Am J Physiol Cell Physiol 285: C1142–C1149, 2003.
First published July 23, 2003; 10.1152/ajpcell.00566.2002.

Partial inhibition of SERCA is responsible for extracellular Ca^{2+} dependence of AlF_4^--induced $[Ca^{2+}]_i$ oscillations in rat pancreatic acinar cells

Seon Ah Chong, Soo Young Hong, Seok Jun Moon, Jee Won Park, Jeong-Hee Hong, Jeong Mi An, Syng-Ill Lee, Dong Min Shin, and Jeong Taeg Seo

Department of Oral Biology and Oral Science Research Center, Brain Korea 21 Project for Medical Science, Yonsei University College of Dentistry, Seoul, Korea

Submitted 5 December 2002; accepted in final form 9 July 2003

Chong, Seon Ah, Soo Young Hong, Seok Jun Moon, Jee Won Park, Jeong-Hee Hong, Jeong Mi An, Syng-Ill Lee, Dong Min Shin, and Jeong Taeg Seo. Partial inhibition of SERCA is responsible for extracellular Ca^{2+} dependence of AlF_4^--induced $[Ca^{2+}]_i$ oscillations in rat pancreatic acinar cells. *Am J Physiol Cell Physiol* 285: C1142–C1149, 2003. First published July 23, 2003; 10.1152/ajpcell.00566.2002.—AlF_4^- is known to generate oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by activating G proteins in many cell types. However, in rat pancreatic acinar cells, AlF_4^--evoked $[Ca^{2+}]_i$ oscillations were reported to be dependent on extracellular Ca^{2+}, which contrasts with the $[Ca^{2+}]_i$ oscillations induced by cholecystokinin (CCK). Therefore, we investigated the mechanisms by which AlF_4^- generates extracellular Ca^{2+}-dependent $[Ca^{2+}]_i$ oscillations in rat pancreatic acinar cells. AlF_4^--induced $[Ca^{2+}]_i$ oscillations were stopped rapidly by the removal of extracellular Ca^{2+} and were abolished on the addition of 20 mM caffeine and 2 μM thapsigargin, indicating that Ca^{2+} influx plays a crucial role in maintenance of the oscillations and that an inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store is also required. The amount of Ca^{2+} in the intracellular Ca^{2+} store was decreased as the AlF_4^--induced $[Ca^{2+}]_i$ oscillations continued. Measurement of $^{45}Ca^{2+}$ influx into isolated microsomes revealed that AlF_4^- directly inhibited sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). The activity of plasma membrane Ca^{2+}-ATPase during AlF_4^- stimulation was not significantly different from that during CCK stimulation. After partial inhibition of SERCA with 1 nM thapsigargin, 20 pM CCK-evoked $[Ca^{2+}]_i$ oscillations were dependent on extracellular Ca^{2+}. This study shows that AlF_4^- induces $[Ca^{2+}]_i$ oscillations, probably by inositol 1,4,5-trisphosphate production via G protein activation but that these oscillations are strongly dependent on extracellular Ca^{2+} as a result of the partial inhibition of SERCA.

cholecystokinin; plasma membrane adenosine 5′-triphosphatase; G proteins; caffeine

CALCIUM PLAYS A PIVOTAL ROLE in the regulation of a diverse range of cellular functions, such as muscle contraction, secretion, synaptic plasticity, cell proliferation, and cell death (2). Many hormones and neurotransmitters increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by mobilizing Ca^{2+} from intracellular stores and by inducing an influx from the extracellular space (4, 17). In electrically nonexcitable cells, such as pancreatic acinar cells, Ca^{2+} mobilization from internal stores is primarily triggered by the binding of inositol 1,4,5-trisphosphate (IP_3), which is produced from phosphatidylinositol 4,5-bisphosphate by the activation of phospholipase C (1). However, Ca^{2+} should enter the cells through the plasma membrane to maintain the Ca^{2+} signal and to refill intracellular Ca^{2+} stores. The main route for Ca^{2+} influx is via the capacitative Ca^{2+} entry (CCE) channels, which are activated by depletion of Ca^{2+} stores (16).

Physiological concentrations of hormones and neurotransmitters usually evoke $[Ca^{2+}]_i$ oscillations, which are generated primarily by repetitive Ca^{2+} release from IP_3-sensitive Ca^{2+} stores. This phenomenon does not generally require Ca^{2+} influx; thus, in rat pancreatic acinar cells, low concentrations of cholecystokinin (CCK) induce $[Ca^{2+}]_i$ oscillations, which continue for some time in the absence of extracellular Ca^{2+} (20, 24).

Direct activation of G proteins by AlF_4^- has also been shown to induce $[Ca^{2+}]_i$ oscillations in a variety of cell types (12, 18, 25). AlF_4^- binds to the α-subunit of heterotrimeric G proteins and forms a complex with Gα·GDP, which leads to the activation of G proteins and downstream signaling cascades (3). Therefore, the characteristics of AlF_4^--evoked $[Ca^{2+}]_i$ oscillations are thought to be similar to those of agonist-evoked oscillations. However, in rat pancreatic acinar cells, a fundamental difference was reported between AlF_4^-- and CCK-evoked $[Ca^{2+}]_i$ oscillations; i.e., removal of Ca^{2+} from the perfusate resulted in a rapid termination of AlF_4^--evoked $[Ca^{2+}]_i$ oscillations (25). This result suggested that, in contrast to CCK-evoked $[Ca^{2+}]_i$ oscillations, $[Ca^{2+}]_i$ oscillations caused by AlF_4^- acutely depend on extracellular Ca^{2+}. Because $[Ca^{2+}]_i$ oscillations caused by CCK and AlF_4^- are known to be generated through the same signaling pathway leading from G proteins to IP_3 generation, the different sensitivities to extracellular Ca^{2+} of AlF_4^-- and CCK-

Address for reprint requests and other correspondence: J. T. Seo, Dept. of Oral Biology, Yonsei University College of Dentistry, Shinchon-dong 134, Seodaemun-gu, Seoul 120-752, Korea (E-mail: jeong@yumc.yonsei.ac.kr).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

evoked $[Ca^{2+}]_i$ oscillations remain to be explained. Therefore, in the present study, we examined the mechanisms by which AlF_4^- generates extracellular Ca^{2+}-dependent $[Ca^{2+}]_i$ oscillations in rat pancreatic acinar cells.

METHODS

Isolation of acinar cells from rat pancreas. Pancreatic acinar cells were prepared by a modification of a method described previously (10). Briefly, male Sprague-Dawley rats (150–250 g) were anesthetized with diethyl ether and killed by decapitation. The pancreata were immediately removed and trimmed of fat on ice. Acinar cells were isolated using 50 U/ml collagenase and suspended in a HEPES-buffered physiological solution containing 104 mM NaCl, 4.5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 25 mM HEPES-Na, 15 mM HEPES free acid, 15 mM D-glucose, MEM amino acids (1×), 1% bovine serum albumin (BSA), soybean trypsin inhibitor (4 mg/ml), and 2 mM L-glutamine (adjusted to pH 7.4 and gassed with 100% O_2). The project was approved by the Institutional Animal Care and Use Committee of Yonsei University Medical Center.

$[Ca^{2+}]_i$ measurements. Cells were loaded with fura 2 by incubation with 2 μM fura 2-acetoxymethyl ester in HEPES-buffered solution equilibrated with 100% O_2 for 40 min at room temperature. They were washed twice and resuspended in an HCO_3^--buffered solution containing (in mM) 110 NaCl, 4.5 KCl, 1.0 NaH_2PO_4, 1.0 $MgSO_4$, 1.5 $CaCl_2$, 25 $NaHCO_3$, 5 HEPES-Na, 5 HEPES free acid, and 10 D-glucose and equilibrated with 95% O_2-5% CO_2 to give pH 7.4. The cells were allowed to attach to a coverslip, which formed the base of a cell chamber mounted on the stage of an inverted microscope. Once the cells had adhered to the coverslip, they were continuously superfused with the HCO_3^--buffered solution at a flow rate of 2 ml/min. In experiments involving caffeine, 10 mM NaCl was replaced by 20 mM caffeine. In Ca^{2+}-free solutions, $CaCl_2$ was omitted and 1 mM EGTA was added. Because Al^{3+} binds to EGTA with high affinity, 1 mM EGTA-containing Ca^{2+}-free solutions were supplemented with 1 mM $AlCl_3$ to allow a free Al^{3+} concentration of 100 nM. Free Al^{3+} concentration was calculated using Maxchelator software (C. Patton, Stanford University, Stanford, CA). All experiments were carried out at 37°C. $[Ca^{2+}]_i$ was measured by spectrofluorometry (Photon Technology International, Brunswick, NJ), with excitation at 340 and 380 nm and emission at 510 nm. The values of $[Ca^{2+}]_i$ were calculated using the equation previously described (6).

Isolation of microsomes from rat skeletal muscle. The microsomal fraction of the rat skeletal muscle was obtained using a modification of a method described previously (9). Fresh skeletal muscles were dissected and then cut into small pieces. Tissues were homogenized in ice-cold solution containing 10 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, 2 mM DTT, 0.25 M sucrose, 0.03% soybean trypsin inhibitor, 100 μM PMSF, leupeptin (1 μg/ml), and aprotinin (4 μg/ml). Homogenates were centrifuged at 8,000 *g* for 20 min at 4°C. The supernatants were centrifuged at 100,000 *g* for 90 min at 4°C, and the resulting microsomal pellet was resuspended in 10 mM Tris·HCl (pH 7.5), 0.15 M KCl, 2 mM DTT, 20 μM $CaCl_2$, and 0.03% soybean trypsin inhibitor.

$^{45}Ca^{2+}$ uptake assay. $^{45}Ca^{2+}$ uptake was determined using the Millipore filtration technique described previously (27). Microsomes (20 μg protein/ml) were incubated at 37°C in a $^{45}Ca^{2+}$ uptake medium containing 20 mM MOPS (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, and 2 μCi of $^{45}Ca^{2+}$ in the absence or presence of 5 mM NaF + 100 nM $AlCl_3$, 5 mM NaF + 50 μM $AlCl_3$, and 10 μM thapsigargin. The reaction was started by addition of 3 mM ATP and terminated at different times by vacuum filtration (0.45-μm Millipore filters). The filters were then washed with 2 mM $LaCl_3$ and 150 mM KCl, and the radioactivity remaining in the filters was determined by scintillation counting.

Values are means ± SE. The statistical significance of differences between averaged data was assessed using the unpaired Student's *t*-test.

Materials. BSA, MEM amino acids, DMSO, L-glutamine, caffeine, NaF, $AlCl_3$, CCK, EGTA, collagenase (type IV), ATP, PMSF, leupeptin, aprotinin, DTT, soybean trypsin inhibitor, and MOPS were purchased from Sigma (St. Louis, MO); $^{45}Ca^{2+}$ from NEN Life Science Products (Boston, MA); and fura 2-acetoxymethyl ester and thapsigargin from Molecular Probes (Eugene, OR).

RESULTS

AlF_4^--induced $[Ca^{2+}]_i$ oscillations were acutely dependent on extracellular Ca^{2+}. Application of AlF_4^- (5 mM NaF + 100 nM $AlCl_3$) to rat pancreatic single acinar cells evoked $[Ca^{2+}]_i$ oscillations. The pattern of $[Ca^{2+}]_i$ oscillations evoked by AlF_4^- was similar to that evoked by 20 pM CCK, although they were of longer latency: 16.6 ± 1.9 and 3.8 ± 0.4 min for AlF_4^- and 20 pM CCK, respectively (n = 16 each; Fig. 1, *A* and *B*). The mean amplitude of the $[Ca^{2+}]_i$ oscillations induced by AlF_4^- was 83.5 ± 10.5 nM (n = 16), which was significantly lower than that induced by 20 pM CCK (149.2 ± 29.3 nM, n = 16, $P < 0.05$). However, the most distinctive difference between AlF_4^-- and CCK-evoked $[Ca^{2+}]_i$ oscillations was that the AlF_4^--evoked $[Ca^{2+}]_i$ oscillations rapidly stopped on removal of extracellular Ca^{2+}, whereas 20 pM CCK-evoked oscillations continued for long periods of time after withdrawal of perfusate Ca^{2+}. The numbers of Ca^{2+} peaks observed after removal of perfusate Ca^{2+} are shown in Fig. 1*C*. The mean number of Ca^{2+} peaks observed after removal of perfusate Ca^{2+} during AlF_4^- stimulation was 0.63 ± 0.18 (n = 16), which was significantly less than the number observed during CCK stimulation (5.38 ± 0.52, n = 16, $P < 0.01$).

AlF_4^--induced $[Ca^{2+}]_i$ oscillations were also dependent on the IP_3-sensitive Ca^{2+} store. Because the $[Ca^{2+}]_i$ oscillations induced by AlF_4^- were found to be more dependent on extracellular Ca^{2+} than those induced by CCK, we next investigated whether AlF_4^--evoked $[Ca^{2+}]_i$ oscillations were independent of the IP_3-sensitive intracellular Ca^{2+} store and mainly generated through Ca^{2+} channels on the plasma membrane. In rat parotid acinar cells, agonist- and thapsigargin-induced $[Ca^{2+}]_i$ oscillations were reported to be generated through CCE channels on the plasma membrane without any involvement of IP_3-sensitive Ca^{2+} channels (5). To test the dependence of AlF_4^--evoked $[Ca^{2+}]_i$ oscillations on IP_3-sensitive Ca^{2+} stores, we first introduced 20 mM caffeine, an inhibitor of IP_3 receptors and IP_3 production (14, 19), which is known to block the $[Ca^{2+}]_i$ oscillations generated through IP_3-sensitive Ca^{2+} channels, but not the $[Ca^{2+}]_i$ oscillations generated through Ca^{2+} channels on the plasma membrane (5).

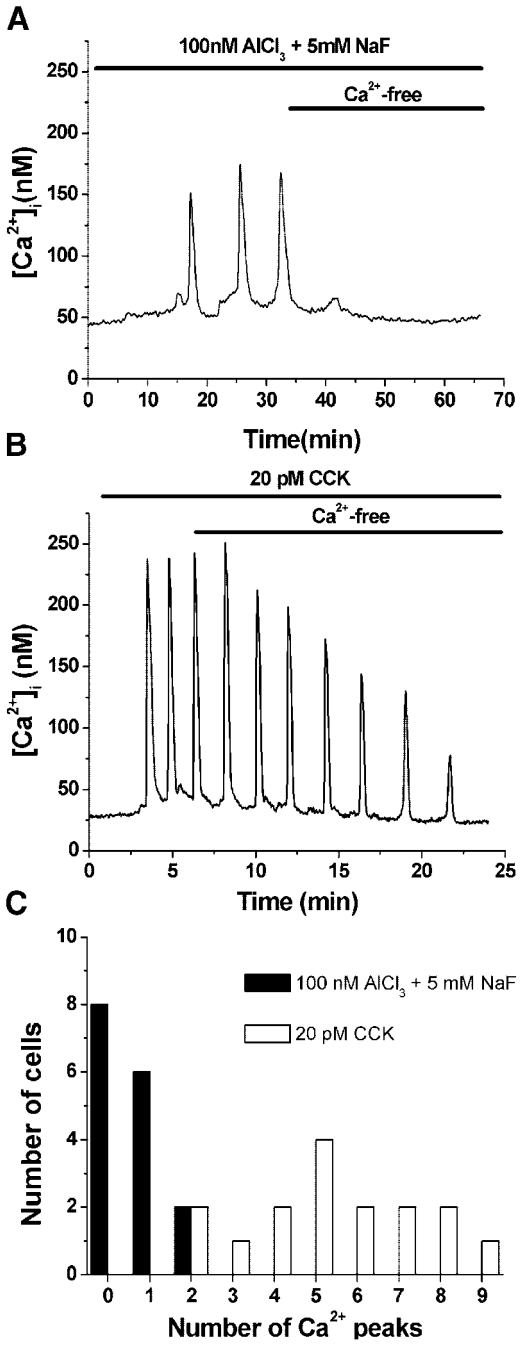

Fig. 1. Effect of extracellular Ca^{2+} on cholecystokinin (CCK)- and AlF_4^--evoked oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Fura 2-loaded rat pancreatic acinar cells were exposed to AlF_4^- (5 mM NaF + 100 nM $AlCl_3$, *A*) or 20 pM CCK (*B*), and Ca^{2+} was removed from the perfusate during $[Ca^{2+}]_i$ oscillation, as shown by horizontal bars (n = 16 each). *C*: number of Ca^{2+} peaks evoked after withdrawal of perfusate Ca^{2+} in the presence of AlF_4^- or CCK.

Caffeine at 20 mM completely blocked AlF_4^--evoked $[Ca^{2+}]_i$ oscillations (n = 4; Fig. 2*A*), suggesting that generation of $[Ca^{2+}]_i$ oscillations in response to AlF_4^- requires Ca^{2+} release through IP_3-sensitive Ca^{2+} channels. In addition, the oscillations were acutely discontinued after the intracellular Ca^{2+} store was depleted by 2 μM thapsigargin (n = 4; Fig. 2*B*). Furthermore, AlF_4^- induced one to three spikes in Ca^{2+}-free solution, suggesting that $[Ca^{2+}]_i$ oscillations can be initiated independently of Ca^{2+} influx (n = 4; Fig. 2*C*). These results imply that the IP_3-sensitive Ca^{2+} store is essential for initiation and maintenance of the $[Ca^{2+}]_i$ oscillations evoked by AlF_4^-.

AlF_4^- decreased the amount of Ca^{2+} in the Ca^{2+} store, probably by inhibiting sarco/endoplasmic reticulum Ca^{2+} ATPase activity. Because AlF_4^--evoked $[Ca^{2+}]_i$ oscillations were not sustained after perfusate Ca^{2+} was withdrawn, it can be assumed that the amount of

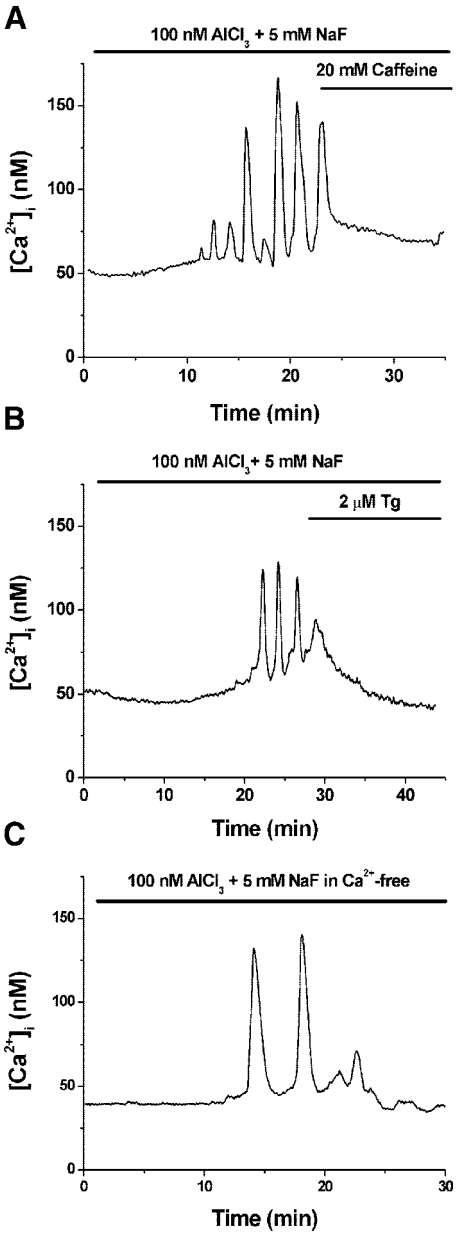

Fig. 2. Involvement of inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} stores in generation of AlF_4^--evoked $[Ca^{2+}]_i$ oscillations. Fura 2-loaded rat pancreatic acinar cells were exposed to AlF_4^- (5 mM NaF + 100 nM $AlCl_3$), and 20 mM caffeine (*A*) or 2 μM thapsigargin (Tg, *B*) was added during $[Ca^{2+}]_i$ oscillation, as shown by horizontal bars (n = 4 each). *C*: fura 2-loaded rat pancreatic acinar cells were exposed to AlF_4^- in the absence of perfusate Ca^{2+} (n = 4).

Ca^{2+} in the store was not sufficient to maintain the oscillations in the absence of a supply of Ca^{2+} from the extracellular space. Therefore, we measured the size of the Ca^{2+} store during CCK or AlF_4^- stimulation by using thapsigargin to measure the amount of Ca^{2+} released from the store. Accordingly, we changed the perfusate to a Ca^{2+}-free solution containing 2 μM thapsigargin during $[Ca^{2+}]_i$ oscillation. During AlF_4^- stimulation, the amplitude of the $[Ca^{2+}]_i$ increase caused by changing the perfusate to 2 μM thapsigargin-containing Ca^{2+}-free solution was 139.3 ± 13.9 nM ($n = 4$) when the perfusate was changed just after the $[Ca^{2+}]_i$ oscillations had been initiated, but this was reduced to 42.2 ± 3.3 nM ($n = 4$, $P < 0.05$) when the perfusate was changed after the fourth peak (Fig. 3*A*, *B*, and *E*). In contrast to the marked decrease in peak size during AlF_4^- stimulation, the amplitudes of the $[Ca^{2+}]_i$ increases caused by the perfusate changes were not significantly different during stimulation with 20 pM CCK: 173.5 ± 18.3 nM just after the initiation of the $[Ca^{2+}]_i$ oscillations and 178.5 ± 13.1 nM just after the fourth peak ($n = 4$ each; Fig. 3, *C–E*).

Because sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is the major contributor to the maintenance of Ca^{2+} concentrations in the store, we examined whether AlF_4^- inhibits SERCA activity. The SERCA activity on the isolated microsomes may be represented by the rate of $^{45}Ca^{2+}$ uptake into the microsomes. Therefore, we measured the $^{45}Ca^{2+}$ uptake at 2-min intervals in the absence or presence of AlF_4^-. The rate of $^{45}Ca^{2+}$ uptake was reduced by ~23% by pretreatment with 5 mM NaF + 100 nM $AlCl_3$ ($n = 8$; Fig. 4). In addition, pretreating the microsomes with 5 mM NaF + 50 μM $AlCl_3$ decreased the rate of $^{45}Ca^{2+}$ uptake by 58% ($n = 8$), indicating that AlF_4^- directly inhibited the Ca^{2+}-ATPase activity dose dependently. $AlCl_3$ (50 μM) in the absence of NaF did not inhibit the $^{45}Ca^{2+}$ uptake ($n = 3$). Thapsigargin (10 μM) reduced Ca^{2+}-ATPase activity by 91% ($n = 8$), suggesting that

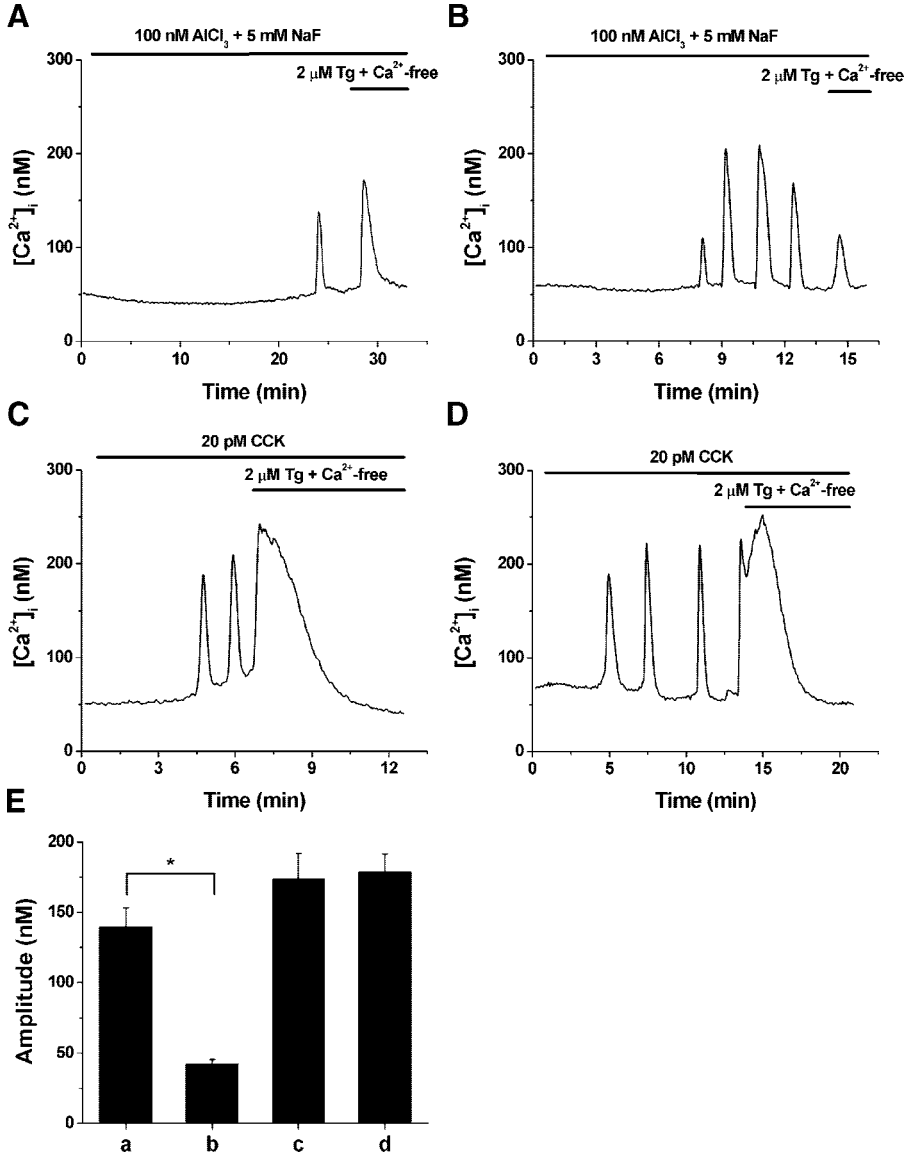

Fig. 3. Comparison of amount of Ca^{2+} in the Ca^{2+} stores during stimulations with AlF_4^- and CCK. $[Ca^{2+}]_i$ oscillations were induced by AlF_4^- (5 mM NaF + 100 nM $AlCl_3$, $n = 4$ each; *A* and *B*) and 20 pM CCK ($n = 4$ each; *C* and *D*), and then the perfusate was changed to a Ca^{2+}-free solution containing 2 μM thapsigargin. The solution was changed just after oscillations (*A* and *C*) or after the 4th peak (*B* and *D*). *E*: amplitudes of $[Ca^{2+}]_i$ increases caused by solution change were measured to compare amounts of Ca^{2+} in the store as follows: just after initiation of $[Ca^{2+}]_i$ oscillations during AlF_4^- stimulation (*a*), just after the 4th peak during AlF_4^- stimulation (*b*), just after initiation of $[Ca^{2+}]_i$ oscillations during CCK stimulation (*c*), and just after the 4th peak during CCK stimulation (*d*). Values are means ± SE. $^*P < 0.05$.

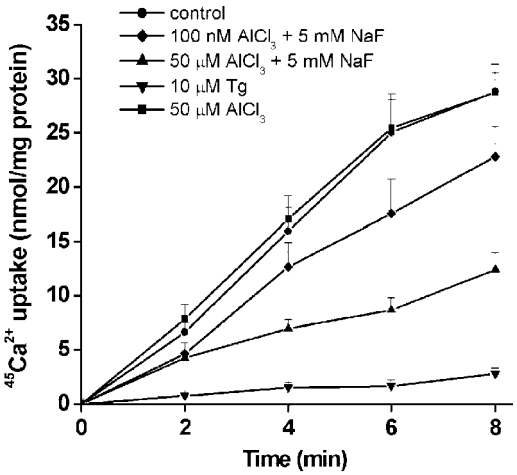

Fig. 4. Inhibition of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity by AlF_4^-. Activity of SERCA in the absence ($n = 8$, control) or presence of 5 mM NaF + 100 nM $AlCl_3$ ($n = 8$), 5 mM NaF + 50 μM $AlCl_3$ ($n = 8$), 10 μM thapsigargin ($n = 8$), and 50 μM $AlCl_3$ ($n = 3$) was determined by measuring the rate of $^{45}Ca^{2+}$ uptake into microsomes. Values are means ± SE.

the ATPase activity detected in this experiment was mainly SERCA activity.

Rate of Ca^{2+} efflux was increased by AlF_4^- and CCK to a similar extent. We also tested whether the rate of Ca^{2+} efflux was affected by AlF_4^-. The rate of Ca^{2+} efflux was measured by calculating the rate of Ca^{2+} decline at the same $[Ca^{2+}]_i$ after an acute change of perfusate to a 2 μM thapsigargin-containing Ca^{2+}-free solution during AlF_4^- or CCK stimulation. The rate of Ca^{2+} efflux was 0.89 ± 0.12 nM/s ($n = 8$) during stimulation with 20 pM CCK (Fig. 5*A*) and 0.8 ± 0.18 nM/s ($n = 9$) during stimulation with AlF_4^- (Fig. 5*B*). The efflux rate of Ca^{2+} was 0.44 ± 0.09 nM/s ($n = 5$) in the absence of stimulation (Fig. 5*C*). Therefore, the rate of Ca^{2+} efflux was higher during AlF_4^- stimulation than in the absence of stimulation, but it was not significantly different from that observed during CCK stimulation.

Partial inhibition of SERCA made CCK-evoked $[Ca^{2+}]_i$ oscillations more dependent on extracellular Ca^{2+}. To test whether the partial inhibition of SERCA was responsible for dependence of the $[Ca^{2+}]_i$ oscillations on extracellular Ca^{2+}, cells were exposed to a low concentration of thapsigargin before the addition of CCK. As shown in Fig. 6, $[Ca^{2+}]_i$ increased slightly after addition of 1 nM thapsigargin. Moreover, in the presence of 1 nM thapsigargin, 20 pM CCK induced $[Ca^{2+}]_i$ oscillations of lower amplitude (97.2 ± 10.8 nM, $n = 11$) than those induced by 20 pM CCK alone (149.2 ± 29.3 nM, $n = 10$, $P < 0.05$). When Ca^{2+} was removed from the perfusate, $[Ca^{2+}]_i$ oscillations rapidly stopped ($n = 11$). These results suggest that a reduced Ca^{2+} loading in the Ca^{2+} store, due to the partial inhibition of SERCA, may be responsible for the extracellular Ca^{2+} dependence of $[Ca^{2+}]_i$ oscillations in response to AlF_4^-.

DISCUSSION

In the present study, we observed that AlF_4^- (5 mM NaF + 100 nM $AlCl_3$) evoked $[Ca^{2+}]_i$ oscillations and that maintenance of these oscillations was strongly dependent on Ca^{2+} influx in rat pancreatic acinar cells. This observation is in contrast to the CCK-evoked $[Ca^{2+}]_i$ oscillations, which primarily depend on the release of Ca^{2+} stored in the IP_3-dependent intracellular Ca^{2+} store. The different responses to CCK and AlF_4^- have been reported by Yule and Williams (25),

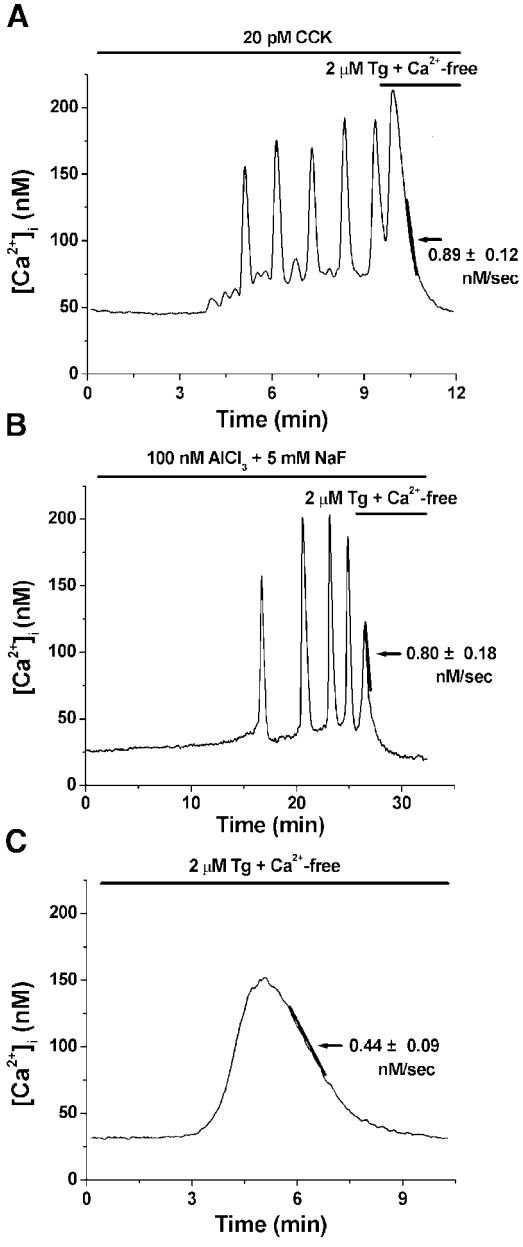

Fig. 5. Rate of Ca^{2+} efflux during AlF_4^- and CCK stimulations determined by measuring the rate of Ca^{2+} decline at the same $[Ca^{2+}]_i$ after acute change of perfusate to 2 μM thapsigargin-containing Ca^{2+}-free solution. Rate of Ca^{2+} efflux was 0.89 ± 0.12 ($n = 8$) and 0.8 ± 0.18 nM/s ($n = 9$) during 20 pM CCK (*A*) and AlF_4^- (5 mM NaF + 100 nM $AlCl_3$) stimulations (*B*), respectively, and 0.44 ± 0.09 nM/s ($n = 5$) in the absence of stimulation (*C*).

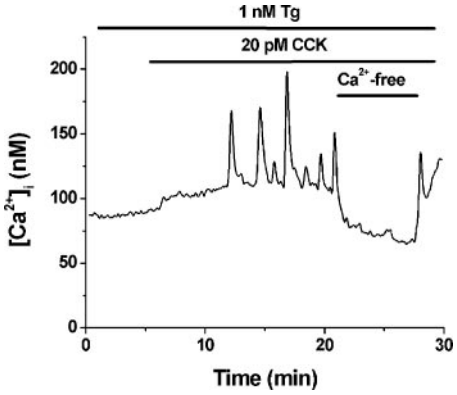

Fig. 6. Increased dependence of CCK-evoked $[Ca^{2+}]_i$ oscillations on extracellular Ca^{2+} by partial inhibition of SERCA activity. Fura 2-loaded rat pancreatic acinar cells were treated with 1 nM thapsigargin before stimulation with 20 pM CCK, and then Ca^{2+} was removed during $[Ca^{2+}]_i$ oscillation. Trace is representative of 11 independent experiments.

but the cause of the difference has not been explained. Because AlF_4^- is known to mobilize Ca^{2+} by activating G proteins, the downstream targets of Ca^{2+}-mobilizing receptor stimulation (12, 18), the characteristics of AlF_4^--evoked $[Ca^{2+}]_i$ oscillations should be identical to those of CCK-evoked oscillations, if this is the only mechanism of the generation of $[Ca^{2+}]_i$ oscillations. However, we found that the Ca^{2+} responses to AlF_4^- or CCK were different, in terms of the extracellular Ca^{2+} dependence of $[Ca^{2+}]_i$ oscillations. Therefore, our observations and those of Yule and Williams suggest that AlF_4^- acts on additional target molecules that play a critical role in the generation of extracellular Ca^{2+}-dependent $[Ca^{2+}]_i$ oscillations.

We first examined whether AlF_4^- induced repetitive Ca^{2+} influx by acting at the plasma membrane. Membrane-linked $[Ca^{2+}]_i$ oscillations have been previously reported in guinea pig ileal smooth muscle cells stimulated with AlF_4^- (7). In this cell type, AlF_4^- (10 μM Al_3^+ + 1 mM F^-) evoked $[Ca^{2+}]_i$ oscillations by causing the periodic opening and closing of voltage-dependent Ca^{2+} channels in the plasma membrane. AlF_4^- inhibits plasma membrane Ca^{2+}-ATPase (PMCA), causing membrane depolarization, which leads to the opening of voltage-operated Ca^{2+} channels. However, in pancreatic acinar cells in which voltage-operated Ca^{2+} channels are not functional, the mechanism for the generation of $[Ca^{2+}]_i$ oscillations does not seem to be the same as that in smooth muscle cells. However, guinea pig ileal smooth muscle cells are not unique in terms of generating "membrane-type $[Ca^{2+}]_i$ oscillations." In rat salivary acinar cells, high concentrations of Ca^{2+}-mobilizing agonists or thapsigargin were also shown to generate membrane-type $[Ca^{2+}]_i$ oscillations through CCE channels (5). To test whether AlF_4^--induced $[Ca^{2+}]_i$ oscillations originate from the plasma membrane in rat pancreatic acinar cells, we treated cells with caffeine during $[Ca^{2+}]_i$ oscillation. High concentrations of caffeine (>10 mM) are known to block IP_3 receptors (14, 21) and to inhibit IP_3 production in mouse pancreatic acinar cells (19). Therefore, if AlF_4^- does not require an IP_3-sensitive intracellular Ca^{2+} store to generate $[Ca^{2+}]_i$ oscillations and simply acts on the plasma membrane, caffeine should not have an inhibitory effect on the generation of $[Ca^{2+}]_i$ oscillations, as shown previously (5). However, 20 mM caffeine immediately blocked AlF_4^--evoked $[Ca^{2+}]_i$ oscillations. In addition, we also found that depletion of the IP_3-sensitive Ca^{2+} store with the use of thapsigargin stopped the $[Ca^{2+}]_i$ oscillations. These results indicate that cyclic release of Ca^{2+} from the IP_3-sensitive Ca^{2+} store is essential for generation of $[Ca^{2+}]_i$ oscillations. However, these results do not necessarily mean that Ca^{2+} influx is not the primary target of AlF_4^- action. Recent studies have indicated that elevation of $[Ca^{2+}]_i$ activates IP_3 channel gating in the absence of IP_3 (23). If AlF_4^- activates the Ca^{2+} influx pathway, this may trigger Ca^{2+} release from IP_3-sensitive Ca^{2+} stores. To test this possibility, we simply exposed the cells to AlF_4^- in the absence of perfusate Ca^{2+} and found that $[Ca^{2+}]_i$ oscillations were initiated, even in the absence of Ca^{2+} influx. Because AlF_4^- has been known to activate heterotrimeric G proteins by forming a Gα-GDP-AlF_4^- complex, which resembles Gα·GTP, these results imply that the generation of IP_3 via activation of $G_{q/11}$ proteins and subsequent activation of IP_3 receptors play a fundamental role in initiation and maintenance of $[Ca^{2+}]_i$ oscillations and that the plasma membrane is not the primary target of AlF_4^- in terms of generating $[Ca^{2+}]_i$ oscillations.

The second possible explanation for the strong dependence of AlF_4^--induced $[Ca^{2+}]_i$ oscillations on Ca^{2+} influx is that AlF_4^- may reduce the activity of SERCA and the size of the intracellular Ca^{2+} store. During $[Ca^{2+}]_i$ oscillations induced by CCK in rat pancreatic acinar cells, Ca^{2+} released from the store is mainly resequestered into the store by SERCA and only partly expelled into the extracellular space by PMCA (20, 22). However, if SERCA activity is reduced, more Ca^{2+} is pumped out of the cells and the store becomes depleted during $[Ca^{2+}]_i$ oscillations. In this case, $[Ca^{2+}]_i$ oscillations could be maintained only with the aid of Ca^{2+} influx through the CCE pathways. Our data indeed showed that the activity of SERCA was directly inhibited by ~23% with AlF_4^- at the concentration we used and that the amount of Ca^{2+} in the store decreased as the AlF_4^--induced $[Ca^{2+}]_i$ oscillations continued. Therefore, it seems that a 23% reduction in SERCA activity has a profound effect on the reuptake of released Ca^{2+} by the store; i.e., reuptake of Ca^{2+} by the store decreases, and thus Ca^{2+} efflux increases. However, despite the 23% reduction in activity, SERCA may still allow refilling of the stores with Ca^{2+}, probably during the interspike phase, which results in maintenance of $[Ca^{2+}]_i$ oscillations. A higher concentration of AlF_4^- (5 mM NaF + 50 μM $AlCl_3$) inhibited SERCA activity by 58% and induced a tonic increase in $[Ca^{2+}]_i$ (data not shown), indicating that the strong inhibition of SERCA with a high concentration of AlF_4^- causes a rapid depletion of the Ca^{2+} store and fails to recharge the store.

In contrast to SERCA, our study showed that the rate of Ca^{2+} efflux, which might represent PMCA ac-

tivity, was increased by AlF_4^-. This is in good agreement with a previous report in which it was found that Ca^{2+}-mobilizing hormones activate PMCA activity in rat pancreatic acinar cells by activating protein kinase C (13). In our study, the increased activity of PMCA by CCK stimulation was similar to that achieved by AlF_4^- stimulation, suggesting that activation of PMCA per se does not play a crucial role in making $[Ca^{2+}]_i$ oscillations dependent on extracellular Ca^{2+}. Although it was reported that AlF_4^- directly inhibits PMCA in pig stomach smooth muscle and erythrocytes (11), the direct effect of AlF_4^-, at the concentration we used, on PMCA appeared to be minimal in rat pancreatic acinar cells; i.e., no significant difference was observed between the rates of Ca^{2+} efflux during AlF_4^- and CCK stimulation, although the Ca^{2+} efflux rate was slightly lower during AlF_4^- stimulation than during CCK stimulation.

Having confirmed the inhibition of SERCA by AlF_4^-, we sought to determine whether the partial inhibition of SERCA was responsible for dependence of the oscillations on extracellular Ca^{2+}. Because thapsigargin is known to inhibit SERCA specifically and dose dependently, we used 1 nM thapsigargin to inhibit SERCA partially. Previously, 0.1–1 nM thapsigargin was reported to inhibit SERCA activity by 20–30% in mouse cardiac tissue homogenates (8). In our system, 1 nM thapsigargin often induced a slight increase in $[Ca^{2+}]_i$, suggesting that this concentration of thapsigargin caused partial inhibition of SERCA. Comparing the amplitude of the spikes evoked by 20 pM CCK in the absence and in the presence of 1 nM thapsigargin, we found that the amplitude of spikes decreased by 35% when thapsigargin was present. A similar observation was made by Petersen and colleagues (15) in mouse pancreatic acinar cells. They reported that partial inhibition of SERCA by 500 pM thapsigargin in mouse pancreatic acinar cells decreased the mean amplitude of IP_3-induced $[Ca^{2+}]_i$ oscillations by ~26%. Our study and that of Petersen and colleagues may imply that the reduced amount of Ca^{2+} in the stores caused by partial SERCA inhibition results in less Ca^{2+} release per spike. However, the most striking effect of the partial inhibition of SERCA on CCK-induced $[Ca^{2+}]_i$ oscillations was the immediate termination of $[Ca^{2+}]_i$ oscillations on withdrawal of the perfusate Ca^{2+}. Partial inhibition of SERCA may decrease the rate of Ca^{2+} reuptake into the store, and thus Ca^{2+} would be preferentially pumped out by PMCA. Therefore, if SERCA is partially inhibited, the amount of Ca^{2+} available in the store for the next spike would be reduced and a greater Ca^{2+} influx would be needed to recharge the stores to maintain $[Ca^{2+}]_i$ oscillations; i.e., $[Ca^{2+}]_i$ oscillations become critically dependent on the extracellular Ca^{2+} by partial inhibition of SERCA.

With consideration that SERCA is a crucial element involved in the regulation of intracellular Ca^{2+} homeostasis, a decrease in SERCA activity would be expected to cause a profound impairment of Ca^{2+} signaling. However, in the present study, we showed that $[Ca^{2+}]_i$ oscillations were maintained, even in the condition of decreased SERCA activity, and that the maintenance of $[Ca^{2+}]_i$ oscillations was attributed to functional compensation by Ca^{2+} influx. The plasticity of Ca^{2+} signaling appears to be an important feature to maintain intracellular Ca^{2+} homeostasis and normal cell functions. In support of this, Zhao and colleagues (26) reported a remarkable plasticity and adaptability of Ca^{2+} signaling and Ca^{2+}-dependent cellular functions in $SERCA2^{+/-}$ mice. In their study, although the rate of Ca^{2+} uptake was ~50% slower into the internal stores of the permeabilized pancreatic acini from $SERCA2^{+/-}$ mice than into the store of wild-type mice, agonist-stimulated exocytosis was identical in these cell types. Therefore, even if SERCA activity is reduced, normal physiology can be ensured by plasticity of Ca^{2+} signaling.

In conclusion, the strong dependence of AlF_4^--induced $[Ca^{2+}]_i$ oscillations on Ca^{2+} influx is probably due to the reduction of the activity of SERCA and the size of the intracellular Ca^{2+} stores.

DISCLOSURES

This work was supported by the Research Fund of Yonsei University College of Dentistry for 2001. S. A. Chong was a graduate student supported by BK21 Project for Medical Science, Yonsei University.

REFERENCES

1. **Berridge MJ.** Inositol trisphosphate and calcium signalling. *Nature* 361: 315–325, 1993.
2. **Berridge MJ, Lipp P, and Bootman MD.** The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1: 11–21, 2000.
3. **Bigay J, Deterre P, Pfister C, and Chabre M.** Fluoride complexes of aluminum or beryllium act on G-proteins as reversibly bound analogues of the γ-phosphate of GTP. *EMBO J* 6: 2907–2913, 1987.
4. **Clapham DE.** Calcium signaling. *Cell* 80: 259–268, 1995.
5. **Foskett JK and Wong DC.** $[Ca^{2+}]_i$ inhibition of Ca^{2+} release-activated Ca^{2+} influx underlies agonist- and thapsigargin-induced $[Ca^{2+}]_i$ oscillations in salivary acinar cells. *J Biol Chem* 269: 31525–31532, 1994.
6. **Grynkiewicz G, Poenie M, and Tsien RY.** A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985.
7. **Himpens B, Missiaen L, Droogmans G, and Casteels R.** AlF_4^- induces Ca^{2+} oscillations in guinea-pig ileal smooth muscle. *Pflügers Arch* 417: 645–650, 1991.
8. **Ji Y, Loukianov E, and Periasamy M.** Analysis of sarcoplasmic reticulum Ca^{2+} transport and Ca^{2+} ATPase enzymatic properties using mouse cardiac tissue homogenates. *Anal Biochem* 269: 236–244, 1999.
9. **Machkrill JJ, Challiss RAJ, O'Connell DA, Lai FA, and Nahorski SR.** Differential expression and regulation of ryanodine receptor and *myo*-inositol 1,4,5-trisphosphate receptor Ca^{2+} release channels in mammalian tissues and cell lines. *Biochem J* 327: 251–258, 1997.
10. **Matozaki T, Goke B, Tsunoda Y, Rodriguez M, Martinez J, and Williams JA.** Two functionally distinct cholecystokinin receptors show different modes of action on Ca^{2+} mobilization and phospholipid hydrolysis in isolated rat pancreatic acini. Studies using a new cholecystokinin analog, JMV-180. *J Biol Chem* 265: 6247–6254, 1990.
11. **Missiaen L, Wuytack F, De Smedt H, Vrolix M, and Casteels R.** AlF_4^- reversibly inhibits "P"-type cation-transport ATPases, possibly by interacting with the phosphate-binding site of the ATPase. *Biochem J* 253: 827–833, 1988.
12. **Moon C, Fraser SP, and Djamgoz MB.** G-protein activation, intracellular Ca^{2+} mobilization and phosphorylation studies of

membrane currents induced by AlF_4^- in *Xenopus* oocytes. *Cell Signal* 9: 497–504, 1997.
13. **Muallem S, Pandol SJ, and Beeker TG.** Calcium mobilizing hormones activate the plasma membrane Ca^{2+} pump of pancreatic acinar cells. *J Membr Biol* 106: 57–69, 1988.
14. **Parker I and Ivorra I.** Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in *Xenopus* oocytes. *J Physiol* 433: 229–240, 1991.
15. **Petersen CC, Petersen OH, and Berridge MJ.** The role of endoplasmic reticulum calcium pumps during cytosolic calcium spiking in pancreatic acinar cells. *J Biol Chem* 268: 22262–22264, 1993.
16. **Putney JW Jr.** A model for receptor-regulated calcium entry. *Cell Calcium* 7: 1–12, 1986.
17. **Putney JW Jr, Broad LM, Braun FJ, Lievremont JP, and Bird GS.** Mechanisms of capacitative calcium entry. *J Cell Sci* 114: 2223–2229, 2001.
18. **Thomas D, Lipp P, Tovey SC, Berridge MJ, Li W, Tsien RY, and Bootman MD.** Microscopic properties of elementary Ca^{2+} release sites in non-excitable cells. *Curr Biol* 10: 8–15, 2000.
19. **Toescu EC, O'Neill SC, Petersen OH, and Eisner DA.** Caffeine inhibits the agonist-evoked cytosolic Ca^{2+} signal in mouse pancreatic acinar cells by blocking inositol trisphosphate production. *J Biol Chem* 267: 23467–23470, 1992.
20. **Tsunoda Y, Stuenkel EL, and Williams JA.** Oscillatory mode of calcium signaling in rat pancreatic acinar cells. *Am J Physiol Cell Physiol* 258: C147–C155, 1990.
21. **Wakui M, Kase H, and Petersen OH.** Cytoplasmic Ca^{2+} signals evoked by activation of cholecystokinin receptors: Ca^{2+}-dependent current recording in internally perfused pancreatic acinar cells. *J Membr Biol* 124: 179–187, 1991.
22. **Williams JA.** Intracellular signaling mechanisms activated by cholecystokinin-regulating synthesis and secretion of digestive enzymes in pancreatic acinar cells. *Annu Rev Physiol* 63: 77–97, 2001.
23. **Yang J, McBride S, Mak DO, Vardi N, Palczewski K, Haeseleer F, and Foskett JK.** Identification of a family of calcium sensors as protein ligands of inositol trisphosphate receptor Ca^{2+} release channels. *Proc Natl Acad Sci USA* 99: 7711–7716, 2002.
24. **Yule DI, Lawrie AM, and Gallacher DV.** Acetylcholine and cholecystokinin induce different patterns of oscillating calcium signals in pancreatic acinar cells. *Cell Calcium* 12: 145–151, 1991.
25. **Yule DI and Williams JA.** Mastoparan induces oscillations of cytosolic Ca^{2+} in rat pancreatic acinar cells. *Biochem Biophys Res Commun* 177: 159–165, 1991.
26. **Zhao XS, Shin DM, Liu LH, Shull GE, and Muallem S.** Plasticity and adaptation of Ca^{2+} signaling and Ca^{2+}-dependent exocytosis in $SERCA2^{+/-}$ mice. *EMBO J* 20: 2680–2689, 2001.
27. **Zhong L and Inesi G.** Role of the S3 stalk segment in the thapsigargin concentration dependence of sarco-endoplasmic reticulum Ca^{2+} ATPase inhibition. *J Biol Chem* 273: 12994–12998, 1998.