depletion of the agonist-sensitive intracellular Ca^{2+} pool is *sufficient*, but do not show that it is *necessary*, to activate the Ca^{2+} uptake pathway. In the present study, we assessed the effects of inhibiting mobilization of intracellular Ca^{2+} on the activation of the divalent cation uptake mechanism. We directly demonstrate that muscarinic stimulation-induced divalent cation influx requires depletion of the 1,4,5-IP_3-releasable, intracellular Ca^{2+} store.

2. MATERIALS AND METHODS

2.1. *Materials*

Collagenase (type CLSPA) was purchased from Worthington Biomedical, Malvern, PA. Hyaluronidase (type I-S) and bovine serum albumin (type V) were from Sigma, St. Louis, MO. Earle's minimal essential medium was purchased from Biofluids, Rockville, MD. Fura-2/AM and ionomycin were from Molecular Probes, Eugene, OR. The D-myo-inositol 1,4,5-IP [^{3}H]assay system was from Amersham, Arlington Heights, IL. TMB-8 (8-(diethylamino)octyl-3,4,5-trimethoxybenzoate) was purchased from Aldrich Chem, Milwaukee, WI. Dantrolene (1-{[5-(*p*-nitrophenyl)furfurylidene]amino} hydantoin sodium hydrate) was from Norwich Eaton Pharmaceuticals, Norwich, NY. Thapsigargin was from LC Services Corp., Woburn, MA. All other chemicals were of the highest grade available

2.2. *Preparation of sublingual mucous acini*

Male, Wistar strain rats (150–250 g; Charles River, Kingston Facil-

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ity, NY) were euthanized by exsanguination after exposure to CO_2. Sublingual glands were removed and placed in ice-cold digestion medium which consisted of Earle's minimal essential medium containing 1% BSA, 50 U/ml collagenase and 0.02 mg/ml hyaluronidase. The glands were minced in 2 ml of the digestion medium, and then placed in 10 ml of the same medium, incubating at 37°C in a Dubnoff shaker with continuous gassing (95% O_2/5% CO_2, humidified) and agitation (80 cycles/min). The mince was dispersed by gently pipetting 10 times with a 10 ml plastic pipette at 15 min intervals. After 45 min digestion, the digestion medium was replaced with fresh medium by centrifugation at $400 \times g$ for 30 s. After a total of 1.5 hr of digestion, the preparation was washed 3 times with physiological salt solution (PSS) containing 0.05% BSA and resuspended in the same medium. The PSS consisted of (mM): 110 NaCl, 25 $NaHCO_3$, 20 HEPES, 10 glucose, 5.4 KCl, 1.2 $CaCl_2$, 0.8 $MgSO_4$, 0.4 KH_2PO_4, 0.33 NaH_2PO_4, adjusted to pH 7.4 with NaOH. For the nominally Ca^{2+} free solution, $CaCl_2$ was omitted.

2.3 *Determination of* $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined as previously reported [8,15]. The sublingual cells were loaded with fura-2 by incubation of the cells with 2 mM fura-2/AM for 30 min at room temperature. Then the cells were centrifuged at $400 \times g$ for 15 s and rinsed with PSS containing 0.05% BSA. The fura-2-loaded cells were kept at room temperature with continuous shaking and gassing (95% O_2/5% CO_2, humidified) until use. For $[Ca^{2+}]_i$ measurement, 0.5 ml of cell suspension was centrifuged at $400 \times g$ for 15 s and resuspended in PSS without BSA to promote attachment to a coverslip mounted in a perfusion chamber. The superfusion was conducted at 23°C. A pinhole turret and a Nikon fluor × 40 oil immersion objective (NA 1.3) were used to isolate five to eight cells on the stage of a Nikon inverted microscope. The fura-2 fluorescence was monitored using an AR-CM fluorometer (SPEX Industries, Edison, NJ). Excitation wavelengths were 340/380 nm and the emission wavelength was 505 nm. The fluorescence ratios were converted to $[Ca^{2+}]_i$ by *in situ* calibrations. To obtain the minimum fluorescence (F_{min}), acini were superfused with Ca^{2+}-free PSS containing 3 mM EGTA, 10 mM carbachol and 5 mM ionomycin. The maximum fluorescence (F_{max}) was obtained by superfusion with PSS containing 1.2 mM Ca^{2+} and 5 mM ionomycin. Ratios were converted to $[Ca^{2+}]_i$ as described by Grynkiewicz et al [16] using 224 nM as the K_d for Ca^{2+}.

2.4. *Measurement of* Mn^{2+} *influx*

The procedure for the determination of Mn^{2+} influx was the same as described previously [8,17]. Briefly, the fura-2-loaded sublingual cells were attached to a coverslip which had been mounted in a perfusion chamber, and then superfused as described above for $[Ca^{2+}]_i$ determinations. Mn^{2+} influx was monitored according to the following protocol: acini were stimulated for 5 min with 10 μM CCh in a Ca^{2+}-free solution, 10 μM atropine was then added to inhibit the muscarinic receptor, and 5 min later 50 μM Mn^{2+} was added to initiate the quench of the fura-2 fluorescence signal. The fluorescence signals at the Ca^{2+}-insensitive 360 nm excitation wavelength were obtained by collecting the 505 nm emission wavelength.

2.5. *Determination of* IP_3 *and* IP_4 *turnover and 1,4,5-*IP_3 *content*

The generation of IP_3 and IP_4 was determined by anion exchange chromatography using a formate resin column as previously described [2]. Briefly, sublingual acini were labelled with [^{3}H]*myo*-inositol in PSS for 90 min at 37°C. Acini were rinsed 3 times with PSS containing 10 mM unlabeled *myo*-inositol, resuspended in the same medium, and incubated for an additional 60 min. During the final 15 min, 10 mM LiCl was added. Acini were then stimulated with 10 mM CCh and the reaction stopped with an equal volume of ice-cold 10% perchloric acid containing 2.5 g/l phytic acid. Samples were placed on ice for 15 min, then centrifuged at $500 \times g$ for 1 min. 200 ml of 10 mM EDTA (pH 7.4) was added to 800 μl of supernatant. An equal volume of a mixture of Freon:tri-*n*-octylamine (1:1) was added to neutralize and extract the inositol phosphates. Samples of the upper phase were then applied to columns and eluted with the following solutions: 20 ml of water, 15 ml of borax, and 15 ml each of following ammonium formate solutions: 0.2 M, 0.4 M, 0.8 M and 1.0 M. Samples were then collected for scintillation counting and normalized according to protein content.

Since the separation technique for inositol phosphates does not identify the different IP_3 isoforms, 1,4,5-IP_3 content was determined using a radioligand assay kit. To prepare sublingual acini for 1,4,5-IP_3 content determinations, samples were mixed with an equal volume of 1 M ice-cold trichloroacetic acid. Samples were left on ice for ~15 min and then centrifuged at 2000 rpm for 5 min. The supernatant was vortexed with an equal volume of diethyl ether and the ether phase discarded (3 ×). Samples were neutralized with 0.5 M $NaHCO_3$ prior to assaying 1,4,5-IP_3 content.

2.6. *Statistics*

All results are presented as mean ± S.E.M. Comparisons were made using the unpaired Student's *t*-test.

3. RESULTS AND DISCUSSION

Calcium plays a critical role in the muscarinic-evoked secretory response in salivary glands by regulating ion permeabilities, intracellular pH and cell volume [8,19–22]. Release of Ca^{2+} from an intracellular pool, triggered by the second messenger inositol 1,4,5-trisphosphate (IP_3) [23], results in a dramatic increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by a lower sustained phase of elevated $[Ca^{2+}]_i$ produced by Ca^{2+} influx [1,8,15,24]. After agonist stimulation ceases, Ca^{2+} continues to enter the cytoplasm via the Ca^{2+} entry pathway located in the plasma membrane until the intracellular Ca^{2+} store is refilled [7,25]. Thus, muscarinic-induced Ca^{2+} entry is an essential factor in the secretory response in salivary glands. The nature of the transport mechanism involved in this process remains at present poorly defined, although, it is thought that depletion of the intracellular Ca^{2+} pool is required [8,9,11,14,25]. In the present study, we show that inhibiting Ca^{2+} release from the intracellular pool prevents the activation of the Ca^{2+} influx pathway.

3.1. *Inhibition of the muscarinic-stimulated increase in* $[Ca^{2+}]_i$ *with TMB-8 and dantrolene*

TMB-8 and dantrolene (DTL) inhibit the stimulation-induced increase in $[Ca^{2+}]_i$ in muscle, colonic, and fibroblast cells by blocking the release of Ca^{2+} from the sarcoplasmic reticulum and endoplasmic reticulum [26,27,28]. To determine the effects of these inhibitors on muscarinic-stimulated Ca^{2+} mobilization in sublingual mucous acini, cells were loaded with the Ca^{2+}-sensitive dye, fura-2. Stimulation with the muscarinic agonist, carbachol (CCh; 10 mM), induced a rapid increase in $[Ca^{2+}]_i$ of 5.6-fold, from the resting concentration of 51.2 ± 6.7 to 286.7 ± 14.8 nM, followed by a sustained increase of 4.1-fold (207.6 ± 15.1 nM, $n = 10$). Pretreatment with TMB-8 (50 mM) for 2 min almost completely abolished the CCh-induced $[Ca^{2+}]_i$ increase (initial transient = 63.3 ± 4.4 nM, sustained = 58.6 ± 1.5 nM; Fig. 1). Inhibition of the CCh-stimulated $[Ca^{2+}]_i$ increase by TMB-8 was concentration-dependent

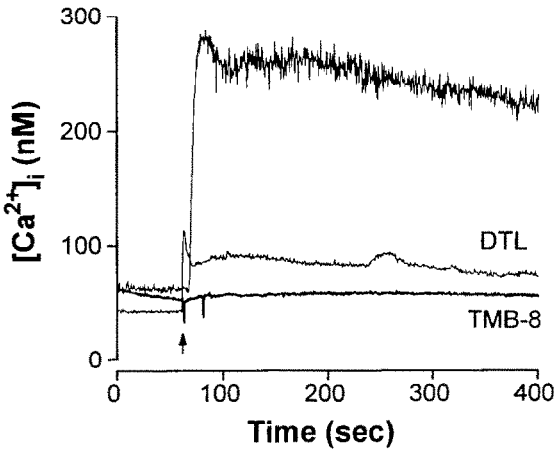

Fig. 1 Effects of TMB-8 and dantrolene on the CCh-induced increase in $[Ca^{2+}]_i$. Rat sublingual acinar cells were loaded with fura-2/AM (2 μM) for 30 min at room temperature, and $[Ca^{2+}]_i$ was determined as described in section 2. Fura-2-loaded acini were stimulated with 10 μM CCh at the time indicated by the arrows. TMB-8 (50 μM) or dantrolene (25 μM; DTL) was added 2 min prior to CCh stimulation. Traces are representative of at least 5 separate experiments using different cell preparations.

($K_{1/2}$ = 11.1 ± 1.8 mM, n = 4; data not shown). DTL (25 mM) was nearly as effective as TMB-8 at blocking the CCh-induced increase in $[Ca^{2+}]_i$ (initial transient = 91.8 ± 10.5 nM; sustained = 75.7 ± 6.9 nM; Fig. 1).

3.2. *TMB-8 and dantrolene block the muscarinic-induced Mn^{2+} influx*

The results shown in Fig. 1 suggest that blocking the CCh-induced release of the intracellular Ca^{2+} pool inhibits Ca^{2+} influx. The data do not exclude the possibility, however, that Ca^{2+} influx *and* Ca^{2+} efflux may have been simultaneously stimulated, resulting in no significant change in the intracellular $[Ca^{2+}]$. Indeed, Zhang et al. [29] have recently shown that the agonist-induced increase of plasma membrane Ca^{2+}ATPase activity can be independent of the rise in the intracellular $[Ca^{2+}]$ in pancreatic acini. Therefore, to distinguish whether receptor stimulation or depletion of the Ca^{2+} pool is required to activate the cation entry pathway, the effects of TMB-8 and DTL on CCh-induced Mn^{2+} influx were monitored. Mn^{2+} influx is thought to occur in several cell types, including salivary acinar cells, via a divalent cation entry pathway activated by depleting the intracellular Ca^{2+} pool [8,9,30]. In the present study, divalent cation influx was monitored by exploiting the ability of Mn^{2+} to quench the fura-2 fluorescence signal (see section 2).

As shown in Fig. 2A, the fura-2 fluorescence quench rate after CCh-stimulation was ~4-fold faster (20.8 ± 3.7%/min) than in unstimulated cells (5.5 ± 0.5%/min). Since atropine, a muscarinic antagonist that was added 5 min prior to Mn^{2+} addition, did not block Mn^{2+} entry, the participation of receptor-operated Ca^{2+} influx was ruled out (see Fig. 2 legend for details). Addition of TMB-8 or DTL two min prior to CCh stimulation abolished the CCh-stimulated quench of the fura-2 signal by Mn^{2+} (TMB-8 = 6.5 ± 1.5%/min, Fig. 2B; DTL = 5.3 ± 0.3%/min, Fig. 2C). Inhibition of the Ca^{2+} release pathway with TMB-8 or DTL prevented both the muscarinic-induced $[Ca^{2+}]_i$ increase (Fig. 1) and the influx of Mn^{2+} (Fig. 2) strongly suggesting that depletion of the intracellular store activated Ca^{2+} influx. These effects were clearly not due to direct blockade of

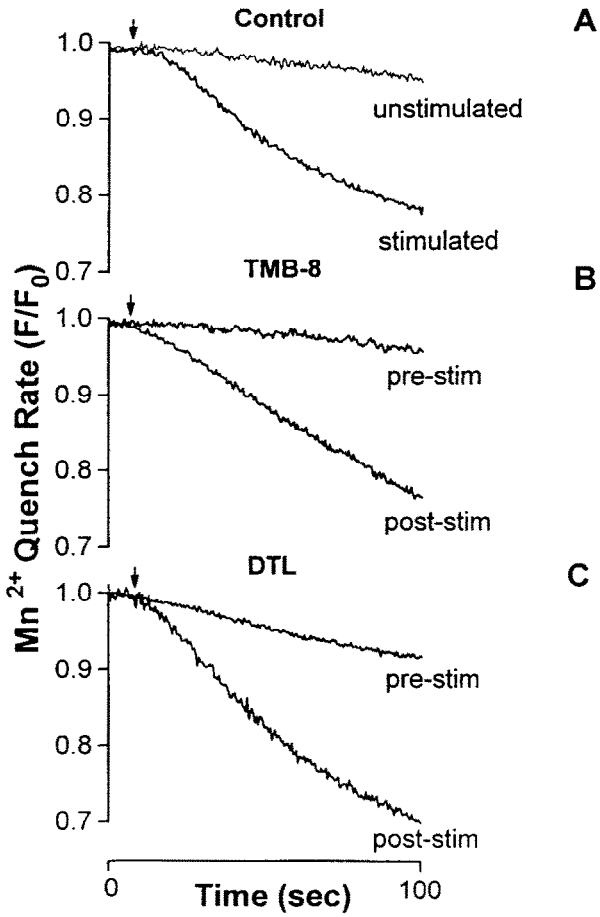

Fig 2. Effects of TMB-8 and dantrolene on CCh-activated quench of fura-2 fluorescence by Mn^{2+} influx. Fura-2-loaded acini were stimulated in a Ca^{2+}-free solution with 10 μM CCh for 5 min, then 10 μM atropine was added to inhibit the muscarinic receptor. Five minutes later in the absence of extracellular Ca^{2+}, 50 μM $MnCl_2$ was added to quench the fura-2 fluorescence signal at the time indicated by the arrows. (A = control) Acini were either unstimulated or stimulated with CCh, stimulated is different from unstimulated by $P < 0.01$. (B = TMB-8) Pre-stimulation, 50 μM TMB-8 was added 2 min *before* CCh; post-stimulation, 50 μM TMB-8 was added 8 min *after* CCh stimulation, pre-stimulated is different from post-stimulated by $P < 0.01$. (C = dantrolene (DTL)) Pre-stimulation, 25 μM DTL was added at 2 min *before* CCh stimulation; post-stimulation, 25 μM DTL was added 8 min *after* CCh stimulation, pre-stimulated is different from post-stimulated by $P < 0.01$. Traces are representative of at least 4 separate experiments using different preparations. Quenching of the fura-2 fluorescence signal by Mn^{2+} is expressed as the fluorescence divided by the original starting fluorescence.

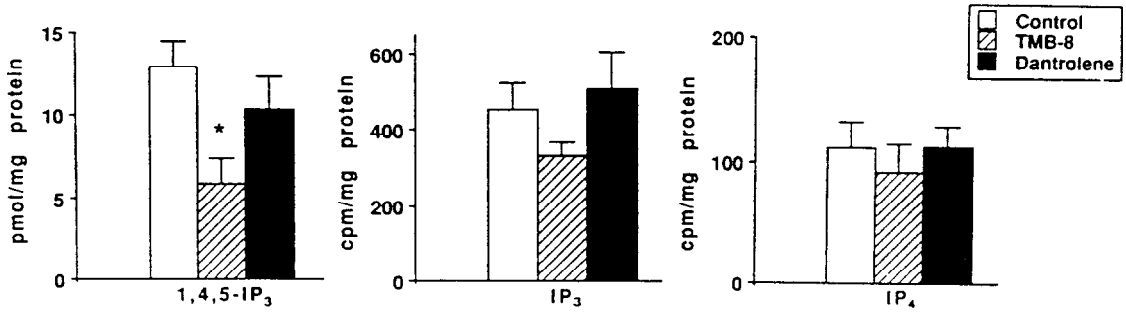

Fig 3 Effects of TMB-8 and dantrolene on the response of 1,4,5-IP_3, IP_3 and IP_4 to muscarinic stimulation in rat sublingual acini. Ten μM carbachol was added to [^{3}H]*myo*-inositol-loaded acini to monitor IP_3 and IP_4 turnover in the presence or absence of TMB-8 and DTL, and the reaction stopped after 30 s Pretreatment with vehicle only, TMB-8 or DTL for two minutes had no significant effect on IP_3 and IP_4 (IP_3: vehicle = 88.0 ± 12.6 cpm/mg protein, TMB-8 = 88.5 ± 22 9, DTL = 73.5 ± 15.3, n = 4, IP_4 vehicle = 52.0 ± 6.3 cpm/mg protein, TMB-8 = 71 1 ± 16 5, DTL = 69.5 ± 12.8, n = 4). 1,4,5-IP_3 content was determined using a radioligand assay kit (see section 2) Pretreatment with vehicle only, TMB-8 or DTL for two minutes had no significant effect on 1,4,5-IP_3 content (1,4,5-IP_3, vehicle = 1.9 ± 0 3 pmol/mg protein, TMB-8 = 2.2 ± 0.3, DTL = 1 9 ± 0.3, n = 3). Each bar illustrates the average response from at least four separate preparations (mean ± SEM). Significantly different from control *,$P < 0.05$.

the divalent cation entry mechanism since addition of either TMB-8 or DTL *after* CCh stimulation did not significantly influence the muscarinic-induced quench of fura-2 fluorescence (TMB- 8 = 15.6 ± 0.6%/min, Fig. 2B; DTL = 23.7 ± 5.3%/min, Fig. 2C). Therefore, CCh-induced Ca^{2+} entry in sublingual mucous acini is apparently dependent on the filling state of the intracellular Ca^{2+} store and is not directly coupled to receptor activation.

3.3. *Effects of TMB-8 and dantrolene on IP_3 and IP_4 generation*

Muscarinic receptor activation in salivary gland acinar cells induces an increase in the cellular content of inositol phosphates [31,32]. 1,4,5-IP_3 then triggers Ca^{2+} release from the intracellular Ca^{2+} store [32], whereas, 1,3,4,5-IP_4, and possibly 1,4,5-IP_3 or both 1,4,5-IP_3 and 1,3,4,5-IP_4 acting in concert, has been suggested to activate Ca^{2+} influx (see [4]). The data in Fig. 2 indicate that TMB-8 and DTL do not directly block the Ca^{2+} entry mechanism; however, this study did not assess the influence of these agents on receptor activation and the resulting generation of inositol phosphates. To explore the possibility that TMB-8 and DTL disturb the coupling of the muscarinic receptor with the generation of inositol phosphates, cellular IP_3, IP_4 and 1,4,5-IP_3 contents were determined. Inositol phosphate turnover was determined after 30 s of stimulation when much of the increase in IP_3 content is due to the generation of the 1,4,5-IP_3 isoform [31]. CCh stimulation resulted in a 5.5-fold increase in the IP_3 level, a 2.3-fold increase in the IP_4 level and 7.2-fold increase in 1,4,5-IP_3 content (Fig. 3). Pretreatment with 50 μM TMB-8 or 25 μM DTL for 2 min did not significantly alter stimulation-induced IP_3 and IP_4 production, although TMB-8, but not DTL, did inhibit 1,4,5-IP_3 level by ~55% (Fig. 3). The observation that inositol phosphate generation is nearly normal in the presence of TMB-8 and DTL indicates that receptor coupling is intact, and in fact, strongly suggests that neither IP_3 nor IP_4 directly regulate Ca^{2+} influx as previously proposed [4].

3.4. *Thapsigargin-induced Ca^{2+} release and Mn^{2+} influx in the presence of TMB-8*

The Ca^{2+}ATPase inhibitor thapsigargin is thought to release Ca^{2+} from the same intracellular site as the 1,4,5-IP_3-sensitive Ca^{2+} store [33]. Consistent with this hypothesis, stimulation-induced increases in $[Ca^{2+}]_i$ with CCh and thapsigargin were not additive (Fig. 4), suggesting that CCh and thapsigargin release Ca^{2+} from the same intracellular Ca^{2+} store in sublingual mucous acini. The Ca^{2+} release induced by inhibition of Ca^{2+}-ATPase with thapsigargin is apparently mediated by passive leakage and not by the IP_3-activated Ca^{2+} channel [33]. To further examine the effects of TMB-8 on the depletion of the intracellular Ca^{2+} store and the subsequent activation of the Ca^{2+} influx mechanism, we examined Ca^{2+} release and Mn^{2+} influx induced by thapsigargin. Thapsigargin (2 μM) induced an increase in $[Ca^{2+}]_i$ from 31.8 ± 7.8 nM to 298.9 ± 25.2 nM (Fig. 5A) comparable to the $[Ca^{2+}]_i$ increase stimulated by CCh (see Fig. 1). TMB-8 did not inhibit the thapsigargin-induced $[Ca^{2+}]_i$ increase (Fig. 5A, 283.3 ± 30.7 nM).

Depletion of the intracellular Ca^{2+} store with thapsigargin markedly enhanced the quench of fura-2 by Mn^{2+} (30.3 ± 5.2%/min, Fig. 5B). TMB-8 had no effect on this process (28.8 ± 3.7%/min, Fig. 5B). Since the Ca^{2+} released by thapsigargin is from the same intracellular pool as the 1,4,5-IP_3-releasable Ca^{2+} store (Fig. 4), it seems likely that thapsigargin-induced Ca^{2+} influx is mediated by the emptying of the same Ca^{2+} store that is activated by CCh. Taken together, these results suggest that TMB-8 and DTL do not interfere with divalent cation influx by inhibiting the generation of inositol phosphates or by blocking a step distal to depletion of the intracellular Ca^{2+} pool. TMB-8 and DTL thus pre-

vent intracellular Ca^{2+} mobilization and Mn^{2+} entry by inhibiting the 1,4,5-IP_3-sensitive, Ca^{2+} release mechanism in rat sublingual mucous acini. These data therefore provide the first direct evidence in salivary acinar cells that the depletion of the intracellular Ca^{2+} pool acts to induce Ca^{2+} influx. These results are comparable to observations made in lacrimal acinar cells [34] and portal vein smooth muscle cells [35] where heparin, an inhibitor of the 1,4,5-IP_3 receptor, was used to block activation of the Ca^{2+} influx mechanism. In contrast, Ca^{2+} influx in ileum smooth muscle cells has been shown to be independent of the emptying of the IP_3-releasable, intracellular pool [36] suggesting that the Ca^{2+} entry mechanism possibly senses the Ca^{2+} transient rather than the filling status of the Ca^{2+} stores.

In summary, previous studies suggested that Ca^{2+} depletion of the intracellular pool was sufficient to activate Ca^{2+} entry. These studies examined this process by monitoring divalent cation uptake *after* emptying the intracellular Ca^{2+} pool. In the present study, we have directly shown, by inhibiting the agonist-induced emptying of the intracellular Ca^{2+} store with TMB-8 and DTL, that pool depletion is necessary to activate the divalent cation influx mechanism. The effects of these agents were not due to direct inhibition of the divalent cation uptake pathway (Fig. 2), repression of inositol phosphate formation (Fig. 3) or related to interference of divalent cation influx at a step distal to depletion of the intracellular Ca^{2+} pool (Fig. 5). Therefore, the observation that divalent cation influx is prevented by direct inhibition of the intracellular Ca^{2+} release mechanism supplies clear evidence that Ca^{2+} uptake in sublingual acini is regulated by the filling state of the 1,4,5-IP_3-releasable intracellular Ca^{2+} store.

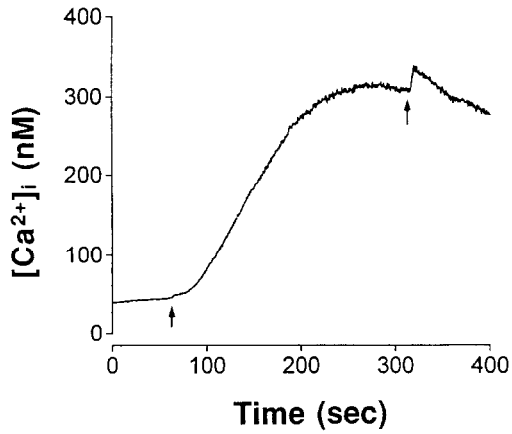

Fig. 4. Thapsigargin and carbachol release Ca^{2+} from the same intracellular pool. Fura-2-loaded acini were stimulated with 2 μM thapsigargin at the time indicated by the first arrow. Four minutes later 10 μM carbachol was added at the second arrow. The trace is representative of 6 separate experiments using different preparations.

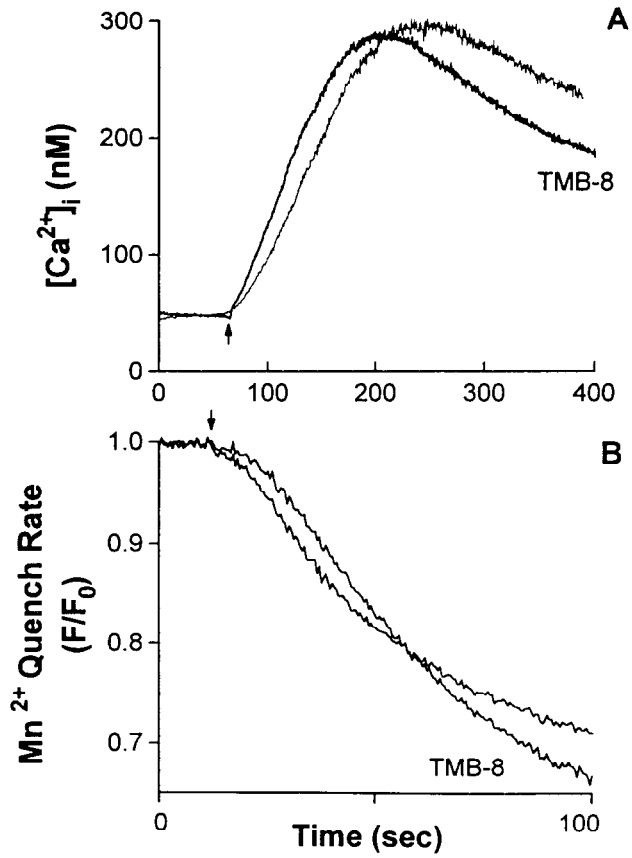

Fig. 5. Effects of TMB-8 on the thapsigargin-induced increase in intracellular $[Ca^{2+}]_i$ and the quench of fura-2 fluorescence by Mn^{2+} influx. (A) Fura-2-loaded acini were stimulated with 2 μM thapsigargin at the time indicated by the arrow. Fifty μM TMB-8 was added 2 min before thapsigargin. Traces are representative of at least 4 separate experiments using different preparations. (B) Fura-2-loaded acini were stimulated with 2 μM thapsigargin for 10 min in a Ca^{2+}-free solution in the presence or absence of TMB-8 added 2 min before thapsigargin, and then 50 μM $MnCl_2$ was added to quench the fura-2 fluorescence signal at the time indicated by the arrow. Quenching of the fura-2 fluorescence signal by Mn^{2+} is expressed as the fluorescence divided by the original starting fluorescence. Traces are representative of at least 5 separate experiments using different preparations.

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