Characterization of the Calcium Signaling System in the Submandibular Cell Line SMG-C6 (44567)

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Abstract. **Establishment of salivary cell lines retaining normal morphological and physiological characteristics is important in the investigation of salivary cell function. A submandibular gland cell line, SMG-C6, has recently been established. In the present study, we characterized the phosphoinositide (PI)-Ca^{2+} signaling system in this cell line. Inositol 1,4,5-trisphosphate(1,4,5-IP_3) formation, as well as Ca^{2+} storage, release, and influx in response to muscarinic, α_1-adrenergic, P2Y-nucleotide, and cytokine receptor agonists were determined. Ca^{2+} release from intracellular stores was strongly stimulated by acetylcholine (ACh) and ATP, but not by norepinephrine (NA), epidermal growth factor (EGF), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα). Consistently, 1,4,5-IP_3 formation was dramatically stimulated by ACh and ATP. ACh-stimulated cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ increase was inhibited by ryanodine, suggesting that the Ca^{2+}-induced Ca^{2+} release mechanism is involved in the ACh-elicited Ca^{2+} release process. Furthermore, ACh and ATP partially discharged the IP_3-sensitive Ca^{2+} store, and a subsequent exposure to thapsigargin (TG) induced further $[Ca^{2+}]_i$ increase. However, exposure to TG depleted the store and a subsequent stimulation with ACh or ATP did not induce further $[Ca^{2+}]_i$ increase, suggesting that ACh and ATP discharge the same storage site sensitive to TG. As in freshly isolated submandibular acinar cells, exposure to ionomycin and monensin following ACh or TG induced further $[Ca^{2+}]_i$ increase, suggesting that IP_3-insensitive stores exist in SMG-C6 cells. Ca^{2+} influx was activated by ACh, ATP, or TG, and was significantly inhibited by La^{3+}, suggesting the involvement of store-operated Ca^{2+} entry (SOCE) pathway. These results indicate that in SMG-C6 cells: (i) Ca^{2+} release is triggered by muscarinic and P2Y-nucleotide receptor agonists through formation of IP_3; (ii) both the IP_3-sensitive and -insensitive Ca^{2+} stores are present; and (iii) Ca^{2+} influx is mediated by the store-operated Ca^{2+} entry pathway. We conclude that Ca^{2+} regulation in SMG-C6 cells is similar to that in freshly isolated SMG acinar cells; therefore, this cell line represents an excellent SMG cell model in terms of intracellular Ca^{2+} signaling.**

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The salivary gland is an excellent model for study of protein, fluid, and electrolyte secretion. Saliva secretion is a two-phase process. The primary saliva is produced by acinar cells and modified by the duct system through a variety of ion transport processes (1). Unlike many major organs, salivary glands are small and incapable of providing large amounts of cells for experimental use. Therefore, the establishment and characterization of immortalized salivary cell lines are of key importance for studying mechanisms of saliva formation and secretion *in vitro*. Although a number of immortalized ductal cell lines have been established and widely used in investigations of ion modification mechanisms in the duct system (2), very few immortalized salivary acinar cell lines are available. This is due primarily to the unique characteristics of acinar cells, which are highly differentiated so that their ability to maintain growth and differentiation, and especially maintenance of their acinar characteristics, is believed to be minimal.

Recently, several rat salivary cell lines, Par-C5 and C10 (PG), and SMG-C6 and C10, have been established using a

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plasmid containing a replication-defective simian virus (SV40) genome, and initial characterization suggests that they are of acinar origin (3, 4). These cell lines maintain the characteristics of acinar cells, including moderate amounts of secretory granules and cellular polarization. Furthermore, all these cell lines express β-adrenergic receptors coupled to cAMP formation, and muscarinic and P2Y receptors coupled to the phosphoinositide (PI) signal transduction pathway (3, 4). Electrophysiological studies indicate that transcellular Cl^- and Na^+ movement is regulated by P2Y and muscarinic agonists in SMG-C6 (5) and Par-C10 cells (6). Recent studies (3–6) have suggested that these cell lines represent excellent cell models for studying fluid and ion transport processes. However, it remains unclear whether these cell lines have similar intracellular signaling systems to freshly isolated salivary cells. Therefore, the purpose of the present study was to characterize the primary signaling system regulating fluid and electrolyte secretion, the PI-Ca^{2+} system, in SMG-C6 cells.

Materials and Methods

Materials. ACh, ATP, bovine serum albumin (BSA) (type V), EGTA, HEPES, ionomycin, NA, and ryanodine were from Sigma (St. Louis, MO). 2-Aminoethoxydiphenyl borate (2APB), IL-6, TG, and TNF-α were purchased from CalBiochem (La Jolla, CA). Basal Eagle Medium amino acids (BEM) were from GIBCO (Grand Island, NY). 1,2-*bis*(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid (BAPTA) and Fura-2/acetoxyl ester (AM) was from Molecular Probes (Eugene, OR). All other chemicals used were of the highest grade available.

Solutions. Physiological salt solution (PSS) consisting of (in m*M*): 110 NaCl, 25 $NaHCO_3$, 20 Hepes, 10 glucose, 5.4 KCl, 0.4 KH_2PO_4, 0.33 NaH_2PO_4, 1.2 $CaCl_2$, 0.8 $MgSO_4$, and 0.01% (w/v) BSA. Solution pH was adjusted to 7.4 after gassing with 95%O_2/5% CO_2 for 45 min. For Ca^{2+}-free PSS, $CaCl_2$ was omitted, and 30 μ*M* BAPTA was added to adjust medium Ca^{2+} concentration $[Ca^{2+}]_o$ to 100 n*M*. The nominally Ca^{2+}-free medium contains 800 n*M* ionic Ca^{2+} (12). Using high-concentration (1 m*M*) EGTA to chelate Ca^{2+} induces depletion of intracellular Ca^{2+}. Incubation of SMG-C6 cells in the Ca^{2+}-free medium containing 1 m*M* EGTA for 3–5 min reduced the basal $[Ca^{2+}]_i$ from 73 ± 4 n*M* (n = 19) to 26 ± 3 n*M* (n = 10; $P < 0.001$). Moreover, the EGTA-containing solution depleted the IP_3-sensitive Ca^{2+} store, resulting in a reduction in Ca^{2+} release response. The $[Ca^{2+}]_i$ increase stimulated by 10 μ*M* ACh was 126 ± 15 n*M* (n = 10) following incubation in Ca^{2+}-free medium containing 30 μ*M* BAPTA and 24 ± 2 n*M* (n = 5; $P < 0.001$) in EGTA (1 m*M*)-containing medium for 5 min. Furthermore, an important advantage of using BAPTA as a Ca^{2+} chelator is that BAPTA, unlike EGTA, has a much (10,000-fold) higher affinity for Ca^{2+} than for Mg^{2+} (7). Thus, using BAPTA as a Ca^{2+} chelator will not affect Mg^{2+} concentration that is a critical factor for cell function including Ca^{2+} mobilization. Therefore, unless specifically indicated, most experiments in Ca^{2+}-free medium were performed in a Ca^{2+}-free medium containing 30 μ*M* BAPTA.

Cell Culture. The rat SMG cell line SMG-C6 established by Quissell *et al.* (3) was routinely grown at 37°C in a humidified 5% CO_2 atmosphere on plastic tissue culture T-75 flasks in DMEM/F12 (1:1) medium containing 2.5% FBS, 20 n*M* sodium selenite, 5 μg/ml transferrin, 1.1 μ*M* hydrocortisone, 0.1 μ*M* retinoic acid, 2 n*M* T3, 8.4 ng/ml cholera toxin, 5 μg/ml insulin, 80 ng/ml EGF, 5 m*M* glutamine, 50 μg/ml gentamicin sulfate, 1 μg/ml amphotericin B, 100 μg/ml penicillin G, and 100 U/ml streptomycin and trace element mixture. The medium was changed twice weekly. Subculture was conducted by washing the cells with fresh, serum-free medium and adding 0.25% trypsin and 1 m*M* EDTA for 5 min. Trypsinized cells were counted, centrifuged, resuspended in fresh medium and seeded to new flasks. For the experiments, the confluent cells were trypsinized, rinsed twice with PSS, resuspended in fresh PSS containing 1% newborn bovine serum and 2% trypsin inhibitors, and incubated at 37°C for 2–3 hr before use. The cell viability was routinely tested by trypan blue exclusion.

Determination of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined using the Ca^{2+} sensitive fluorescent probe Fura-2 as previously described (8, 9). Briefly, trypsinized SMG-C6 cells were incubated at 37°C in PSS containing 2% trypsin inhibitor for 2 hr and then loaded with Fura-2 by incubation with 2 μ*M* Fura-2/AM for 20 min at 37°C in PSS containing 0.01% BSA. After loading, the cells were rinsed twice with PSS containing 0.01% BSA and kept at 37°C. For $[Ca^{2+}]_i$ measurements, a 2-ml aliquot of Fura-2-loaded cells was quickly pelleted, resuspended in fresh medium (1.5×10^6 cells/ml) containing 0.01% BSA, and placed in a 4-ml cuvette. Fura-2 fluorescence was monitored with a PTI Deltascan fluorometer (PTI Inc., S. Brunswick, NJ). The excitation wavelengths used were 340 and 380 nm, and emission wavelength was 505 nm. Calibration of $[Ca^{2+}]_i$ was performed for each measurement trace as previously described (8–10). Briefly, 1 m*M* $CaCl_2$ and 50 μ*M* ionomycin were sequentially added to obtain the limiting ratio for Ca^{2+} saturated form (R_{max}) of Fura-2. Then, 0.0005% digitonin and 10 m*M* EGTA were sequentially added to obtain the limiting ratio for the unbound form (R_{min}) of Fura-2. Fluorescence ratios of the 340/380 nm excitation and 505 nm emission were converted to $[Ca^{2+}]_i$ according to Grynkiewicz *et al.* (11) using 224 n*M* as K_d of Fura-2 for Ca^{2+} at 37°C.

Measurement of Ca^{2+} Influx. Ca^{2+} influx through SOCE activated by depletion of the IP_3-sensitive intracellular store was measured by a method described previously (7, 13–14). Fura-2-loaded cells were stimulated with indicated reagents in Ca^{2+}-free medium for the indicated time, and 1 m*M* Ca^{2+} was then added. The initial portion (first 15 sec) of $[Ca^{2+}]_i$ changes after addition of Ca^{2+} was used to calculate Ca^{2+} influx rate (n*M*/min).

Measurement of 1,4,5-IP_3. 1,4,5-IP_3 formation was determined as previously described (13, 15, 16) using a

radioimmunoassay kit (Amersham, Arlington Heights, IL). Briefly, cells were incubated in PSS at 37°C, and stimulated with ACh or ATP for 0–5 min. The reaction was terminated by adding an equal volume of 1 *M* ice-cold trichloroacetic acid. Samples were left on ice for 15 min, then centrifuged at 6000 rpm in a microfuge at 4°C for 15 min. Trichloroacetic acid was removed by extracting with diethyl ether four times. Samples were then neutralized to pH 7.0 with 0.5 *M* $NaHCO_3$, and 1,4,5-IP_3 concentration was determined according to the procedure recommended by Amersham.

Data Presentation and Statistics. All results are presented as means ± SEM of separate determinations using different cell preparations. Comparisons were made using Student *t* test or the analysis of variance followed by the Newman-Keuls test. *P*-values < 0.05 were considered significant.

Results

$[Ca^{2+}]_i$ Increase in Response to Stimuli. The basal level of $[Ca^{2+}]_i$ in unstimulated SMG-C6 cells was 82 ± 4 n*M* (*n* = 37) with a range of 50 n*M* to 120 n*M*. Stimulation with 10 μ*M* ACh elicited a sharp initial increase in $[Ca^{2+}]_i$. The peak increase was 160 ± 11 n*M* (*n* = 8) in the presence of extracellular Ca^{2+} (from 87 ± 11 n*M* to 247 ± 4 n*M*) and 142 ± 19 n*M* (*n* = 6) in Ca^{2+}-free medium (from 63 ± 8 n*M* to 204 ± 25 n*M*) (Fig. 1A). The sustained increase was 56 ± 5 (*n* = 8) in the presence of external Ca^{2+} and 11 ± 3 n*M* (*n* = 6) in Ca^{2+}-free medium (Fig. 1A).

Stimulation with 2 m*M* ATP, a P2Y-nucleotide receptor agonist, triggered a rapid and large increase in $[Ca^{2+}]_i$. The initial increase was 462 ± 27 n*M* (*n* = 5) in the presence of extracellular Ca^{2+} (from 70 ± 9 n*M* to 532 ± 63 n*M*) and 327 ± 24 n*M* (*n* = 5) in Ca^{2+}-free medium (from 60 ± 7 n*M* to 387 ± 28 n*M*) (Fig. 1B). The sustained $[Ca^{2+}]_i$ increase after ATP was 49 ± 4 n*M* (*n* = 5) in the presence of external Ca^{2+} and 21 ± 2 n*M* in Ca^{2+}-free medium (Fig. 1B). To elucidate which subtype of P2Y-receptors mediates this response to ATP, $[Ca^{2+}]_i$ mobilization stimulated by ATP, UTP, and ADP were compared. The initial $[Ca^{2+}]_i$ increases were identical in response to ATP (100%) and UTP (108 ± 1%, *n* = 5; *P* > 0.05). However, ADP did not induce a substantial response (6.7 ± 0.5%, *n* = 4). These results suggest that the ATP-induced Ca^{2+} response is likely mediated by $P2Y_2$ receptors.

On the other hand, stimulation of α_1-adrenergic receptors with 10μ*M* NA did not result in a substantial increase in $[Ca^{2+}]_i$. The initial increase was only 34 ± 5n*M* in PSS (*n* = 5), and the sustained increase was 31 ± 4n*M*. In Ca^{2+}-free medium, the NA-induced increase was minimal. The initial increase was 16 ± 4 n*M*, and the sustained increase was 8 ± 2 n*M* (*n* = 5) (Fig. 1C).

As shown in Figure 1D, exposure of SMG-C6 cells to the endoplasmic Ca^{2+}-ATPase inhibitor TG (3 μ*M*) induced a gradual increase in $[Ca^{2+}]_i$. The peak increase was 184 ± 19 n*M* (*n* = 5), and the sustained increase at 5 min was 67 ± 8 n*M* (*n* = 5) in PSS. In Ca^{2+}-free medium, a similar peak increase was observed (174 ± 25 n*M*, *n* = 5), but the sustained increase was much smaller (29 ± 4 n*M*, *n* = 5) (Fig. 2). Exposure to 100 n*M* EGF, 200 ng/ml of IL-6 or

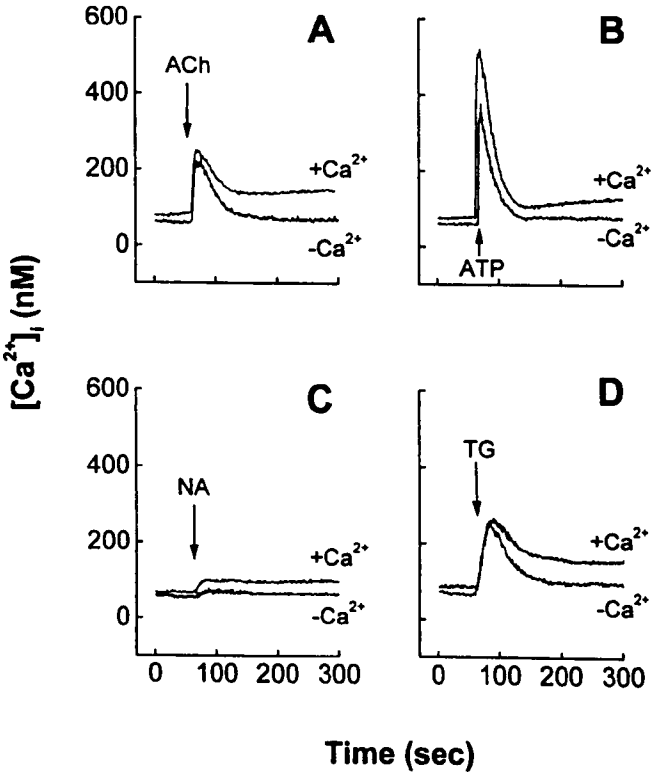

Figure 1. $[Ca^{2+}]_i$ response to acetylcholine, ATP, norepinephrine, and thapsigargin. SMG-C6 cells were loaded with Fura-2 in physiological salt solution at 37°C, and $[Ca^{2+}]_i$ was determined in the presence (+Ca^{2+}) or absence (– Ca^{2+}) of 1.2 m*M* Ca^{2+}. At the time indicated by arrows, 10 μ*M* acetylcholine (ACh), 2 mm ATP (ATP), 10 μ*M* norepinephrine (NA), or 3 μ*M* thapsigargin (TG) was added. Traces are representative of at least five separate experiments.

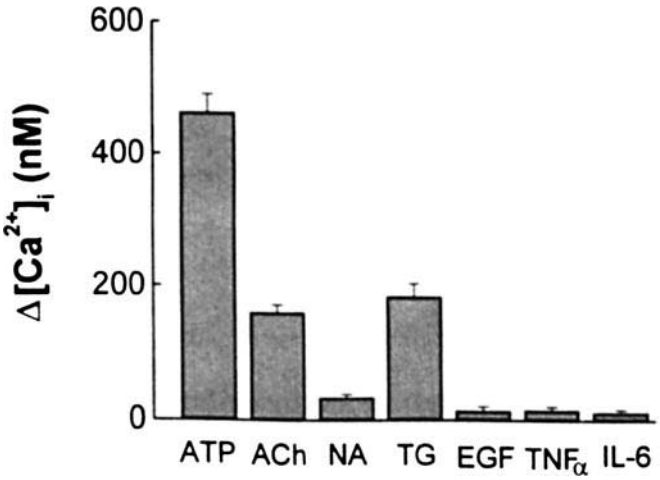

Figure 2. Initial $[Ca^{2+}]_i$ increase in response to various agonists. SMG-C6 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was measured in physiological salt solution containing 1.2 m*M* Ca^{2+}. $[Ca^{2+}]_i$ increase was triggered by addition of 2 m*M* ATP (ATP), 10 μ*M* acetylcholine (ACh), 10 μ*M* norepinephrine (NA), 3 μ*M* thapsigargin (TG), 100 ng/ml of epidermal growth factor (EGF), tumor necrosis factor-α (TNF-α or interleukin-6 (IL-6). Values are means ± SEM of at least five separate experiments.

50 ng/ml of TNF-α did not induce a notable $[Ca^{2+}]_i$ increase (Fig.2).

To test whether ATP and ACh stimulate different intracellular signaling systems that mediate the Ca^{2+} mobilization responses, Fura-2-loaded SMG-C6 cells were simultaneously stimulated with 10 μ*M* ACh and 2 m*M* ATP in Ca^{2+}-free medium. The initial increase in $[Ca^{2+}]_i$ in response to this combined stimulation was 203 ± 10 n*M* (n = 5), which was greater than the increase in response to ACh, but significantly smaller than ATP-stimulated increase (P < 0.002), suggesting that the same signaling system mediates the responses to muscarinic and P2Y agonists.

1,4,5-IP_3 Formation in Response to ACh and ATP. To examine the relationship between 1,4,5-IP_3 formation and initial $[Ca^{2+}]_i$ increase, the time-course of ACh- and ATP-stimulated 1,4,5-IP_3 formation was measured. As shown in Figure 3, 1,4,5-IP_3 content before stimulation was 1.3 ± 0.5 pmoles/10^6 cells (n = 4). Stimulation with 10 μ*M* ACh or 2 m*M* ATP induced a similar time-dependent increase. At 15 sec post-ACh stimulation, the cellular 1,4,5-IP_3 concentration was 14.5 ± 0.5 pmoles/10^6 cells (n = 3), an 11-fold increase. The ATP-stimulated increase in 1,4,5-IP_3 was significantly greater (20.2 ± 2.0 pmoles/10^6 cells (n = 3) (P < 0.05 vs. that stimulated by ACh)). Thereafter, 1,4,5-IP_3 concentrations were maintained at a stable level, ~6–8-fold higher than the unstimulated level (Fig.3).

To determine whether the combination of ACh and ATP stimuli induces a potentiation of 1,4,5-IP_3 formation, cells were simultaneously stimulated with 10 μ*M* ACh and 2 m*M* ATP, as described above. The formation of 1,4,5-IP_3 in response to both ACh and ATP was 12.8 ± 0.8 pmoles/10^6 cells (n = 3) at 30 sec poststimulation (not shown), identical to the responses stimulated by these agonists separately. These results suggest that both muscarinic and P2Y nucleotide receptors are coupled to the same intracellular signaling pathway.

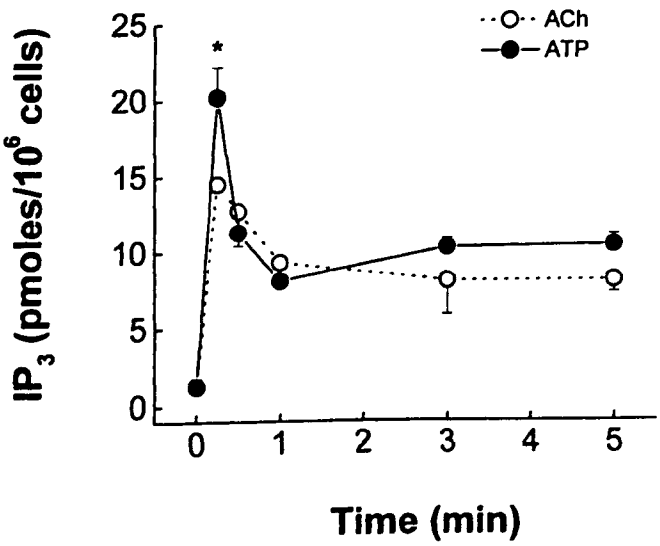

Figure 3. 1,4,5-IP_3 formation in response to acetylcholine and ATP. SMG-C6 cells were incubated in physiological salt solution (PSS) at 37°C and stimulated with 10 μ*M* acetylcholine (ACh) or 2 m*M* ATP (ATP) for 0, 0.25, 0.5, 1, 3, and 5 min. Values are means ± SEM of 3–5 separate experiments using different cell preparations. *P < 0.05 vs ACh-stimulated 1,4,5-IP_3 formation.

Ca^{2+} Release Mechanism. It has been suggested that IP_3-triggered Ca^{2+} release is mediated by two types of receptor channels, the IP_3R channel, and the ryanodine receptor (RyR) channel through the process known as Ca^{2+}-induced Ca^{2+} release (CICR) in pancreatic (17) and salivary cells (18, 19). To elucidate whether the release of IP_3-sensitive Ca^{2+} stores in SMG-C6 cells is through these mechanisms, we examined Ca^{2+} mobilization in response to ACh in the presence of 2-aminoethoxydiphenyl borate (2APB), an inhibitor of IP_3R-mediated Ca^{2+} release (20), or RyR inhibitor ryanodine (18, 21). As shown in Figure 4, ACh (10 μ*M*) stimulation of SMG-C6 cells preincubated with vehicle, dimethylsulfoxide (DMSO), in Ca^{2+}-free medium elicited a rapid initial $[Ca^{2+}]_i$ increase (127 ± 9 n*M*, n = 6) (Fig. 4A). Pretreatment with 100 μ*M* 2APB for 3 min

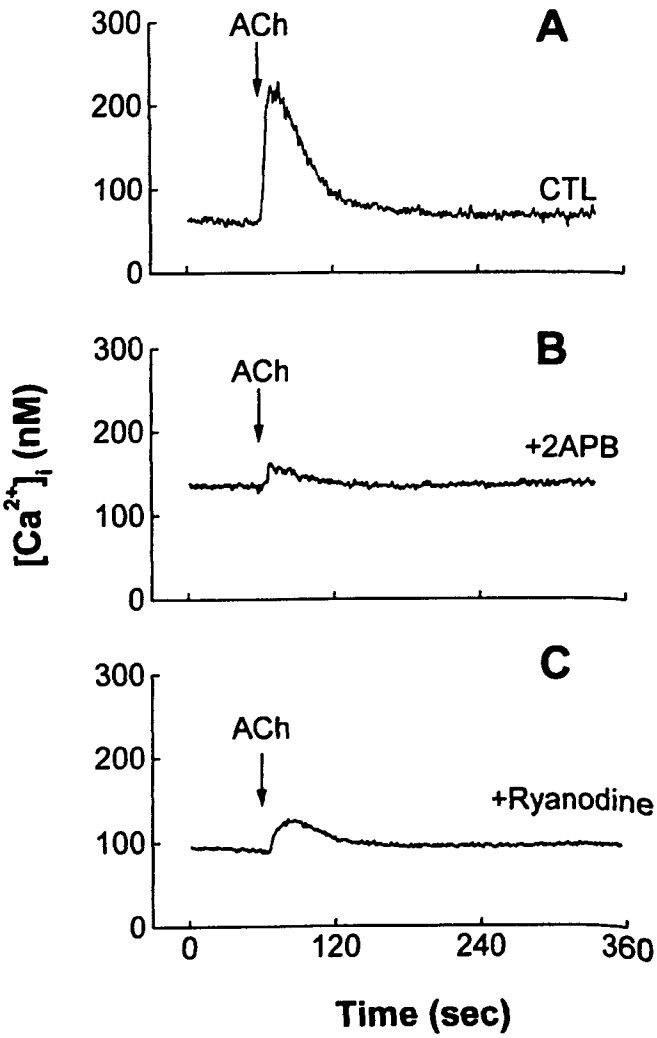

Figure 4. Effects of 2APB and ryanodine on acetylcholine-stimulated $[Ca^{2+}]_i$ increase. SMG-C6 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was determined in Ca^{2+}-free medium. Cells were preincubated with (A) DMSO for 3 min (CTL), (B) 100 μ*M* 2APB for 3 min, or (C) 200 μ*M* ryanodine for 10 min. At the time indicated by arrows, 10 μ*M* acetylcholine (ACh) was added. Traces are representative of at least four separate experiments using different cell preparations.

prevented the ACh-induced $[Ca^{2+}]_i$ increase (27 ± 5 n*M*, *n* = 4; $P < 0.001$) (Fig. 4B). Interestingly, preincubation with 200 μ*M* ryanodine for 10 min also dramatically inhibited the ACh-stimulated initial $[Ca^{2+}]_i$ increase (38 ± 3 n*M*, *n* = 4; $P < 0.001$) (Fig. 4C). These results suggest that both RyR and the CICR mechanism are involved in the process of discharging the IP_3-sensitive Ca^{2+} store.

Ca^{2+} Stores. To examine whether the ACh-, ATP-, and TG-induced initial $[Ca^{2+}]_i$ increases are due to Ca^{2+} release from the same Ca^{2+} storage site, the IP_3-sensitive store, we sequentially stimulated the cells with ACh and TG, ACh and ATP, as well as ATP and TG in Ca^{2+}-free medium. As shown in Figure 5A, after stimulation with 10 μ*M* ACh, a subsequent exposure to 3 μ*M* TG induced another Ca^{2+} release peak. On the other hand, exposure to TG first resulted in a large Ca^{2+} release, and subsequent stimulation with ACh did not induce a substantial further release (Fig. 5B), indicating that the Ca^{2+} store was almost depleted by TG. In a similar pattern, after stimulation with ACh, subsequent stimulation with ATP elicited another Ca^{2+} release peak (Fig. 5C). However, stimulation in reverse order

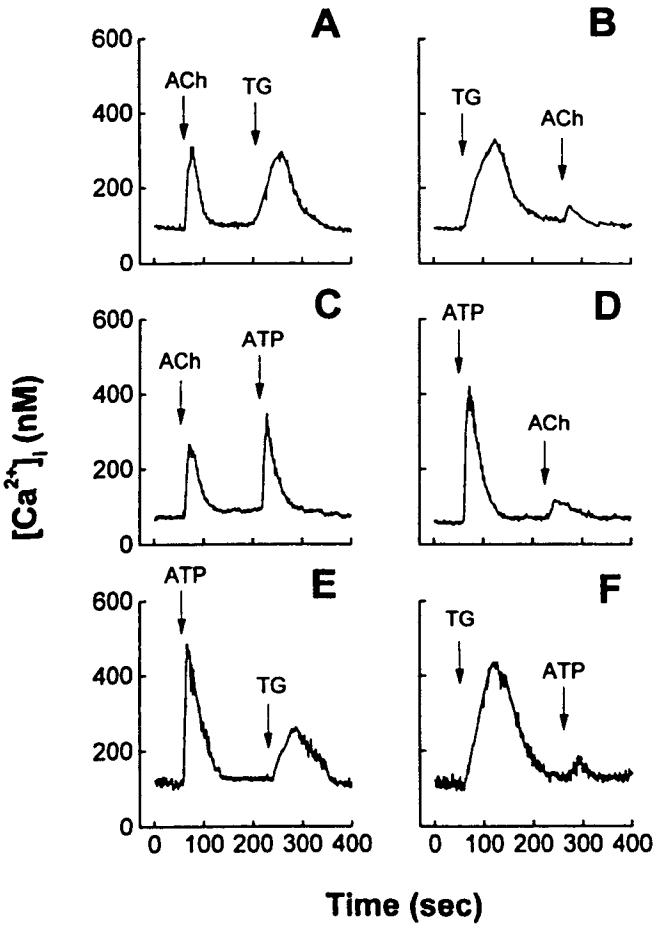

Figure 5. Ca^{2+} release induced by sequential exposure to acetylcholine, thapsigargin, and ATP. SMG-C6 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was determined in Ca^{2+}-free medium containing 30 μ*M* BAPTA. At the time indicated by arrows, 10 μ*M* acetylcholine (ACh), 2 m*M* ATP (ATP), and 3 thapsigargin (TG) were added. Traces are representative of at least five separate experiments using different cell preparations.

(i.e., with ATP first) triggered a large Ca^{2+} release, and subsequent ACh stimulation only induced a small further release (Fig. 5D), suggesting that ACh at this concentration (10 μ*M*) did not deplete the store, and ATP (2 m*M*) induced further release from the store. Interestingly, whereas stimulation with ATP triggered a large release, a subsequent exposure to TG still induced a considerable additional $[Ca^{2+}]_i$ increase (Fig. 5E). Furthermore, when cells were exposed to TG first, the subsequent ATP stimulation did not induce any significant further $[Ca^{2+}]_i$ increase (Fig. 5F). These results suggest either that the TG-sensitive store is larger than the ATP-sensitive one, or that ATP alone cannot deplete the store.

Previous studies (11, 22, 23) have demonstrated that freshly isolated rat SMG acinar cells contain a granule-associated Ca^{2+} store that is insensitive to IP_3 but releasable by ionomycin plus monensin. To examine whether SMG-C6 cells have a similar IP_3-insensitive Ca^{2+} store, the cells were sequentially exposed to TG, ionomycin, and monensin in regular Ca^{2+}-free medium (containing 30 μ*M* BAPTA). As shown in Figure 6A, after the ACh-stimulated $[Ca^{2+}]_i$ increase, 1 μ*M* ionomycin induced another large $[Ca^{2+}]_i$ peak (321 ± 7 n*M*, *n* = 5) followed by a third increase in response to monensin treatment (185 ± 16 n*M*, *n* = 5). Similarly, after TG exposure, ionomycin elicited another $[Ca^{2+}]_i$ increase (183 ± 20 n*M*, *n* = 4), and monensin triggered a further increase (171 ± 16 n*M*, *n* = 5) (Fig. 6B) in Ca^{2+}-free medium, suggesting that IP_3-insensitive Ca^{2+} stores are present in these cells.

To rule out the possibility that the extracellular Ca^{2+} (100 n*M*) in this medium may partially contribute to the $[Ca^{2+}]_i$ increases in response to ionomycin and monensin, the same measurements were conducted in Ca^{2+}-free medium containing 1 m*M* EGTA. As shown in Figure 6, EGTA-containing medium reduced the ACh-induced $[Ca^{2+}]_i$ increase (40 ± 5 n*M*, *n* = 5; $P < 0.01$). The ionomycin- and monensin-induced $[Ca^{2+}]_i$ increases were identical to those in BAPTA-containing medium (Fig. 6A). Similarly, the TG-induced $[Ca^{2+}]_i$ increase was significantly smaller (81 ± 7 n*M*, *n* = 5; $P < 0.002$), and the ionomycin- and monensin-induced $[Ca^{2+}]_i$ increases were comparable to those in BAPTA-containing medium. These results suggest that EGTA (1 m*M*)-containing medium depletes the IP_3-sensitive Ca^{2+} store, and that the $[Ca^{2+}]_i$ increases in response to ionomycin and monensin are due to Ca^{2+} release from intracellular Ca^{2+} stores, and are not derived from Ca^{2+} influx.

Ca^{2+} Influx. As shown in Figure 7, addition of Ca^{2+} after incubation of unstimulated SMG-C6 cells in Ca^{2+}-free medium for 5 min induced a small $[Ca^{2+}]_i$ increase mediated by Ca^{2+} influx. The initial Ca^{2+} influx rate was 56 ± 7 n*M*/min (*n* = 5) (Figs. 7A & 7B). This Ca^{2+} influx was significantly stimulated by 10 μ*M* ACh (196 ± 21 n*M*/min, *n* = 10; $P < 0.001$) (Figs. 7A & 7B). Stimulation with 2 m*M* ATP also induced a significant increase in Ca^{2+} influx (192 ± 28 n*M*/min, *n* = 5; $P < 0.001$) (Fig. 7B). Exposure to 3 μ*M* TG induced a slightly larger Ca^{2+} influx (220 ± 7

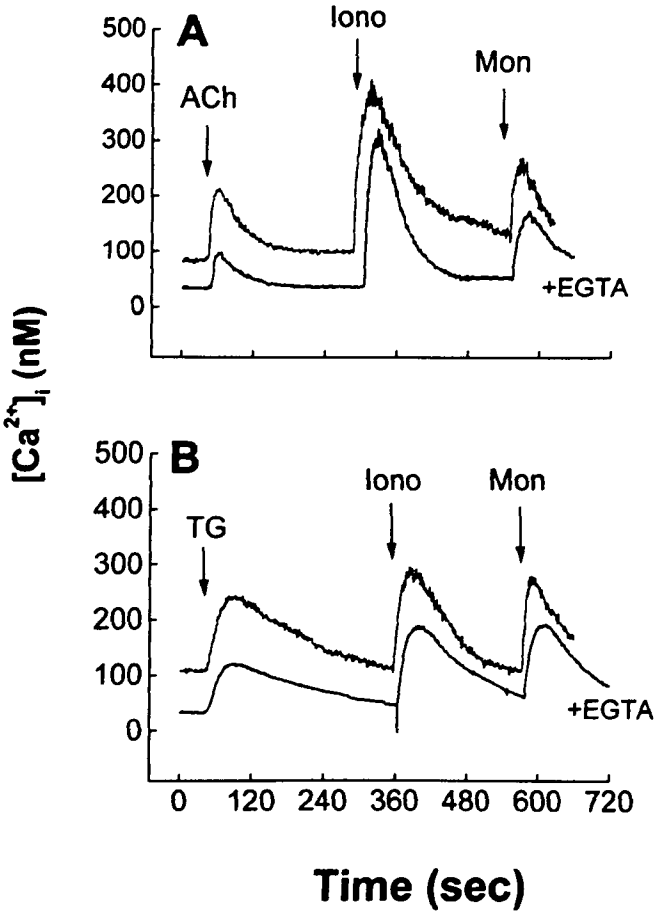

Figure 6. Ca^{2+} release induced by sequential exposure to acetylcholine or thapsigargin, ionomycin, and monensin. SMG-C6 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was monitored at 37°C in Ca^{2+}-free medium containing 30 μ*M* BAPTA or 1 m*M* EGTA (+EGTA). Cells were sequentially exposed to (A) 10 μ*M* acetylcholine (ACh), 1 μ*M* ionomycin (Iono), and 10 μ*M* monensin (Mon), or (B) 3 μ*M* thapsigargin (TG), 1 μ*M* ionomycin (Iono), and 10 μ*M* Monensin (Mon) at the time indicated by the arrows. Traces are representative of at least five separate measurements using different cell preparations.

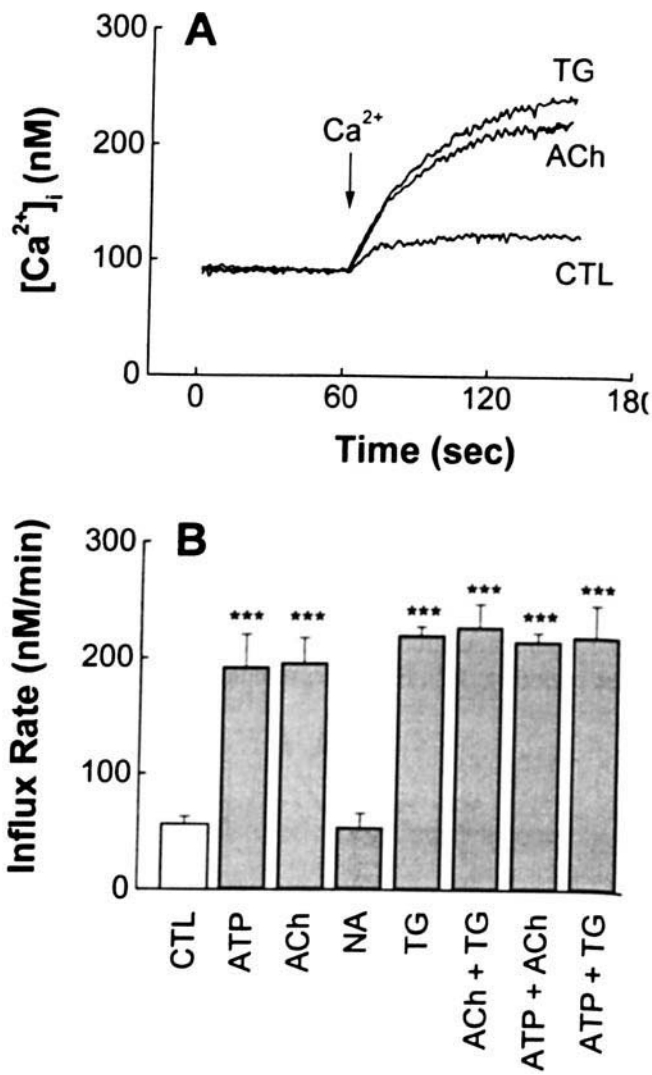

Figure 7. Ca^{2+} influx activated by acetylcholine, ATP, norepinephrine, and thapsigargin. SMG-C6 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was determined in Ca^{2+}-free medium. Cells were exposed to 2 m*M* ATP (ATP), 10 μ*M* acetylcholine (ACh), 10 μ*M* norepinephrine (NA), 3 μ*M* thapsigargin (TG), or a combination of ACh and TG (ACh + TG), ATP and ACh (ATP + ACh), or ATP and TG (ATP + TG) for 5 min, and 1 m*M* Ca^{2+} was added to initiate Ca^{2+} influx. The same volume of vehicle was added in unstimulated control (CTL) cells. Values are means ± SEM of at least five separate measurements using different cell preparations. (A) Representatives of Ca^{2+} influx. (B) The initial rates of Ca^{2+} influx. *** $P < 0.001$ vs unstimulated cells.

n*M*/min, $n = 5$) (Figs. 7A & 7B). However, stimulation with NA did not activate Ca^{2+} influx (54 ± 12 n*M*/min, $n = 5$) (Fig. 7B), which was consistent with the Ca^{2+} release response. These results suggest that Ca^{2+} influx activated by ACh, ATP, and TG is through the SOCE pathway. To test whether Ca^{2+} influx activated by ATP, ACh, and TG is through the same entry pathway, we measured Ca^{2+} influx in the cells stimulated with combinations of ACh and TG, ACh and ATP, or ATP and TG. As shown in Figure 7B, combining these stimuli did not alter the Ca^{2+} influx rate (ACh + TG: 227 ± 20 n*M*/min, $n = 5$; ATP + ACh, 215 ± 7 n*M*/min, $n = 5$; ATP + TG, 219 ± 27 n*M*/min, $n = 5$), suggesting that the same entry pathway mediates the Ca^{2+} influxes.

Since Ca^{2+} influx through the SOCE pathway is activated by depletion of the IP_3-sensitive store, we further examined the effects of inhibiting Ca^{2+} release from this store. Cells were pretreated with 100 μ*M* 2APB or 200 μ*M* ryanodine to block Ca^{2+} release, then stimulated with 10 μ*M* ACh. Both inhibitors significantly attenuated the ACh-elicited Ca^{2+} release (Fig. 8A). The rate (Fig. 8B) and magnitude (Fig. 8C) of Ca^{2+} influx were also proportionally reduced, indicating that the Ca^{2+} influx is through the SOCE pathway.

SOCE is sensitive to La^{3+} (24–26). Therefore, the effect of La^{3+} on ACh-, ATP-, and TG-activated Ca^{2+} influx was examined. As shown in Figure 9, 0.2 m*M* La^{3+} significantly reduced ACh-, ATP-, and TG-stimulated Ca^{2+} influx. The initial influx rates were 42 ± 16 n*M*/min ($n = 5$; $P < 0.001$), 7 ± 4 n*M*/min ($n = 5$; $P < 0.001$) and 108 ± 8 ($n = 4$; $P < 0.001$) in ACh-, ATP-, and TG-stimulated cells, respectively (Fig. 9).

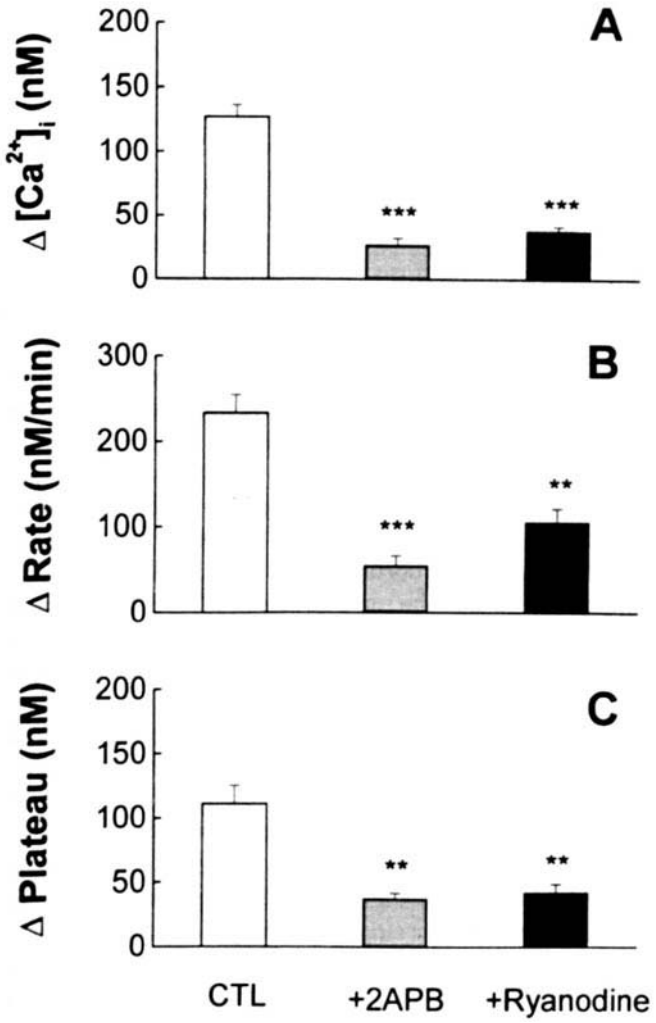

Figure 8. Effects of 2APB and ryanodine on acetylcholine-activated Ca^{2+} influx. SMG-C6 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was determined in Ca^{2+}-free medium. Cells were preincubated with DMSO for 3 min (CTL), 100 μ*M* 2APB for 3 min, or 200 μ*M* ryanodine for 10 min, then stimulated with 10 μ*M* ACh. One m*M* Ca^{2+} was added after stimulation for 5 min. (A) The initial $[Ca^{2+}]_i$ increase, (B) Ca^{2+} influx rate, and (C) magnitude of $[Ca^{2+}]_i$ peak after addition of Ca^{2+} (ΔPlateau) were measured. Values are means ± SEM of at least four separate determinations using different cell preparations. ** and ***, $P < 0.01$ and 0.001 vs control cells.

To examine the relationship between the filling state of the IP_3-sensitive Ca^{2+} store and Ca^{2+} influx, we characterized the TG-induced Ca^{2+} release and influx. As shown in Figure 10, exposure to TG resulted in a concentration-dependent initial $[Ca^{2+}]_i$ increase (EC_{50} = 6 n*M* TG). Ca^{2+} influx rate and the magnitude of $[Ca^{2+}]_i$ increase (ΔPlateau) were also TG concentration-dependent (both EC_{50} = 3 n*M* TG). Furthermore, the same values of EC_{20} (1 n*M* TG) were obtained for initial $[Ca^{2+}]_i$ increase (Ca^{2+} release), influx rate, and ΔPlateau. However, the values of EC_{80} were 31 n*M* TG for $[Ca^{2+}]_i$ increase, 8 n*M* TG for both influx rate and ΔPlateau (Fig. 10). These results suggest that Ca^{2+} influx is initiated by reduction of the IP_3-sensitive Ca^{2+} store, but the maximal influx is probably not dependent on complete depletion of the store.

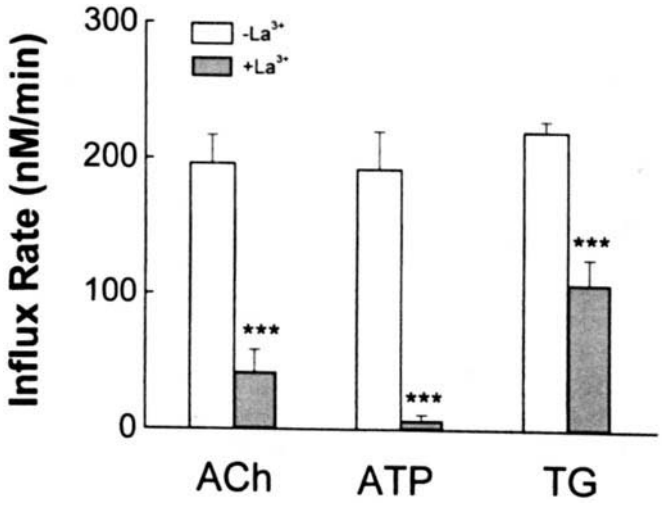

Figure 9. Effect of La^{3+} on Ca^{2+} influx activated by acetylcholine, ATP, or thapsigargin. SMG-C6 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was measured in Ca^{2+}-free medium. Cells were exposed to 10 μ*M* acetylcholine (ACh), 2 m*M* ATP (ATP), or 3 μ*M* thapsigargin (TG) for 4 min, and 0.2 m*M* $LaCl_3$ (+La^{3+}) or vehicle (–La^{3+}) was added. At 5 min postaddition of ACh, ATP, or TG, 1 m*M* Ca^{2+} was added to initiate Ca^{2+} influx. Values are means ± SEM of at least four separate determinations using different cell preparations. *** $P < 0.001$ vs without La^{3+} (–La^{3+}).

Discussion

The results of the present study clearly show that regulation of Ca^{2+} mobilization occurs primarily through muscarinic and P2Y-nucleotide (but not α_1) receptors in SMG-C6 cells. Stimulation of muscarinic and P2Y receptors elicited a rapid Ca^{2+} release from the IP_3-sensitive store, and depletion of this store in turn activated Ca^{2+} influx *via* the SOCE pathway. These results suggest that this cell line retains the physiological Ca^{2+} mobilizing pathway (i.e., plasma membrane muscarinic), and nucleotide receptor agonist stimulation elicits 1,4,5-IP_3 formation and Ca^{2+} release, which in turn activates Ca^{2+} influx through SOCE.

The subtype of the P2Y-nucleotide receptor responsible for the ATP-induced Ca^{2+} release is probably $P2Y_2$ since ATP and UTP induce identical Ca^{2+} releases, and ADP and UDP were not effective over the similar concentration range (3 and the present study). P_2-nucleotide receptors consist of two major receptor classes, P2X and P2Y receptors (27). At least 7 subtypes of P2X receptors have been identified (i.e., $P2X_1$ to $P2X_7$). Some of these receptors are ligand-gated nonselective cation channels, such as $P2X_4$ and $P2X_7$, which lead to Ca^{2+} influx through these cation channels. The Ca^{2+} mobilization in response to ATP in the present study was not mediated by P2X receptors since ATP caused a dramatic increase in 1,4,5-IP_3 formation and a large Ca^{2+} release from intracellular Ca^{2+} stores. P2Y receptors are a large family consisting of at least 5 subtypes, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ (27). Stimulation of these subtypes elicits 1,4,5-IP_3 production and Ca^{2+} mobilization. However, only $P2Y_2$ receptors respond to ATP and UTP equally; $P2Y_1$ and $P2Y_{11}$ do not respond to UTP, and $P2Y_4$ shows a

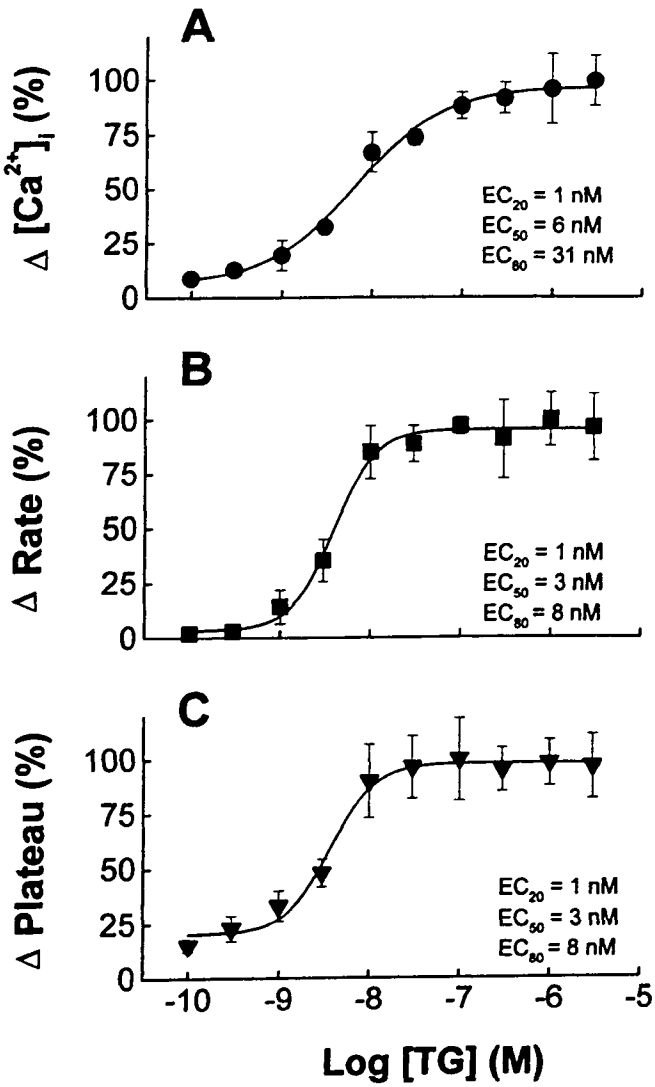

Figure 10. The concentration-response relationship of the initial $[Ca^{2+}]_i$ increase, the rate and magnitude of Ca^{2+} influx activated by thapsigargin. SMG-C6 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was measured in Ca^{2+}-free medium. Cells were exposed to 0.1, 0.3, 1, 3, 10, 30 100, 300, 1000, and 3000 n*M* TG for 5 min, and 1 m*M* Ca^{2+} was then added to initiate Ca^{2+} influx. The net increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$), initial influx rate (ΔRate), and the magnitude of the $[Ca^{2+}]_i$ rise after addition of Ca^{2+} (ΔPlateau) were calculated and plotted. Values are means ± SEM of four separate experiments. Curves are produced by sigmoid (Boltzman) fit.

larger response to UTP than to ATP. Uniquely, UDP is a better agonist of the $P2Y_4$ subtype than UTP and ATP. SMG-C6 cells responded to ATP and UTP equally, but showed a much smaller response to UDP (3) or ADP (3 and the present study), strongly suggesting that the Ca^{2+} mobilization in these cells is mediated by $P2Y_2$ receptors.

Salivary glands express P2-nucleotide receptors and these receptors participate in the regulation of gland function, such as ion transport (28). However, whether salivary cells physiologically express P2Y receptors is much less clear. In immortalized salivary cell lines, $P2Y_2$ receptors are extensively expressed (3, 4, 10, 29, 30). In contrast, freshly isolated SMG acinar cells of adult rats did not show a substantial Ca^{2+} mobilizing response to UTP stimulation, but culturing these cells for 3 hr to 6 days induced significant increases in the Ca^{2+} response (31). Furthermore, unilateral ligation of the main excretory duct of rat SMG glands also induced expression of $P2Y_2$ receptors. The Ca^{2+} mobilization response to the $P2Y_2$ agonist UTP was moderately increased (31). We also observed that SMG acinar cells freshly isolated from adult rats did not show a Ca^{2+} mobilization when stimulated with ATP, but SMG cells from newborn rats responded to ATP, showing a moderate (63% ± 9%) $[Ca^{2+}]_i$ increase (Zhang and Martinez, unpublished data). These studies suggest that expression of P2Y receptors may be associated with development and regeneration after tissue damage (31). Further investigation on this issue is indicated.

The present study also showed that stimulation of α_1-receptors with NA did not induce a substantial Ca^{2+} mobilization in SMG-C6 cells, consistent with the report by Quissell *etal.* (3), who found that total IP (IP+ IP_2+ IP_3) formation and Ca^{2+} increase in response to the α_1-agonists epinephrine and phenylephrine were minimal. This may be due to the lack of the receptor in these cells as reported in most other immortalized salivary cell lines (2). The α_1-receptor appears less likely to be functionally expressed after immortalization than other receptors. Moreover, the coupling between α_1-receptors and the PI signaling system in SMG acinar cells remains controversial. In freshly isolated SMG acinar cells, α_1-receptors may not play a critical role in regulating the Ca^{2+} signal. It has been reported that parotid gland (PG) acinar cells respond strongly to α_1-agonists, manifesting large increases in IP_3 formation and Ca^{2+} mobilization (32, 33), although Soltoff *etal.* (34) observed that stimulation of rat PG acinar cells with 10 μ*M* phenylephrine only slightly (35%) increased $[Ca^{2+}]_i$. In freshly isolated SMG acinar cells, 1,4,5-IP_3 formation and Ca^{2+} release from intracellular stores in response to α_1-receptor stimulation are relatively small (35). Similarly, the immortalized PG cell line Par-C5 retains α_1-receptors, manifesting a moderate $[Ca^{2+}]_i$ increase in response to NA stimulation (Zhang and Martinez, unpublished data). Elucidation of the mechanism mediating this reduced receptor expression following immortalization and the different expression between SMG and PG acinar cells would be of great interest.

The underlying mechanism mediating IP_3-activated Ca^{2+} release in salivary cells remains unclear. Wakui *et al.* (17) suggested a two-store model to explain this process in pancreatic cells. According to this model, 1,4,5-IP_3 first triggers Ca^{2+} release from the IP_3-sensitive store, and the Ca^{2+} released from this store activates RyR channels in the second store leading to further Ca^{2+} release. The results in the present study demonstrated that muscarinic- or $P2Y_2$ agonist–stimulated Ca^{2+} release involves two types of receptors, IP_3R and RyR. This is evidenced by the findings that ACh-stimulated Ca^{2+} release was significantly inhibited not only by 2APB, but also by ryanodine. These results suggest that 1,4,5-IP_3 first elicits Ca^{2+} release, and the in-

crease in $[Ca^{2+}]_i$ activates RyR, leading to further Ca^{2+} release. Nevertheless, whether these receptors are localized at the same store or two separate stores needs further investigation.

Our data also suggest that SMG-C6 cells, similar to freshly isolated acinar cells, have both IP_3-sensitive and -insensitive Ca^{2+} stores. The former is located primarily in the endoplasmic reticulum (ER) and releases Ca^{2+} through IP_3-sensitive channels operating as IP_3R (36, 37). However, the location of the IP_3-insensitive Ca^{2+} stores is less clear. Several organelles have been suggested to store and release Ca^{2+}, including secretory granules (11, 22, 23). Ca^{2+} stored in secretory granules is not discharged by receptor stimulation, or ionomycin, probably because the granules are acidic. It is well established that ionomycin is unable to discharge acidic Ca^{2+} stores unless the pH gradient is collapsed by monensin (38–40). Our results clearly showed that ionomycin induced a large Ca^{2+} release following depletion of the IP_3-sensitive store with TG. Furthermore, addition of monensin following ionomycin resulted in another release of Ca^{2+}, probably from secretory granules. These results indicate that Ca^{2+} stores in immortalized SMG-C6 cells are similar to those in freshly isolated SMG acinar cells.

In SMG acinar cells, depletion of the IP_3-sensitive store activates Ca^{2+} influx through SOCE. The present study demonstrated that Ca^{2+} influx in response to ACh, ATP, or TG in immortalized SMG-C6 cells is also mediated by the same mechanism as in freshly isolated SMG acinar cells. This conclusion is confirmed by the results showing that ACh-, ATP-, or TG-stimulated Ca^{2+} influx was dramatically inhibited by the SOCE pathway blocker La^{3+}. It is noteworthy that both ACh- and ATP-induced Ca^{2+} influxes were slightly smaller (13% and 11%, respectively) than the TG-activated influx. This is consistent with the Ca^{2+} release elicited by these reagents. Since TG induced a complete discharge of the IP_3-sensitive store, the rate of Ca^{2+} influx was proportionally larger. Furthermore, combination of these stimuli (i.e., ACh + TG, ACh + ATP, or ATP + TG) did not induce a larger Ca^{2+} influx than TG alone, suggesting that the Ca^{2+} influx rate reached its maximum by TG treatment. These results also provided further evidence for the same pathway mediating ACh-, ATP-, and TG-stimulated Ca^{2+} influx.

It has been widely recognized that the opening of the SOCE pathway or channel is controlled by the filling state of the IP_3-sensitive Ca^{2+} store. The data of the present study suggested that the initiation of Ca^{2+} influx is signaled by discharging the IP_3-sensitive store as the same EC_{20} (1 n*M* TG) was observed for Ca^{2+} release (reflected by the initial $[Ca^{2+}]_i$ increase), Ca^{2+} influx rate, and the magnitude of $[Ca^{2+}]_i$ plateau after addition of Ca^{2+} (Fig. 10). However, the maximal Ca^{2+} influx does not require complete depletion of the IP_3-sensitive store. This was evidenced by different EC_{80} values for Ca^{2+} release, influx rate, and the magnitude (ΔPlateau). EC_{80} for both Ca^{2+} influx rate and magnitude were 8 n*M* TG, but the EC_{80} for Ca^{2+} release was 31 n*M* TG. Furthermore, the maximal influx rate and magnitude were reached by less than 100 n*M* TG, but the maximal Ca^{2+} release (complete depletion of the store) needed 1000 n*M* TG. Two possibilities may underlie this phenomenon. One is that reduction in stored Ca^{2+} by 50% or more produces the maximal signal for Ca^{2+} influx. Therefore, partial emptying of the store is sufficient for the maximal Ca^{2+} entry. Another possibility is that TG discharges not only the IP_3-sensitive store, but also some IP_3-insensitive pools, and the release of Ca^{2+} from these pools is much slower than discharging the IP_3-sensitive store. This is quite possible since the mechanism of Ca^{2+} release by TG is inhibition of Ca^{2+}-ATPase. The prerequisite for this mechanism is that Ca^{2+} sequestration in these pools must have the same type of Ca^{2+}-ATPase as in the endoplasmic reticulum (the IP_3-sensitive store). In this case, the dose-response curve for TG-induced Ca^{2+} release may be extended to the right side, implying the requirement for higher concentrations to reach the maximal release. Further investigations are needed to delineate these mechanisms.

In summary, the present study clearly shows that the Ca^{2+} signaling system in SMG-C6 cells is similar to that in freshly isolated SMG acinar cells, including 1,4,5-IP_3 formation, Ca^{2+} storage, release, and influx in response to muscarinic agonists. However, expression of $P2Y_2$ receptors and the lack of Ca^{2+} mobilization response to α_1-agonists are the major differences between this cell line and freshly isolated cells. Nevertheless, the immortalized SMG-C6 cell line is an excellent cell model for studying the phosphoinositide-Ca^{2+} signaling system in SMG cells.

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