Modulation of cytosolic free calcium levels by extracellular phosphate and lanthanum

(cholecystokinin/pancreatic acini/IM-9 lymphocytes)

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ABSTRACT The effects of extracellular phosphate and lanthanum on cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) levels were studied in isolated rat pancreatic acini. In the presence of 1.28 mM Ca^{2+} and 1.0 mM phosphate, the mean resting $[\text{Ca}^{2+}]_i$ level was 120 nM. Omission of phosphate from incubation medium significantly lowered this value to 94 nM. The gastrointestinal hormone cholecystokinin octapeptide (CCK-8) rapidly enhanced both $[\text{Ca}^{2+}]_i$ levels and $^{45}\text{Ca}^{2+}$ efflux, irrespective of the presence or absence of phosphate. Lanthanum (0.1 mM), a compound known to block transmembrane Ca^{2+} fluxes, attenuated both actions of CCK-8, but only in the absence of extracellular phosphate. There was a concomitant decrease in amylase secretion induced by 0.1 nM CCK-8 but not by 10 nM CCK-8, without a significant change in cellular ATP levels. The inhibitory actions of lanthanum on CCK-8-stimulated $[\text{Ca}^{2+}]_i$ levels were very rapid and were mimicked only by prolonged incubation of acini in Ca^{2+} -free medium supplemented with EGTA. Omission of phosphate from incubation medium also lowered basal $[\text{Ca}^{2+}]_i$ levels in IM-9 lymphocytes. These findings suggest that extracellular phosphate may modulate resting $[\text{Ca}^{2+}]_i$ levels in pancreatic acini and other cell types and that mobilization of intracellular Ca^{2+} may partly depend on the availability of a lanthanum-sensitive pool of cell-surface Ca^{2+} that is not readily removed by EGTA.

Severe hypophosphatemia is an important clinical entity that may lead to functional and metabolic disturbances in numerous tissues. These include rhabdomyolysis, congestive heart failure, neurological abnormalities, acute respiratory failure, platelet and leukocyte dysfunction, hemolytic anemia, glucose intolerance, renal bicarbonate wasting, increased calcium mobilization from bone, and hypercalciuria (1-4). Despite the recognized importance of extracellular phosphate *in vivo*, little is known of the mechanisms by which phosphate depletion leads to cellular dysfunction. The primary causes for the widespread manifestations of severe hypophosphatemia are presumed to be depletion of cellular ATP stores and of other critical phosphorus-containing compounds (1). Although intracellular Ca^{2+} is known to be of major importance for proper cellular function, the possibility that phosphate depletion might alter cell function by modulating cytosolic Ca^{2+} levels has not been investigated. The purpose of the present study, therefore, was to evaluate the relationship between extracellular phosphate and cytosolic free calcium $[\text{Ca}^{2+}]_i$ levels. In order to correlate the observed changes in $[\text{Ca}^{2+}]_i$ with subsequent biological activity, isolated rat pancreatic acini were primarily used in the present study.

MATERIALS AND METHODS

Preparation of Isolated Pancreatic Acini. Isolated pancreatic acini were prepared from overnight-fasted male Sprague-Dawley rats (180-250 g), as previously described (5, 6).

Hepes-Ringer (HR) buffer, pH 7.4, used in experiments, contained 10 mM Hepes, 1.28 mM CaCl_2 , 141 mM NaCl, 4.7 mM KCl, 1.13 mM MgCl_2 , 11.1 mM glucose, 1 mg of bovine serum albumin per ml, and 0.01% soybean trypsin inhibitor (5). Sodium phosphate (1.0 mM) was present when specified. Whenever phosphate was omitted from HR buffer, it was replaced with 1.0 mM NaCl. Unless otherwise indicated, HR buffer was supplemented with Eagle's minimum essential medium amino acids (5).

Cell Culture. IM-9 lymphocytes were obtained from the American Type Culture Collection (ATCC CCL 159, Rockville, MD). Cells were propagated in suspension at 37°C in a 5% CO_2 /95% air atmosphere, using RPMI 1640 medium supplemented with antibiotics and 10% fetal bovine serum.

Quin-2 Measurements. $[\text{Ca}^{2+}]_i$ levels were determined fluorometrically, using quin 2 tetraakis(acetoxymethyl ester) (quin 2-AM; Calbiochem) (7). Pancreatic acini and IM-9 lymphocytes were incubated in a shaking water bath (60 cycles/min) at 37°C for 30 and 90 min, respectively, in HR buffer containing 40 μM quin 2-AM. Cells were then rinsed three times in HR buffer devoid of quin 2-AM and amino acids, resuspended in the same rinse buffer, and maintained at 23°C until just prior to the fluorescent determination (7). Except when incubated with EGTA, acini were maintained at this temperature for up to 90 min without any significant change in the fluorescent signal. Fluorescence (F) was determined following centrifugation ($10,000 \times g$ for 2 sec) and resuspension of cells in the rinse buffer at 37°C (7). The quin-2 signal was recorded on a temperature-controlled (37°C) Aminco-Bowman model SPF-125 fluorometer (excitation, 339 nm; emission, 492 nm), using slight magnetic stirring to keep the cells in suspension. Additions were carried out through a porthole after a 4- to 5-min incubation at 37°C to assure a stable fluorescent signal. Maximal fluorescence (F_{max}) was determined after lysis of cells with digitonin (50 μM). To prevent lanthanum from quenching the extracellular quin-2 signal after cell lysis in phosphate-free HR buffer, phosphate (1.0 mM) was added back to the lysate. Minimal fluorescence (F_{min}) was determined after adding 4 mM EGTA and Tris base (final pH, 8.5) to the lysed cell suspension. $[\text{Ca}^{2+}]_i$ was calculated using a K_d of 115 nm for the quin-calcium complex (7): $[\text{Ca}^{2+}]_i = 115 \times [(F - F_{\text{min}})/(F_{\text{max}} - F)]$. Cell viability, monitored by Trypan blue exclusion, was not affected by quin 2-AM.

Biological Activity Studies. To measure amylase secretion, $^{45}\text{Ca}^{2+}$ efflux, and cellular ATP levels, acini were incubated at 37°C as previously reported (5, 6). Amylase was assayed using amylose azure and expressed as units of activity (μmol of maltose released per min) per mg of acinar cell protein (5). We have previously shown that under the present incubation conditions CCK-8 does not alter cellular protein content (8). Protein was assayed by the method of Lowry *et al.* (9), using bovine serum albumin as a standard. $^{45}\text{Ca}^{2+}$ efflux was measured as previously reported after loading acini for 60 min

Table 1. Effect of extracellular phosphate on cytosolic free calcium levels

	Extracellular phosphate, mM	Cytosolic free calcium, nM
Pancreatic acini	1.0	128 ± 9 (24)
	None added	95 ± 5 (26)*
IM-9 lymphocytes	1.0	141 ± 5 (6)
	None added	108 ± 6 (6)*

Cells were incubated at 23°C with or without 1.0 mM phosphate for periods ranging from 1 to 90 min. Cells were then centrifuged and resuspended in the respective buffers at 37°C for 4–5 min before determination of the fluorescent signal. Values are means ± SEM from the number of determinations in parentheses.

* $P < 0.005$ when compared with the corresponding values in the presence of phosphate.

at 37°C with 2 μCi of $^{45}\text{Ca}^{2+}$ per ml (1 Ci = 37 GBq) (5, 10). ATP levels (nmol per mg of protein) were measured using luciferin-luciferase (11). Synthetic CCK-8 was a gift from Miguel Ondetti (Squibb, New Brunswick, NJ).

Statistical Analysis. Statistical analysis was done by Student's t test. Data were also analyzed by analysis of variance using an established computer program (Apple Macintosh Plus) to do multiple comparisons. Data were considered statistically significant at $P < 0.05$.

RESULTS

Effect of Omission of Extracellular Phosphate on Cytoplasmic Free Ca^{2+} Levels. Following quin 2-AM loading, pancreatic acini and IM-9 lymphocytes were resuspended in HR buffer containing either 1.0 mM phosphate or no added

phosphate. The resting mean $[\text{Ca}^{2+}]_i$ levels in pancreatic acini were 128 nM in the presence of phosphate and 95 nM in its absence ($P < 0.005$; Table 1). The corresponding values in IM-9 lymphocytes were 141 nM and 108 nM, respectively ($P < 0.005$; Table 1). These values were not significantly altered by 0.1 mM lanthanum (data not shown).

CCK-8, when added at a concentration of 0.1 nM (data not shown) or 10 nM (Fig. 1A and B), rapidly enhanced the quin-2 fluorescent signal in pancreatic acini. The percent increase in $[\text{Ca}^{2+}]_i$ when acini were incubated in complete HR buffer (Fig. 2A) was 77 ± 11 (mean ± SEM, $P < 0.005$) for 0.1 nM CCK-8, and 100 ± 12 (mean ± SEM, $P < 0.005$) for 10 nM CCK-8. Omission of phosphate from incubation medium (Fig. 1C), or addition of lanthanum (0.1 mM) to complete HR buffer (Fig. 1E), did not significantly alter the effects of CCK-8 on either quin-2 fluorescence or on the calculated percent increase in $[\text{Ca}^{2+}]_i$ (Fig. 2A). However, in the absence of extracellular phosphate, lanthanum (0.1 mM) markedly attenuated the increase in $[\text{Ca}^{2+}]_i$ induced by either 0.1 nM or 10 nM CCK-8 (Figs. 1F and 2C).

Effect of Chelation of Extracellular Calcium on Cytoplasmic Free Ca^{2+} Levels. When pancreatic acini were incubated for 10 min at 23°C in the absence of extracellular Ca^{2+} and phosphate, but in the presence of EGTA (0.1 mM), the ability of CCK-8 to increase the quin-2 fluorescent signal was not altered (Fig. 1D). However, prolonged incubation of acini with EGTA prior to addition of CCK-8 resulted in a progressive decrease in the strength of the signal. Complete loss of stimulation by CCK-8 occurred following 90 min of incubation with EGTA at 23°C (Fig. 1G).

Effect of Omission of Extracellular Phosphate on Biological Actions of CCK-8. CCK-8 exerts a biphasic effect on amylase

FIG. 1. Effect of CCK-8 on quin-2 fluorescent signal. Unless otherwise specified, pancreatic acini were incubated for 5 min at 23°C in HR buffer in the presence (A, B, and E) or absence (C, D, F, and G) of 1.0 mM phosphate and in the presence (E, F) or absence (A–D, G) of 0.1 mM lanthanum. Acini were then incubated for 4–5 min at 37°C before making the additions indicated by the arrows. In D and G Ca^{2+} was also omitted from the phosphate-free HR buffer and was replaced with 0.1 mM EGTA. Acini were then incubated at 23°C for 10 min (D) or 90 min (G) prior to the 37°C incubation. Digitonin was added at a concentration of 50 μM . Each tracing is representative of at least three other tracings.

FIG. 2. Effect of lanthanum on CCK-8-mediated biological actions. Pancreatic acini were incubated at 37°C in the absence (open bars; circles) or presence (solid bars; triangles) of 0.1 mM lanthanum. (A and C) The percent increase in $[Ca^{2+}]_i$ following the indicated additions was determined as in Fig. 1, in the presence (A) and absence (C) of 1.0 mM phosphate. Values are means $\pm$ SEM from five to twelve experiments. (B) Amylase release was determined in the absence of phosphate in control (CON) and CCK-8-treated pancreatic acini. Lanthanum was added 10 min before addition of CCK-8, and incubations were stopped 30 min later. Values are means $\pm$ SEM from three experiments. (D) Amylase release was determined in the absence of phosphate and in the absence ($\circ$, Δ) or presence ($\bullet$, $\blacktriangle$) of 10^{-10} M (0.1 nM) CCK-8. Lanthanum was added 10 min before addition of CCK-8. Values are means $\pm$ SEM from three experiments. *, $P < 0.02$; **, $P < 0.005$ when compared with corresponding values in the absence of lanthanum.

release in pancreatic acini—maximal stimulation occurring at 0.1–0.3 nM CCK-8 (5). Higher (supramaximal) concentrations of CCK-8 exert a submaximal stimulatory effect termed restricted stimulation (12). In contrast, CCK-8-mediated $^{45}Ca^{2+}$ efflux, an indirect measure of cellular Ca^{2+} mobilization (13), is maximal at a concentration of 10 nM (11). In the present study, omission of phosphate from the incubation medium did not significantly alter the actions of CCK-8 on either amylase release or $^{45}Ca^{2+}$ efflux (data not shown). However, in the absence of extracellular phosphate, 0.1 mM lanthanum decreased amylase release induced by a maximally effective concentration of CCK-8 (0.1 nM) to a level comparable to that observed with 10 nM CCK-8 (Fig. 2B). This effect was significant within the first 10 min of incubation (Fig. 2D). Further, lanthanum significantly decreased the effect of 0.1 nM CCK-8 on $^{45}Ca^{2+}$ efflux (Table 2) but did not alter either the stimulatory effect of a supramaximal concentration (10 nM) of CCK-8 on amylase release (Fig. 2B) or cellular ATP levels (Table 3).

DISCUSSION

The resting $[Ca^{2+}]_i$ level in eukaryotic cells is dependent on the rate of Ca^{2+} influx and efflux and on the buffering capacity of intracellular organelles (14). Increases in $[Ca^{2+}]_i$ may be due to enhanced Ca^{2+} influx or to release of Ca^{2+} from intracellular stores (14, 15). In the present study, incubation at 37°C of pancreatic acini and IM-9 lymphocytes in phosphate-free buffer resulted in a rapid and significant decrease in the resting $[Ca^{2+}]_i$ levels. Prolonged incubation of either cell type at 23°C did not further decrease $[Ca^{2+}]_i$. Lanthanum, an agent that displaces cell-surface Ca^{2+} (16), did not alter resting $[Ca^{2+}]_i$, irrespective of the presence or

absence of phosphate in the medium. Further, CCK-8-mediated stimulation of $[Ca^{2+}]_i$ was not altered by incubating acini in phosphate-free medium. These findings suggest that regulation of resting $[Ca^{2+}]_i$ levels by extracellular phosphate is partly a temperature-dependent process that is not necessarily linked to either surface-bound or organelle-sequestered Ca^{2+} pools.

Ca^{2+} -mobilizing hormones such as CCK-8 raise $[Ca^{2+}]_i$ levels by generating inositol 1,4,5-trisphosphate (IP_3) (15, 17), which then releases Ca^{2+} from intracellular stores (18–20). Some of these hormones (21), including CCK-8 (10, 22), also raise $[Ca^{2+}]_i$ levels by enhancing Ca^{2+} influx. This effect is relatively minor in the exocrine pancreas (23) and is completely blocked by lanthanum (10). In the present study, addition of lanthanum to acini incubated in phosphate-free

Table 2. Effect of lanthanum on $^{45}Ca^{2+}$ efflux

Addition	$^{45}Ca^{2+}$ Ratio
None	0.31 ± 0.014
Lanthanum (0.1 mM)	0.31 ± 0.001
CCK-8 (0.1 nM)	$0.10 \pm 0.011^*$
CCK-8 (0.1 nM) + lanthanum (0.1 mM)	$0.15 \pm 0.012^{*+}$

Pancreatic acini were incubated for 60 min at 37°C in HR buffer containing $^{45}Ca^{2+}$ (2 μ Ci/ml). Acini were then centrifuged, rinsed, and resuspended at 37°C (zero time) in phosphate-free buffer containing the indicated additions and devoid of radioactivity. Incubation was stopped 20 min later by rapid filtration of acini. Data are expressed as the ratio of $^{45}Ca^{2+}$ remaining in acini at 20 min by comparison with $^{45}Ca^{2+}$ present in acini at zero time. Values are means $\pm$ SEM of 3 experiments.

* $P < 0.005$ when compared with the respective control values.

⁺ $P < 0.05$ when compared with the corresponding value in the absence of lanthanum.

Table 3. Lack of lanthanum effect on cellular ATP levels

Addition	ATP level, nmol per mg of protein
None	13.70 ± 0.26
Phosphate (1.0 mM)	15.34 ± 0.38
Lanthanum (0.1 mM)	17.73 ± 1.59
CCK-8 (0.1 nM)	13.59 ± 1.15
CCK-8 (0.1 nM) + phosphate (1.0 mM)	15.17 ± 0.41
CCK-8 (0.1 nM) + lanthanum (0.1 mM)	16.12 ± 0.66
CCK-8 (10 nM)	13.79 ± 0.18
CCK-8 (10 nM) + phosphate (1.0 mM)	12.68 ± 0.34
CCK-8 (10 nM) + lanthanum (0.1 mM)	15.09 ± 0.99

Pancreatic acini were incubated for 10 min at 37°C in HR buffer containing 1.0 mM phosphate and 0.1 mM lanthanum only when indicated. Additions of CCK-8 were made next, and incubations stopped 30 min later. Values are means ± SEM from three experiments.

medium markedly attenuated the CCK-8-mediated increase in $[Ca^{2+}]_i$. This decrease was considerably greater than would be expected by simply blocking Ca^{2+} influx. Further, the effect of lanthanum was much more rapid than that observed following incubation of acini with EGTA, indicating that chelation of extracellular Ca^{2+} , even in the absence of extracellular phosphate, does not block the rise in $[Ca^{2+}]_i$. These findings support the hypothesis that pancreatic acini have a tightly bound pool of cell-surface Ca^{2+} (22) and suggest that this Ca^{2+} pool may help trigger the mobilization of Ca^{2+} from intracellular stores.

Lanthanum is known to readily quench the quin-2 signal (24). However, it is unlikely that this was the cause for the lanthanum-mediated inhibition of the fluorescent signal under the incubation conditions employed in the present study. First, the quin-2 signal was generated inside the cell, whereas lanthanum does not penetrate the pancreatic acinar cell (22, 25). Second, lanthanum did not alter resting $[Ca^{2+}]_i$ levels. Third, in the absence of phosphate, lanthanum attenuated CCK-8-induced $^{45}Ca^{2+}$ efflux as well as the secretory action of a maximally effective concentration (0.1 nM) of CCK-8, indicating that there was decreased mobilization of cellular Ca^{2+} stores.

Lanthanum failed to block the action of 10 nM CCK-8 on secretion, while markedly attenuating the CCK-8-induced rise in $[Ca^{2+}]_i$. This observation is in agreement with previous reports that secretion mediated by supramaximal concentrations of CCK-8 (10 nM) is not altered by chelation of extracellular Ca^{2+} (6). Further, the present findings suggest that the secretory effects of CCK-8 may be less dependent on a marked rise in $[Ca^{2+}]_i$ than is generally accepted, but that a small component of the secretory action of 0.1 nM CCK-8 is highly dependent on the rise in $[Ca^{2+}]_i$. The secretory response that was not blocked by lanthanum may either be mediated by elevations of local zones of $[Ca^{2+}]_i$, or by additional signals that have a decreased requirement for Ca^{2+} following receptor activation by CCK-8.

Lanthanum did not block the effects of CCK-8 on amylase release, $[Ca^{2+}]_i$, or $^{45}Ca^{2+}$ efflux when phosphate was present in the medium. It is possible that the formation of a phosphate-lanthanum complex precluded lanthanum from displacing cell surface Ca^{2+} . Alternatively, it is possible that in the absence of extracellular phosphate there is depletion of certain critical phosphate stores. This depletion may cause a decrease in phospholipid synthesis, as previously reported in lymphocytes

(26), thereby allowing lanthanum to more effectively lower $[Ca^{2+}]_i$ levels. It is unlikely that lanthanum acted by inhibiting cellular energy metabolism inasmuch as lanthanum does not inhibit oxidative phosphorylation in the exocrine pancreas (27) and did not lower cellular ATP levels in the present study in spite of the absence of extracellular phosphate.

Taken together, the present observations suggest that caution should be exercised in comparing published $[Ca^{2+}]_i$ values in various cell types, unless the extracellular phosphate concentration is considered. Further, our findings raise the possibility that low phosphate levels *in vivo* may lead to altered cell function by modulating $[Ca^{2+}]_i$ levels.

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