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Effects of Serum on Calcium Mobilization in the Submandibular Cell Line A253

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Abstract The effects of serum on inositol 1,4,5-trisphosphate (IP_3) formation and Ca^{2+} mobilization in the human submandibular cell line A253 were studied. Exposure of A253 cells to fetal bovine serum (FBS) elicited a 3.3-fold increase in IP_3 formation and a concentration-dependent transient increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), which was similar in Ca^{2+}-containing and Ca^{2+}-free media. Newborn bovine serum (NBS), but not bovine serum albumin (BSA), induced a similar response. The Ca^{2+} release triggered by FBS was significantly (88%) reduced by the phospholipase C inhibitor U73122, indicating that Ca^{2+} release induced by FBS is through the PLC pathway. Pretreatment with the tyrosine kinase inhibitor genistein abolished the FBS- and NBS-induced Ca^{2+} release, suggesting that tyrosine kinase plays an important role in mediating the Ca^{2+} release. Pre-exposure to ATP or thapsigargin (TG) significantly reduced the FBS-induced $[Ca^{2+}]_i$ increase, indicating that Ca^{2+} release caused by FBS is from the TG- or ATP-sensitive Ca^{2+} store. While FBS exposure elicited a large Ca^{2+} release, it reduced Ca^{2+} influx. Furthermore, FBS significantly inhibited the Ca^{2+} influx activated by the depletion of intracellular stores by ATP or TG. These results suggest that (1) serum elicits Ca^{2+} release from ATP- and TG-sensitive stores, which is mediated by IP_3; (2) the serum-induced Ca^{2+} release may be modulated by a tyrosine kinase-associated process; and (3) serum strongly inhibits Ca^{2+} influxes including the store depletion-activated Ca^{2+} influx. J. Cell. Biochem. 73:458–468, 1999.

Key words: fetal bovine serum; newborn bovine serum; 1,4,5-IP_3; Ca^{2+} release; Ca^{2+} influx; U73122; genistein

Serum, such as fetal bovine serum (FBS), is a well-known factor providing multiple stimuli for cell growth and proliferation [Gallie and Traugh, 1994] and is an essential component of many tissue culture media. Serum is also an important factor in maintaining cell function in many cell lines, and its deprivation can induce striking alterations in cell function. For example, removal of FBS from culture medium can cause a significant increase in the intracellular Na^+ and a decrease in intracellular K^+ in fibroblasts [Hopp et al., 1987]. Moreover, the mitogenic effect of serum may be responsible for some pathological alterations as well. Serum proteins leaking into the brain due to breakdown of blood-brain barrier in head injury, stroke, multiple sclerosis, or other pathological conditions, can result in proliferation of astrocytes and other cells to form a glial scar [Skoff, 1975].

The mechanism of the mitogenic action of serum on cells remains undefined. An increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is a critical messenger mediating or regulating the cell proliferation and growth in a variety of cell types. Ca^{2+} is required for the movement of quiescent cells into the proliferative cycle as well as for movement of proliferating cells from different stages through mitosis [Means, 1994; Reddy, 1994; Berridge, 1995]. Ca^{2+} regulates several aspects of cell division including nuclear envelope breakdown and reformation, cleavage furrow formation and growth, and cell plate formation [Hepler, 1994]. The controlling actions of Ca^{2+} on cell proliferation are probably

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through the regulatory effects on gene expression. A large number of studies have demonstrated that Ca^{2+} regulates early response genes in various cell types including c-*fos*, c-*jun*, *junB*, *zif268*, and *nur77* [Roche and Prentki, 1994; Rosen et al., 1995]. Ca^{2+} also regulates the expression of late response genes such as the genes encoding some hormones, neuropeptides, ion channels and cytokines [Rosen et al., 1995]. Obviously, the mitogenic action of serum on cells is at least partially mediated through its effect on Ca^{2+} mobilization.

A large number of studies have shown that serum induces Ca^{2+} mobilization in many cell types [Tigyi et al., 1990; Amin et al., 1991; Hopp et al., 1992; He and Curry, 1993; Nadal et al., 1995; Wu et al., 1996; Nunez and Garcia-Sancho, 1996; Catinot et al., 1997]. For example, rabbit serum can induce Ca^{2+} release from intracellular stores in *Xenopus* oocytes [Tigyi et al., 1990]. Human serum can induce a similar effect in human platelets [Amin et al., 1991] and in rat neurons and glia [Nunez and Garcia-Sancho, 1996]. Nevertheless, several critical issues regarding Ca^{2+} mobilization in response to serum are still controversial. First, the intracellular mediator of the serum-induced Ca^{2+} release is unclear. It was observed that exposure to serum caused an increase in the formation of inositol 1,4,5-trisphosphate (IP_3) in platelets [Amin et al., 1991] and in *Xenopus* oocytes [Tigyi et al., 1990]. However, the FBS-induced IP_3 formation was much smaller than that induced by carbachol (CCh), while the Ca^{2+} releases elicited by FBS and CCh were comparable in HSG cells [Wu et al., 1996]. Furthermore, the Ca^{2+} pool released by serum is still unknown. Wu et al. [1996] observed that FBS discharged an intracellular store which was distinct from the CCh-sensitive one, but is part of the thapsigargin (TG)-sensitive one in HSG cells. Finally, the effect of serum on Ca^{2+} influx across the plasma membrane is unclear. Stimulation of blastocyst cells with serum enhanced uptake and incorporation of [^{3}H]uridine and this effect was associated with an increase in Ca^{2+} influx [Fishel, 1980]. However, in another study, exposure of HSG cells to FBS did not induce Ca^{2+} influx but seemed to potentiate the CCh-activated Ca^{2+} influx [Wu et al., 1996]. By contrast, serum did not activate Ca^{2+} influx in *Xenopus* oocytes [Tigyi et al., 1990] and in fibroblasts [Hopp et al., 1992]. Since the intracellular mediator, Ca^{2+} store and influx pathway are critical elements in the phosphoinositol-Ca^{2+} signal transduction pathway, elucidation of the effects of serum on these mechanisms is of great significance.

The present study was therefore conducted to examine the effects of FBS and newborn bovine serum (NBS), two widely used sera in tissue culture, on IP_3 formation, Ca^{2+} release, intracellular stores and Ca^{2+} influx in the submandibular duct cell line A253. This cell line was established from a human submandibular gland tumor [Giard et al., 1973] and has been used to study the mechanisms of saliva modification [Patton and Wellner, 1993], chloride transport [Rooms, 1998], and intracellular signal transduction pathways [Sugita et al., in press]. Comparisons of elemental composition between A253 cells and mouse submandibular cells indicate that A253 cells have identical Na, K, Cl, Ca, Mg, S, and P concentrations to that in striated duct cells, but the elemental compositions are less comparable between A253 cells and acinar or granular duct cells [Rooms, 1998]. A253 cells also have a unique receptor profile. Salivary duct cells show a Ca^{2+} mobilizing response to β_2-adrenergic stimulation [Xu et al., 1995]. By contrast, most cell lines do not demonstrate a β-adrenergic agonist-induced increase in $[Ca^{2+}]_i$. However, we have recently demonstrated a large Ca^{2+} release in response to isoproterenol in A253 cells [Zhang et al., 1997a], indicating that this cell line retains a Ca^{2+} response to β-stimulation. Similar to other salivary cell lines, A253 cells demonstrate P_2-purinergic receptors coupled with the intracellular phosphoinositol-calcium pathway [Zhang et al., 1997a]. However, A253 cells lack muscarinic cholinergic receptors [Marmmary et al., 1989; Zhang et al., 1997a]. Increasing evidence [Marmmary et al., 1989; Zhang et al., 1997a; Rooms, 1998; Sugita et al., in press] indicates that A253 is a useful salivary duct cell line for study of the mechanism of ion transport and signal transduction. Further characterization of this cell line, including comparison of the responses to various stimuli with other cell lines, is unquestionably required and beneficial. Therefore, we used this cell line to examine the Ca^{2+} mobilization in response to serum. Our results show that FBS and NBS have specific effects on Ca^{2+} mobilization in this cell line.

MATERIALS AND METHODS

Materials

ATP, bovine serum albumin (BSA, type V), digitonin, dimethylsulfoxide (DMSO), EGTA, HEPES, ionomycin, and TG were purchased from Sigma Chemical Co. (St. Louis, MO). Fura-2/acetoxymethylester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR). Fetal bovine serum and newborn bovine serum were from GIBCO (Grand Island, NY) and were inactivated by heating at 60°C for 60 min. U73122 and genistein were from CalBiochem (La Jolla, CA). All other chemicals used were of the highest grade available.

Solutions

Unless otherwise stated, all experiments used a modified Earle-Hanks' physiological salt solution (PSS) consisting of (in mM): 110 NaCl, 5.4 KCl, 1.2 $CaCl_2$, 0.8 $MgSO_4$, 0.4 KH_2PO_4, 0.33 NaH_2PO_4, 25 $NaHCO_3$, 20 Hepes, and 10 glucose. pH was adjusted to 7.4 after gassing with 95% air/5% CO_2 for at least 45 min. For the Ca^{2+}-free PSS, $CaCl_2$ was omitted.

Cell Culture

The A253 cell line, which was established from a human submandibular gland tumor by Giard et al. [1973], was obtained from the American Type Culture Collection (Rockville, MD) and routinely grown at 37°C in an atmosphere of 5% CO_2 in air on plastic tissue culture T-75 flasks in McCoy's 5A medium (GIBCO, Grand Island, NY) containing 10% NBS, 100 µg/ml penicillin and 100 U/ml streptomycin sulfate. Medium was changed twice weekly. Subculture was conducted by washing the cells with fresh serum-free medium and exposing to 0.25% trypsin and 1 mM EDTA for 5 min. Trypsinized cells were counted, centrifuged, resuspended in fresh medium and seeded to new flasks. For experiments, the confluent A253 cells were trypsinized, rinsed twice with PSS, resuspended in fresh PSS containing 1% NBS and 2% trypsin inhibitors and incubated at 37°C for 2 h before use.

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

$[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent indicator fura-2 as previously described [Zhang et al., 1997a; Martinez and Zhang, 1998]. Trypsinized A253 cells were loaded with fura-2 by incubation in 2 µM fura-2/AM for 20 min at 37°C in PSS containing 0.01% BSA. After loading, the cells were rinsed twice and kept at 37°C. A 2-ml aliquot of fura-2-loaded cells (1.5×10^6 cells/ml) was centrifuged at 50*g* for 2 min, resuspended in fresh medium containing 0.01% BSA, and placed in a 4-ml cuvette. $[Ca^{2+}]_i$ measurement was performed using a PTI Deltascan fluorometer (PTI, S. Brunswick, NJ). The excitation wavelengths used were 340 and 380 nm, and the emission wavelength was 505 nm. Calibration of $[Ca^{2+}]_i$ was performed for each measurement trace by addition of 1 mM $CaCl_2$ and 50µM ionomycin to obtain the limiting ratio for the Ca^{2+} saturated form (R_{max}) of fura-2. Then, 0.0005% digitonin and 10 mM 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. [1985] using 224 nM as the K_d of fura-2 for Ca^{2+} at 37°C.

All Ca^{2+} influx experiments were conducted using Ca^{2+}-free PSS. Cells were exposed to indicated reagents for 5 min, and 1 mM Ca^{2+} was then added. The initial linear portion of $[Ca^{2+}]_i$ changes after addition of Ca^{2+} was used to calculate Ca^{2+} influx rate (nM/min) [Boulay et al., 1997; Wells et al., 1997; Zhang et al., 1997a].

Measurement of Medium Ca^{2+} Concentrations

Medium ionic Ca^{2+} concentrations ($[Ca^{2+}]_o$) was measured using fura-2 free acid. Briefly, 1.2 µM fura-2 (potassium salt) was added to nominally Ca^{2+}-free medium with or without a supplement of 0.1 mM EGTA and 50 µM $CaCl_2$. $[Ca^{2+}]_o$ was monitored at 37°C as described above. Calibration was performed for each determination by sequentially adding 1 mM $CaCl_2$ and 10 mM EGTA. Calculation of the Ca^{2+} concentrations from fluorescence ratios of the 340/380-nm excitation and 505-nm emission was performed as described above.

Determination of IP_3 Formation

IP_3 formation was measured as previously described [Zhang et al., 1997b; Mörk et al., 1999], using a radioimmunoassay (RIA) kit (Amersham, Arlington Heights, IL). A253 cells were stimulated with indicated agents. Unstimulated controls received the same volume of vehicle (water). The reaction was initiated by addition of reagents or vehicle and lasted for 30 s. 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 6,000 rpm in a microfuge at 4°C for 5 min. The supernatants were mixed by vortexing with an equal volume of diethyl ether and the ether phase discarded. This step was repeated three times. Samples were then neutralized to pH 7.0 with 0.5 M $NaHCO_3$ before IP_3 content was assayed. The level of IP_3 was then determined according to the procedure recommended by Amersham.

Data Presentation and Statistics

The results are presented as means ±S.E. of separate experiments using different cell preparations. Comparisons were made using the unpaired Student's t-test or the analysis of variance (ANOVA), followed by the Newman-Keuls test. *P* values of <0.05 were considered significant.

RESULTS

$[Ca^{2+}]_i$ Increase in Response to Serum

Exposure of A253 cells to inactivated FBS in PSS induced a rapid increase in $[Ca^{2+}]_i$, which was comparable in the presence and absence of extracellular Ca^{2+} (Fig.1A). In Ca^{2+}-containing solution (1.2 mM Ca^{2+}), exposure to 0.38% FBS induced a 200 ± 21 nM net increase in $[Ca^{2+}]_i$ (from 103 ± 10 nM to 303 ± 28 nM, n = 5). Similarly, in nominally Ca^{2+}-free medium (0.8 μM Ca^{2+}), the same exposure elicited a 182 ± 19 nM net increase (n = 12). After the initial increase, $[Ca^{2+}]_i$ was rapidly returned to the unstimulated level in both 1.2 mM Ca^{2+} and 0.8 μM Ca^{2+} media. These results suggest that the serum-induced $[Ca^{2+}]_i$ increase is primarily due to Ca^{2+} release from intracellular stores. The increase in $[Ca^{2+}]_i$ in response to FBS was concentration-dependent with a maximal increase of 409 nM and the 50% effective concentration (EC_{50}) of 0.38% FBS (Fig.1B).

Since NBS is widely used as a surrogate for FBS in tissue culture and BSA is commonly used in cell preparations, the effects of NBS and BSA on $[Ca^{2+}]_i$ were also examined. As shown in Figure 2, 0.38% NBS induced a rapid and large increase in $[Ca^{2+}]_i$ (net increase: 334 ± 41 nM, from 69 ± 7 nM to 403 ± 40 nM, n = 6). Similar to the FBS-induced change, $[Ca^{2+}]_i$ was also rapidly returned to basal level. By contrast, the same concentration of BSA did not result in any substantial alteration in $[Ca^{2+}]_i$ (net increase 14 ± 2 nM, from 76 ± 7 nM to 90 ± 6 nM, n = 5) (Fig. 2).

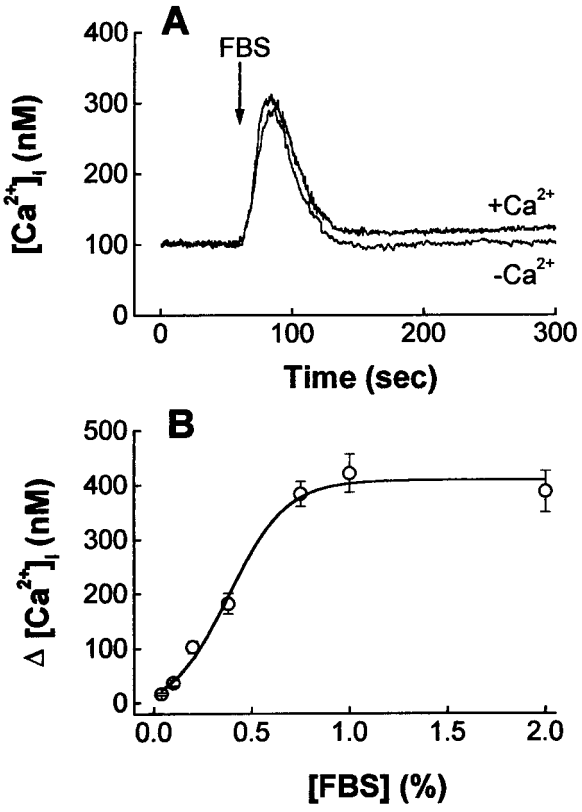

Fig. 1. FBS-induced $[Ca^{2+}]_i$ Increase. Fura-2-loaded A253 cells were suspended in physiological salt solution (PSS) containing 1.2 mM Ca^{2+} (+*Ca^{2+}*) or nominally Ca^{2+}-free PSS (−*Ca^{2+}*) ($[Ca^{2+}]_o$= 0.8 μM) and $[Ca^{2+}]_i$ was monitored. Cells were separately exposed to different concentrations of FBS. **A:** $[Ca^{2+}]_i$ changes in response to 0.38% FBS. Each trace is representative of separate experiments (+Ca^{2+}, n = 5; −Ca^{2+}, n = 12). **B:** The concentration-dependence of FBS-induced $[Ca^{2+}]_i$ increase. Values are mean ±S.E.M. of five to seven separate experiments. The curve is produced by sigmoid curve (Boltzman) fit. The maximal $[Ca^{2+}]_i$ increase was 409 nM, and the EC_{50} was 0.38% FBS.

The Messenger Mediating Ca^{2+} Release in Response to Serum

The intracellular mediator of Ca^{2+} release in response to serum has not been clearly defined, although some studies observed that serum stimulated an increase in the formation of IP_3 [Tigyi et al., 1990; Amin et al., 1991]. To examine whether IP_3 is the second messenger mediating the increase in $[Ca^{2+}]_i$ in response to serum exposure in A253 cells, we measured changes in cellular content of IP_3. Because A253 cells do not have muscarinic receptors [Marmmary et al., 1989; Zhang et al., 1997a] and IP_3 formation can be activated by stimulation of P_2-purinergic receptors with ATP [Zhang et al.,

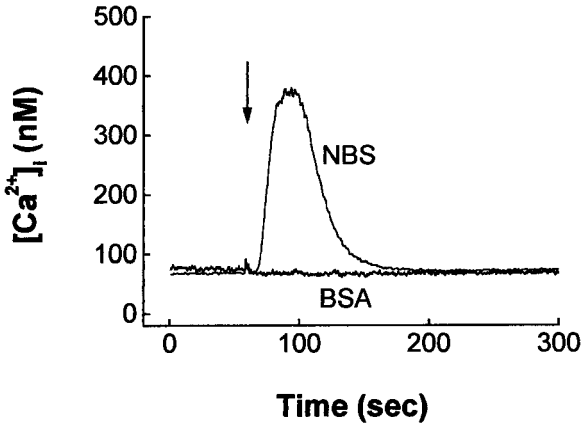

Fig. 2. $[Ca^{2+}]_i$ Changes in response to NBS and BSA. Fura-2-loaded A253 cells were suspended in PSS (1.2 mM Ca^{2+}) and $[Ca^{2+}]_i$ was monitored. Cells were exposed to 0.38% NBS or 0.38% BSA at the time indicated by the arrow. Each trace is representative of separate experiments using different cell preparations (NBS, n = 6; BSA, n =5).

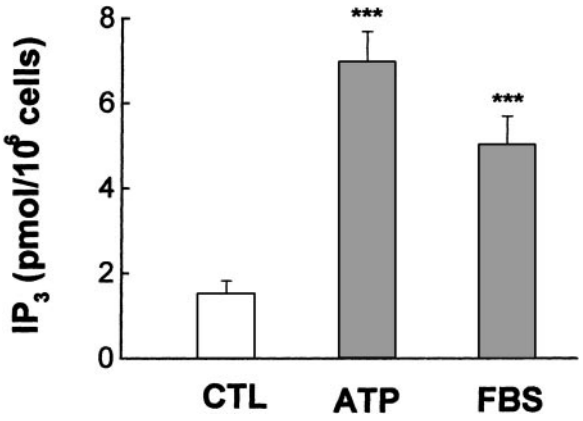

Fig. 3. Effects of ATP and FBS on the formation of 1,4,5-IP_3. A253 cells were exposed to 200 μM ATP, 0.38% FBS or the same volume of water (*CTL*) for 30 sec, and the reaction was then stopped and cellular 1,4,5-IP_3 (*IP_3*) levels were measured with a radioimmunoassay system. Values are means ±S.E.M. of four separate experiments. ***, $P < 0.005$ compared with CTL.

1997a], we compared the effects of serum with that of ATP on IP_3 formation. As shown in Figure 3, the IP_3 content in unstimulated cells was 1.5 ± 0.3 pmoles/10^6 cells (n = 4). Stimulation with 200 μM ATP induced a significant increase in IP_3 formation (7.0 ± 0.7 pmoles/10^6 cells, n = 4, $P < 0.005$). Exposure to 0.38% FBS induced a similar increase (5.0 ± 0.7 pmoles/10^6 cells, n = 4, $P < 0.005$) (Fig. 3). These results suggest that IP_3 is the mediator in the Ca^{2+} release induced by serum.

To confirm that the Ca^{2+} mobilization in response to FBS is mediated by IP_3, the effect of phospholipase C (PLC) inhibitor U73122 [Heemskerk et al., 1997] on the FBS-induced $[Ca^{2+}]_i$ increase was examined. Consistent with the effect of serum on IP_3 formation, pre-exposure of the cells to 2 μM U73122 for 3 min almost completely abolished the FBS-induced Ca^{2+} release (net increase: 16 ± 3 nM, n = 5; $P < 0.001$ vs FBS only) (Fig. 4), indicating that the Ca^{2+} release in response to FBS is mediated by IP_3 produced by PLC activation.

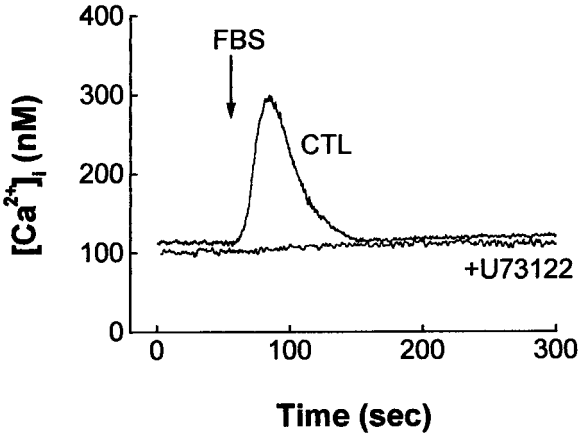

Fig. 4. Effect of U73122 on FBS-induced $[Ca^{2+}]_i$ Increase. Fura-2-loaded A253 cells were suspended in nominally Ca^{2+}-free medium ($[Ca^{2+}]_o$ = 0.8 μM) in the absence (*CTL*) or presence (+*U73122*) of 2 μM U73122, and exposed to 0.38% FBS at the time indicated by the arrow. Each trace is representative of separate experiments (CTL, n = 6; +U73122, n = 5).

Mechanism of PLC Activation

PLC can be activated not only by G-proteins that couple with PLC isoforms, but also by protein tyrosine kinases (PTKs) [Archuleta et al., 1993; Clapham, 1995]. To further examine the mechanism by which serum activates PLC, the effect of the PTK inhibitor genistein on ATP-induced Ca^{2+} release was compared with that on serum-induced Ca^{2+} release. Pretreatment with 20 μM genistein for 30 min almost completely abolished the FBS-induced $[Ca^{2+}]_i$ increase (16 ± 4 nM, n = 5; $P < 0.001$ vs vehicle control) (Fig. 5A). By contrast, the same pretreatment with genistein did not affect the ATP-elicited $[Ca^{2+}]_i$ increase (Fig. 5B). In the similar pattern, the NBS-induced Ca^{2+} release was also prevented by pretreatment with 20 μM genistein (Fig. 6). These results indicate that PLC activation in response to serum is probably through PTKs.

A253 cells express a large number of cell surface epidermal growth factor (EGF) recep-

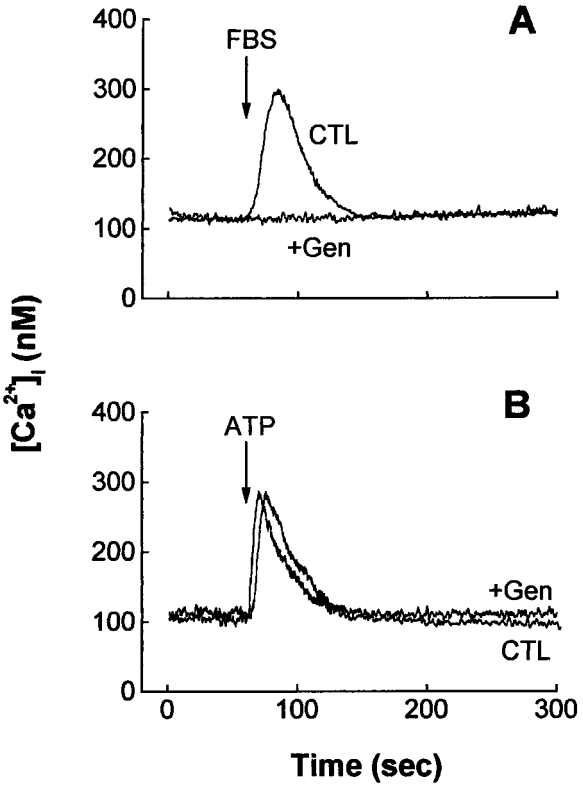

Fig. 5. Effects of genistein on FBS- and ATP-induced $[Ca^{2+}]_i$ Increase. Fura-2-loaded A253 cells were suspended in nominally Ca^{2+}-free medium ($[Ca^{2+}]_o$ = 0.8 μM) and $[Ca^{2+}]_i$ was monitored. Cells were pretreated with 0.5% (v/v) vehicle (DMSO; *CTL*) or 20 μM genistein (+*Gen*) for 30 min and then exposed to 0.38% FBS **(A)** or 200 μM ATP **(B)** at the time indicated by the arrow. Each trace is representative of separate experiments (A: +DMSO, n = 5; +Gen, n = 5; B: CTL, n = 14; +Gen, n = 5).

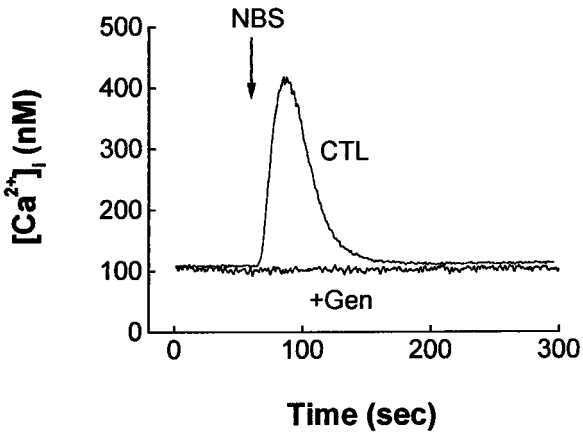

Fig. 6. Effects of genistein on NBS-induced $[Ca^{2+}]_i$ Increase. Fura-2-loaded A253 cells were suspended in nominally Ca^{2+}-free medium ($[Ca^{2+}]_o$ = 0.8 μM) and $[Ca^{2+}]_i$ was monitored. Cells were pretreated with 0.5% (v/v) vehicle (*CTL*) or 20 μM genistein (+*Gen*) for 30 min and then exposed to 0.38% NBS at the time indicated by the arrow. Each trace is representative of five separate experiments.

tors [Reiss et al., 1991]. Since EGF receptors are coupled with PTKs, the possibility that serum-induced IP_3 formation and Ca^{2+} release is mediated by receptor PTKs was examined by exposing cells to EGF. Stimulation of A253 cells with 10 nM or 100 nM EGF did not induce any substantial increase in $[Ca^{2+}]_i$. The net increase in $[Ca^{2+}]_i$ was 35 ± 3 nM (n = 5) in response to 100 nM EGF (not shown). These results indicate that the serum-induced Ca^{2+} mobilization is not caused by stimulation of EGF receptors.

Intracellular Store Sensitive to Serum

A253 cells have a IP_3-sensitive Ca^{2+} store, which can be partially discharged by ATP stimulation. This store appears to be also sensitive to the endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin (TG), since pre-exposure to either of these two drugs partially, but not completely, inhibited the release of Ca^{2+} by subsequent exposure to another drug [Zhang et al., 1997a]. To explore the storage site of the FBS-induced Ca^{2+} release, A253 cells were sequentially exposed to 3 μM TG and 0.38% FBS or to 2 mM ATP and 0.38% FBS. As shown in Figure 7A, stimulation with ATP elicited a rapid, large Ca^{2+} release. After $[Ca^{2+}]_i$ returned to the unstimulated level, addition of 0.38% FBS caused a further increase in $[Ca^{2+}]_i$ (76 ± 5 nM, n = 9), which was significantly smaller than that induced by FBS only ($P < 0.001$). Similarly, pre-exposure to 3 μM TG significantly reduced the $[Ca^{2+}]_i$ increase triggered by subsequent exposure to 0.38% FBS (40 ± 8 nM, n = 5; $P < 0.001$ vs FBS only) (Fig.7B). These results indicate that the FBS-induced Ca^{2+} release is from the IP_3- and TG-sensitive Ca^{2+} store(s).

Effects of Serum on Ca^{2+} Influx

Ca^{2+} influx plays an important role in the regulation of $[Ca^{2+}]_i$. Therefore, the effects of FBS on Ca^{2+} influx was measured by a sensitive method described by us [Zhang et al., 1997a; Wells et al., 1997] and by others [Boulay et al., 1997]. A253 cells were incubated in Ca^{2+}-free medium in the presence or absence of serum for 5 min, and 1 mM Ca^{2+} was then added to initiate Ca^{2+} influx. As shown in Figure 8A, unstimulated cells (0% FBS) had a Ca^{2+} influx rate of 126 ± 18 nM/min (n = 5) after incubation in Ca^{2+}-free medium for 5 min. Exposure to

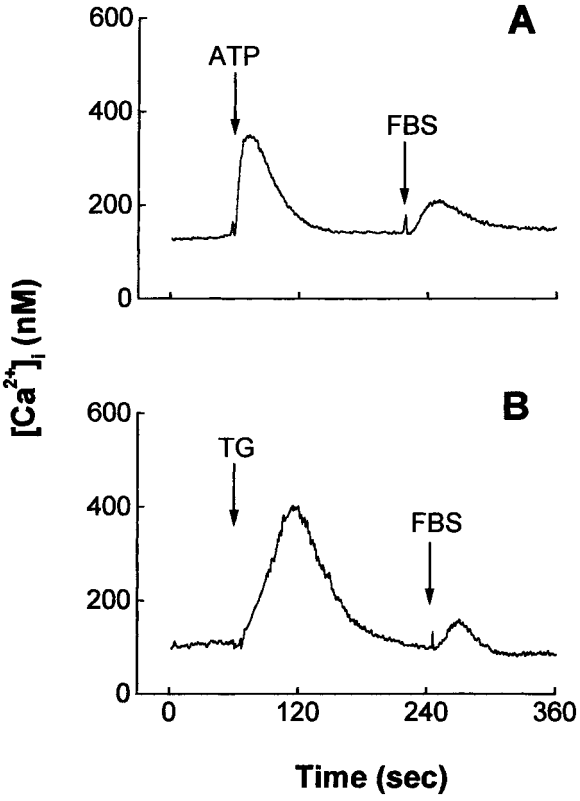

Fig. 7. Influence of ATP and TG on FBS-induced Ca^{2+} Release. Fura-2-loaded A253 cells were suspended in nominally Ca^{2+}-free medium ($[Ca^{2+}]_o$ = 0.8 μM) and $[Ca^{2+}]_i$ was monitored. Cells were exposed to 2 mM ATP **(A)** or 3 μM TG **(B)** for about 3 min, and when $[Ca^{2+}]_i$ was returned to the pre-stimulated levels, 0.38% FBS was added. Each trace is representative of separate experiments (A: n = 9; B: n = 5).

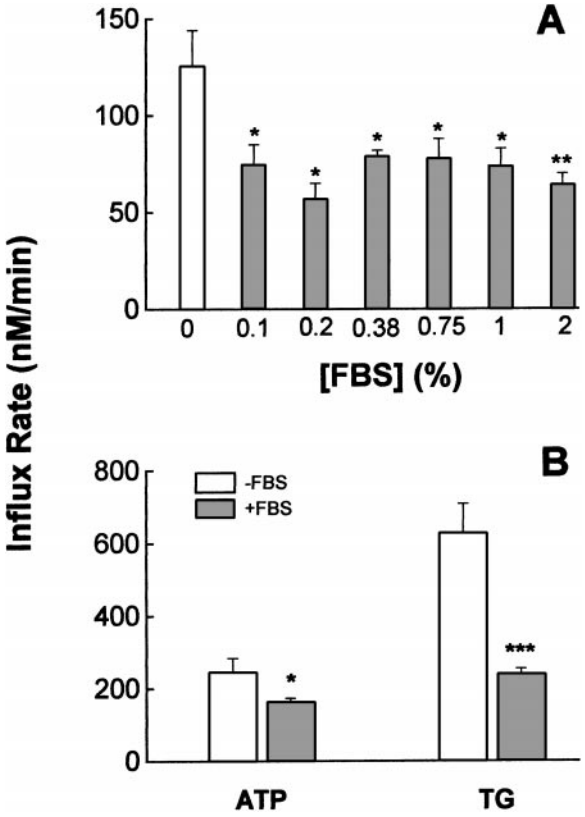

Fig. 8. Effects of FBS on Ca^{2+} Influx. Fura-2-loaded cells were suspended in nominally Ca^{2+}-free medium ($[Ca^{2+}]_o$ = 0.8 μM) and $[Ca^{2+}]_i$ was monitored. **A:** Ca^{2+} influx after exposure to FBS. Cells were exposed to 0, 0.1%, 0.2%, 0.38%, 0.75%, 1%, or 2% FBS for 5 min, and 1 mM Ca^{2+} was then added. Values are means ±S.E.M. of at least five separate experiments. **B:** Effect of FBS on ATP- and TG-activated Ca^{2+} influx. Cells were exposed to 2 mM ATP or 3 μM TG for 3 min, then to 0.38% FBS or vehicle for 3 min; 1 mM Ca^{2+} was then added. Values are means ±S.E.M. (ATP: CTL, n = 9; +FBS, n = 9. TG: CTL, n = 5; +FBS, n = 5).

0.1–2% FBS for 5 min significantly reduced the influx rate (all $P < 0.05$) (Fig. 8A). An comparable result was observed with NBS. The Ca^{2+} influx rate in cells exposed to 0.38% NBS for 5 min in Ca^{2+}-free medium was identical to that exposed to FBS (78 ± 7 nM/min, n = 6) (not shown). These results suggest that serum inhibits its Ca^{2+} entry mechanisms.

To examine whether serum affects the store-regulated Ca^{2+} entry pathway, i.e., the capacitative entry [Putney, 1990], IP_3- and TG-sensitive Ca^{2+} stores were emptied by pretreatment with ATP or TG, and followed by a subsequent exposure to FBS. Stimulation of A253 cells with 200 μM ATP induced a potentiated Ca^{2+} influx (218 ± 17 nM/min, n = 9; $P < 0.01$ vs unstimulated cells) (Fig. 8B). Exposure to both ATP and FBS significantly reduced the influx rate (164 ± 8 nM/min, n = 9; $P < 0.05$ vs ATP only) (Fig. 8B). In a similar pattern, exposure to TG (3 μM) induced a 626 ± 82 nM/min Ca^{2+} influx (n = 5). However, exposure to both TG and 0.38% FBS significantly diminished the rate of Ca^{2+} influx (239 ± 15 nM/min, n = 5; $P < 0.002$) (Fig. 8B). These results indicate that serum inhibits Ca^{2+} influx activated by ATP or by TG.

Next, we examined the possibility that serum may reduce extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$), which reduces the driving force of Ca^{2+} influx. As shown in Figure 9, $[Ca^{2+}]_o$ in nominally Ca^{2+}-free solution was 800 ± 15 nM (n = 4). The addition of FBS to final concentrations of 0.5%, 1%, and 2% elevated $[Ca^{2+}]_o$ to 1.51 ± 0.13, 2.19 ± 0.16, and 5.17 ± 0.65 μM, respectively (Fig. 9).

DISCUSSION

Although serum can induce Ca^{2+} mobilization in several cell types, the intracellular me-

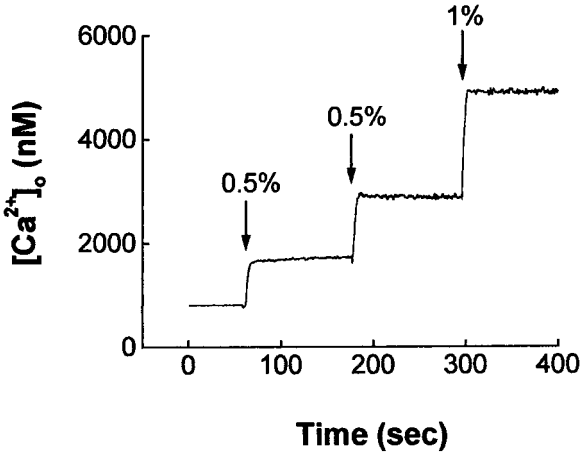

Fig. 9. Effect of FBS on medium Ca^{2+} concentration. The medium ionic Ca^{2+} concentration ($[Ca^{2+}]_o$) in nominally Ca^{2+}-free solution was measured as described under Materials and Methods. The solution contained the same components as physiological salt solution (PSS), except $CaCl_2$ was omitted. $[Ca^{2+}]_o$ was monitored by using the potassium salt of fura-2 (1.2 μM). FBS was added at the time indicated by the arrows. The trace is representative of four separate experiments.

diator(s) are still not clearly defined. There are few, if any, studies on the effect of serum on Ca^{2+} mobilization in salivary cells. Our results in this study clearly show that the two widely used sera, FBS and NBS, elicit a rapid and large Ca^{2+} release from the IP_3- and TG-sensitive Ca^{2+} store(s), possibly mediated by IP_3 formation by PLC. More interestingly, our results also indicate that serum inhibits Ca^{2+} influx, including the store-regulated Ca^{2+} entry.

Serum can elicit Ca^{2+} release in several cell types including human platelets [Amin et al., 1991] and rat neurons and glia [Nunez and Sancho, 1996], but it is still unclear whether serum can also induce a similar Ca^{2+} release in salivary cells. Wu et al. [1996] exposed the human submandibular cell line HSG to 10% FBS, resulting in a large Ca^{2+} release which was comparable to the release elicited by the muscarinic agonist carbachol. The present study observed that FBS and NBS triggered a rapid and large Ca^{2+} release similar to that stimulated by the P_2-purinergic agonist ATP in A253 cells. In contrast to native duct cells, the A253 cell line lacks muscarinic receptors [Marmmary et al., 1989; Zhang et al., 1997a] but discharge IP_3-sensitive Ca^{2+} store in response to extracellular ATP [Zhang et al., 1997a; Sugita et al., in press]. Therefore, we compared the effect of serum with that of ATP, instead of carbachol. The results of the Ca^{2+} release induced by serum observed in these two cell lines in the studies by us and by Wu et al. [1996] are consistent. These results indicate that serum is an effective factor to release Ca^{2+}. Further investigation to compare this effect in cultured cells and freshly isolated salivary cells is of great physiological significance.

The intracellular signal or second messenger mediating the Ca^{2+} release by serum has not been clearly elucidated. Amin et al. [1991] observed that human serum albumin can cause an increase in IP_3 formation and in $^{45}Ca^{2+}$ release in human platelets. However, while FBS and carbachol induced a similar Ca^{2+} release, the IP_3 increase induced by serum was only 20% of that induced by carbachol in HSG cells [Wu et al., 1996]. The results of the present study show that serum-stimulated Ca^{2+} release is mediated by IP_3 in A253 cells. This conclusion comes from two sets of evidence. One is that FBS exposure resulted in a significant increase in IP_3 formation, which is comparable to that induced by ATP. Previous studies [Zhang et al., 1997a; Sugita et al., in press] have shown that ATP triggers a large Ca^{2+} release and a rapid increase in IP_3 formation in this cell line. Another is that inhibition of PLC by U73122 abolished the Ca^{2+} release in response to FBS. The difference between our results and those of Wu et al. may be attributable to the different cell lines used in these studies. Although both HSG and A253 cell lines are submandibular duct cells originated from human tissue, A253 cells have been reported to have characteristics of striated duct cells [Roomans, 1998], but HSG cells have characteristics of intercalated duct cells [Shirasuna et al., 1981]. It may, therefore, possibly reflect the difference in the regulation of PLC activity between intercalated duct cells and striated duct cells.

FBS- or NBS-induced, but not ATP-stimulated, Ca^{2+} release was inhibited by the PTK inhibitor genistein (Figs. 5, 6). These results suggest that the serum-elicited IP_3 formation or Ca^{2+} release is probably mediated by a PTK-regulated process. It is well known that receptor PTKs, such as those activated by growth factor receptors, can activate PLCγ, leading to the formation of IP_3 [Clapham, 1995]. PLCγ can also be activated by nonreceptor PTKs [Archuleta et al., 1993; Holsapple et al., 1996]. It is likely that serum-induced IP_3 formation is mediated by PLCγ activated by PTKs. However,

the activation of PTKs is not through stimulation of EGF receptors in this cell line. Although A253 cells express a large number of EGF receptors [Reiss et al., 1991], direct stimulation of these receptors with EGF did not elicit a Ca^{2+} mobilization response in the present study. This is consistent with previous studies, which indicate that the active component responsible for Ca^{2+} mobilization in serum is not a protein or a peptide, but seems to be a polar lipid [Nadal et al., 1995], or lysophosphatidates [Tigyi et al., 1990; Tigyi and Miledi, 1992], or glutamate bound to serum albumin [Nunez and Garcia-Sancho, 1996]. Further investigations to identify and characterize the active component(s) in serum are required.

The site of Ca^{2+} store sensitive to serum has not been defined in any cell types. In HSG cells, FBS appears to discharge a Ca^{2+} store that is part of the TG-sensitive pool, but distinct from the carbachol-sensitive pool [Wu et al., 1996]. Our results have clearly shown that the FBS-induced Ca^{2+} release is from the IP_3-sensitive and TG-sensitive store(s). In a previous study [Zhang et al., 1997a], we observed that the A253 cells have two Ca^{2+} stores that are sensitive to ATP and TG. These stores are largely, but not totally, overlapped judged by the observations that exposure to one of ATP and TG cannot completely prevent the subsequent release by another. In the present study, exposure to ATP or TG significantly reduced the FBS-induced Ca^{2+} release, indicating that FBS-induced Ca^{2+} release is primarily from these Ca^{2+} stores.

Another interesting effect of serum is on Ca^{2+} influx. Although Ca^{2+} influx plays a critical role in fluid and electrolyte secretion in salivary cells [Melvin et al., 1991], the effect of serum on Ca^{2+} entry pathways remains controversial. It was observed that exposure to serum enhanced Ca^{2+} influx in blastocyst [Fishel, 1980], but did not induce Ca^{2+} influx in *Xenopus* oocytes and fibroblasts [Tigyi et al., 1990; Hopp et al., 1992; Wu et al., 1996]. In the present study, although serum triggered a large Ca^{2+} release, it did not activate Ca^{2+} influx in A253 cells. In fact, FBS strongly inhibited Ca^{2+} influx. This was observed under two experimental conditions. First, exposure cells to FBS in the presence of 1.2 mM extracellular Ca^{2+} did not maintain a sustained elevation in $[Ca^{2+}]_i$ after the initial $[Ca^{2+}]_i$ increase. Since the sustained increase in $[Ca^{2+}]_i$ is primarily due to Ca^{2+} influx, the lack of the $[Ca^{2+}]_i$ elevation strongly suggests there is not sufficient Ca^{2+} influx. Second, as low as 0.1% FBS significantly blocked Ca^{2+} influx activated by addition of 1 mM Ca^{2+} to the cell suspensions which were incubated in a low (0.8 μM) Ca^{2+} medium for 5 min. The inhibition of Ca^{2+} influx by FBS was not due to chelating of extracellular Ca^{2+}, since FBS did not reduce, but actually added extra Ca^{2+} into the media (Fig. 9).

Depletion of the IP_3- or TG-sensitive Ca^{2+} pool can activate Ca^{2+} influx, i.e., capacitative Ca^{2+} entry [Putney, 1990]. Interestingly, FBS dramatically reduced the ATP- and TG-activated Ca^{2+} entry in A253 cells (Fig. 8B). Obviously, this effect is not due to inhibition of store depletion, since serum itself induces large Ca^{2+} release from the ATP- or TG-sensitive store. The mechanism by which serum blocks Ca^{2+} influx cannot be delineated in the present study. The regulation of Ca^{2+} influx, including the capacitative Ca^{2+} entry, is complex and so far poorly understood. A number of factors have been reported to be involved in the regulatory process of the capacitative entry, such as protein kinase C, PTKs, cAMP, cGMP, and other possible proteins or peptides which may be released by Ca^{2+} stores [Putney and Bird, 1993]. Recently, another model, which interprets the type 3 IP_3 receptor located at the plasma membrane as the capacitative Ca^{2+} entry pathway in some cell types, has been suggested [Putney, 1997]. In the present study, the serum-induced Ca^{2+} increase was abolished with PTK inactivation, indicating that PTKs were not inhibited during serum exposure. In addition, serum elevates cellular IP_3 levels, but suppresses the TG-activated Ca^{2+} influx, which indicates that these cells do not have the Ca^{2+} entry pathway constituted by type 3 IP_3 receptors. Furthermore, it is also doubtful that cAMP or cGMP regulates the inhibition of Ca^{2+} influx during serum exposure. There have been no previous report indicating any alterations in cAMP or cGMP levels in response to serum exposure. In addition, cAMP or cGMP is not directly involved in regulation of Ca^{2+} influx in salivary cells [Martinez and Zhang, 1998].

Although the underlying mechanism by which serum inhibits Ca^{2+} influx is unclear, this inhibition may be of important significance. This inhibition probably prevents a long-lasting elevation of $[Ca^{2+}]_i$ in cells which are grown in a medium containing high concentration (usually

10–15%) of FBS or NBS. It is well demonstrated that long term increase in $[Ca^{2+}]_i$ can induce severe cell damage, even cell death [Trump and Berezesky, 1995; Orreniuns et al., 1996; McConkey and Orrenins, 1997]. Moreover, few, if any, physiological factors block the capacitative Ca^{2+} entry. Since human serum albumin can stimulate IP_3 formation and Ca^{2+} release in platelets [Amin et al., 1991], it is intriguing to know whether this inhibition of serum on Ca^{2+} influx is a physiological process. These issues are currently under further investigation.

In summary, the present study has shown that FBS and NBS activate a large Ca^{2+} release from the IP_3- and the TG-sensitive stores, which is mediated by an increased IP_3 formation. Although serum induces Ca^{2+} release, Ca^{2+} influx including the capacitative entry was strongly inhibited by serum. The stimulation of Ca^{2+} release and the inhibition of Ca^{2+} influx by serum provide at least partial explanation for the mitogenic action of serum.

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