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Cytosolic Free Calcium Spiking Affected by Intracellular pH Change

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The characteristics underlying cytosolic free calcium oscillation were evaluated by superfused dual wavelength microspectrofluorometry of fura-2-loaded single acinar cells from rat pancreas. Application of a physiological concentration of cholecystokinin octapeptide (CCK) (20 p*M*) induced a small basal increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) averaging 34 n*M* above the prestimulation level (69 n*M*) with superimposed repetitive Ca^{2+} spike oscillation. The oscillation amplitude averaged 121 n*M* above the basal increase in $[Ca^{2+}]_i$ and occurred at a frequency of one pulse every 49 s. Although extracellular Ca^{2+} was required for maintenance of high frequency and amplitude of the spikes with increase in basal $[Ca^{2+}]_i$, the primary source utilized for oscillation was intracellular. The threshold of the peak $[Ca^{2+}]_i$ amplitude for causing synchronized and same-sized oscillations was less than 300 n*M*. The $[Ca^{2+}]_i$ oscillation was sensitive to intracellular pH (pH_i) change. This is shown by the fact that the large pH_i shift toward acidification (ΔpH_i decrease, 0.95) led to a basal increase in $[Ca^{2+}]_i$ to the spike peak level with inhibiting Ca^{2+} oscillation. The pH_i shift toward alkalinization (ΔpH_i increase, 0.33) led to a basal decrease in $[Ca^{2+}]_i$ to the prestimulation level, possibly due to reuptake of Ca^{2+} into the Ca^{2+} stores, with inhibiting Ca^{2+} oscillation. Whereas extracellular pH (pH_o) change had only minimal effects on Ca^{2+} oscillation (and/or Ca^{2+} release from intracellular stores), the extra-Ca^{2+} entry process, which was induced by higher concentrations of CCK, was totally inhibited by decreasing pH_o from 7.4 to 6.5. Thus the major regulatory sites by which H^+ affects Ca^{2+} oscillation are accessible from the intracellular space.

INTRODUCTION

Cytosolic free calcium ($[Ca^{2+}]_i$) is now widely believed to play a key role in stimulus–secretion coupling. Five components are found to contribute to calcium mobilization: initial Ca^{2+} release from intracellular stores, sustained Ca^{2+} entry from the extracellular space, Ca^{2+} efflux across the plasma membrane, Ca^{2+} reuptake (and/or refilling) into the stores, and repetitive Ca^{2+} spike oscillation. The final component, Ca^{2+} oscillation, has been found in various cell types, including nonexcitable cells, muscle cells, neuronal cells, and differentiated cells (summarized in Refs. [1–3]). It seems that the digitized form of Ca^{2+} signaling oscillation plays a fundamental role in cellular signal transduction because lower and more physiological concentrations of agonist lead to synchronized periodic Ca^{2+} spikes rather than to an initial large rise in $[Ca^{2+}]_i$ followed by a sustained $[Ca^{2+}]_i$ plateau above the prestimulation level in various cell types. The oscillation is observed in single cells since studies using whole populations are hindered by the mixture of different individual cells in time and duration. The molecular mechanism and biological significance of the oscillatory mode of Ca^{2+} signaling are still unknown, although considerable progress has been made in understanding them, including in pancreatic acinar cells [4–8]. This study examined the characteristics underlying cytosolic free calcium oscillation in single pancreatic acinar cells, using a fura-2 dual wavelength microspectrofluorometer equipped with a superfusion system. The major finding is that cytosolic Ca^{2+} oscillation might be sensitive to intracellular pH change.

MATERIALS AND METHODS

Materials. Cholecystokinin octapeptide, carbamylcholine, NH_4Cl, propionate, and digitonin were obtained from Sigma (St. Louis, MO). Fura-2/AM (acetoxymethyl ester type) and BCECF/AM (acetoxymethyl ester type) were from Molecular Probes (Eugene, OR). Nigericin was from Calbiochem (La Jolla, CA). The physiological salt solutions (PSS) used contained the following (in m*M*): PSS-A—NaCl, 137; KCl, 4.7; $MgCl_2$, 0.57; $CaCl_2$, 1.28; NaH_2PO_4, 1; *N*-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 10; *l*-glutamine, 2; *d*-glucose, 5.5, and 2% MEM essential amino acids (GIBCO, Grand Island, NY) neutralized with NaOH, adjusted at pH 7.4, and equilibrated with 100% O_2; PSS-B—NaCl, 130; K_2HPO_4, 2.5; $MgSO_4$, 1; $CaSO_4$, 1; Hepes, 10; *d*-glucose, 10, adjusted at pH 7.35 and equilibrated with 100% O_2. The calcium-free solution in PSS-B was identical except that $CaSO_4$ was replaced with 1 m*M* ethyleneglycol bis(2-aminoethyl ester)-*N,N,N',N'*-tetraacetic acid (EGTA).

Preparation of cells and dye loading. Isolated pancreatic acini were prepared from fed male Sprague–Dawley rats (weighing 250 g) according to the collagenase digestion procedure described previously [9]. Isolated acini were incubated with 1 μM fura-2/AM for 20 min at 37°C in PSS-A, containing 0.01% soybean trypsin inhibitor (type I-S, Sigma) and 0.1% bovine serum albumin. Fura-2-loaded cells were then

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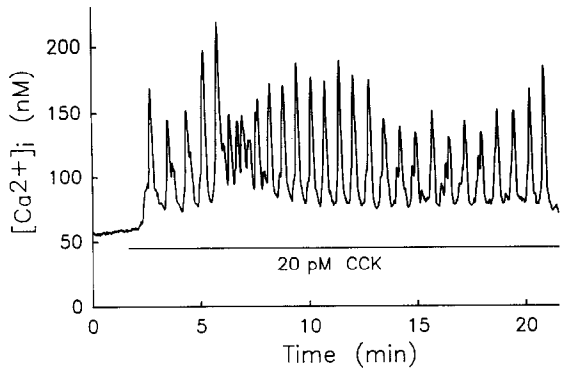

FIG. 1. Typical Ca^{2+} oscillation pattern induced by 20 p*M* cholecystokinin octapeptide (CCK). The data are representative of 32 individual cells. See text for details.

washed by centrifugation at 50*g* and resuspended in fresh PSS-A. Cells were stored as a concentrated suspension at room temperature for up to 3 h. For pH_i measurements, cells were similarly incubated with 2 μM BCECF/AM for 30 min at 37°C, shielded from light, and then handled in a manner identical to that of acini loaded with fura-2.

Measurement of $[Ca^{2+}]_i$. Measurement of fura-2 fluorescence from individual acinar cells and calculation of $[Ca^{2+}]_i$ was performed as previously described [7, 8]. To measure fluorescence of single cells in the superfusion system, a portion of acinar suspension was placed on a thin glass coverslip mounted at the bottom of a rectangular chamber (internal volume 0.1 ml). Acini were superfused at a flow rate of 1 ml/min from a temperature-regulated (37°C) multiple-solution reservoir. The chamber was mounted on an aluminum-blocked 37°C heated stage of a Nicon Diaphot inverted microscope equipped with a 40× oil immersion epifluorescence lens (1.3 NA). Single cells bulging from an acinus were masked using a pinhole diaphragm stopped down to an optical diameter of 10 μm, the size of a single acinar cell without background. The solution used for superfusion was PSS-B, containing 0.05% bovine serum albumin unless otherwise noted. $[Ca^{2+}]_i$ measurements in single cells were obtained using a modular fluorometer system (Spex Fluorog 2). Excitation light from a 450-W xenon lamp was provided by two monochrometers preset at 340 nm (slit 0.5 nm) and 380 nm (slit 0.5 nm), respectively, altered by a rotating chopper mirror. The excitation beam was further deflected to the sample by a dichroic mirror (DM 400). Emitted light was monitored at an emission wavelength of 510 ± 20 nm by a bandpass filter fixed on a photomultiplier tube. The fluorometer and photometer were coupled to a Spex Datamate microcomputer that stored two alternating fluorescence signals in separate memories to obtain a 340/380-nm ratio. Each signal was averaged over 0.5-s intervals and displayed. The fluorescence ratio was calculated off-line and converted to $[Ca^{2+}]_i$ [7, 8]. Autofluorescence determined in single cells was found to contribute less than 3–5% of the emitted signal and was not subtracted.

Measurements of pH_i. Experiments with pH_i in single cells were carried out using excitation wavelengths of 490 nm (slit 1 nm) and 439 nm (slit 1 nm); a neutral density filter (30% transmittance) was inserted in front of each beam to reduce incident light. Excitation beams were further deflected to the sample by the dichroic mirror (DM 500) and emitted light passed through a 520- to 560-nm bandpass filter. For each measurement the fluorescence ratio from the cells was calibrated to yield pH_i by use of an internal calibration method [10]. The cell in the chamber was superfused with 10 μM K^+–H^+ ionophore nigericin solubilized in KCl buffer which contained (in m*M*): KCl, 145; K_2HPO_4, 2.5; $CaSO_4$, 1.0; $MgSO_4$, 1.0, and Hepes, 10. Then the pH of the medium was changed from 6.8 to 7.6 to obtain a pH-to-fluorescence ratio for every measurement. Under these conditions, BCECF fluorescence varied in a linear fashion. Subsequent to pH_i calibration, cellular autofluorescence was measured by superfusion with 75 μM digitonin solubilized in KCl buffer to release tapped intracellular BCECF. Autofluorescence values for each wavelength were subtracted from each averaged emission intensity value at that wavelength prior to obtaining the ratio of fluorescence intensities (percentage of autofluorescence, 8.5% at 490 nm and 12.5% at 439 nm).

RESULTS

Characteristics of Cytosolic Free Calcium Oscillation

Resting $[Ca^{2+}]_i$ in single pancreatic acinar cells was 69 ± 4 n*M* (n = 32). Lower and more physiological concentrations of cholecystokinin octapeptide (CCK, 20 p*M*) induced a small basal increase in $[Ca^{2+}]_i$ (amplitude, 34 ± 3 n*M*) with superimposed repetitive transient increases after a latent period (60–90 s) (Fig. 1). These oscillations were 121 ± 14 n*M* in amplitude above the basal increase in $[Ca^{2+}]_i$; they occurred at a frequency of one pulse every 48.6 ± 3.2 s and were maintained as long as the stimulus was applied (at least up to 50 min).

Although extracellular Ca^{2+} was required for maintenance of both spike amplitude and frequency, the basic source utilized for oscillations was intracellular since elimination of Ca^{2+} from the medium failed to inhibit them for 10 min (Fig. 2). Amplitude and frequency of the Ca^{2+} spike were reduced to 100 ± 17 n*M* and to one pulse every 50.4 ± 8.0 s on the average (n = 8) when extracellular Ca^{2+} was eliminated by EGTA. These changes were reversible on Ca^{2+} readdition by changing the superfusion medium. A small basal increase in $[Ca^{2+}]_i$ was, on the other hand, totally dependent on extracellular Ca^{2+}.

Some reports have suggested that Ca^{2+} may exert its action in a frequency-dependent rather than an amplitude-dependent manner and the frequency of Ca^{2+} spiking also increased when the concentration was raised [1–3]. As shown in Fig. 3, frequency was, however, reciprocally proportional to amplitude that was induced by 1–30 p*M* CCK. A small amplitude decreased the interval of each signal, resulting in a high frequency of spikes.

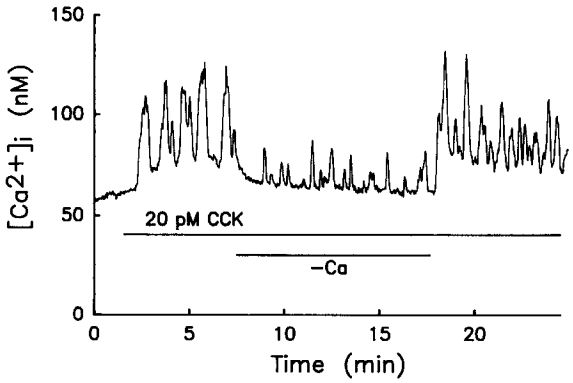

FIG. 2. Typical Ca^{2+} oscillation pattern in the presence and in the absence of extracellular Ca^{2+}. The data are representative of eight separate experiments. See text for details.

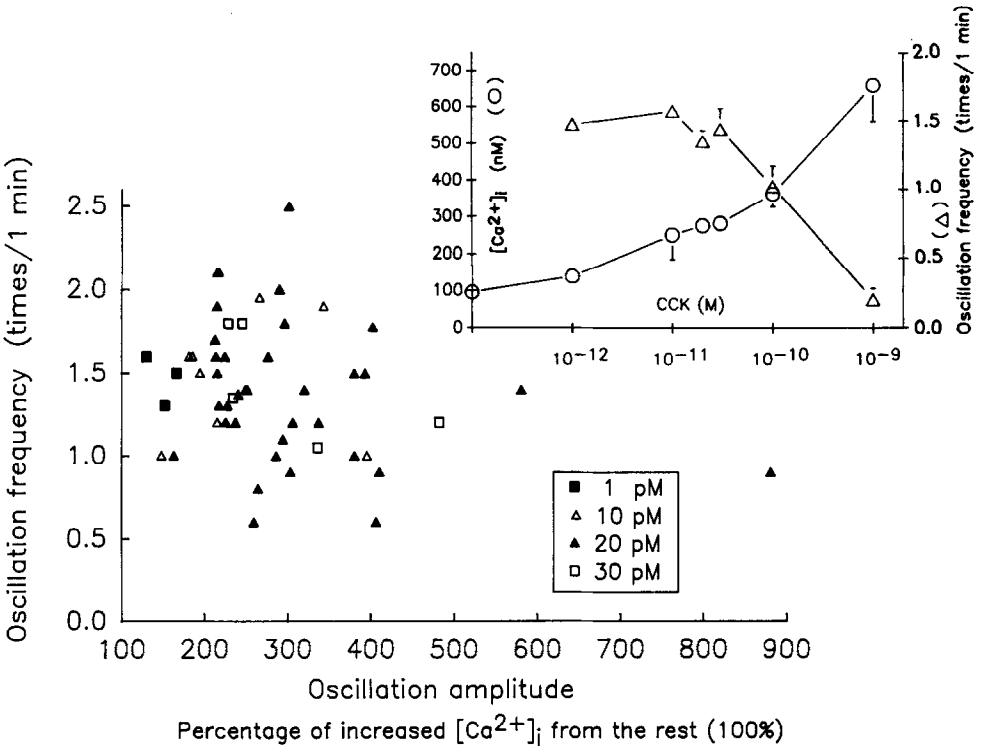

FIG. 3. Amplitude and frequency of Ca^{2+} oscillation induced by various concentrations of CCK. The data were obtained from 50 single cells that caused regular-sized Ca^{2+} oscillation when 1–30 p*M* CCK was applied. The *P* value (amplitude versus frequency) was 0.1047. Oscillation amplitude (percentage of increased $[Ca^{2+}]_i$ from the rest) was calibrated from each individual cell. (Inset) Dose–response curve of CCK on Ca^{2+} mobilization. Amplitude of peak $[Ca^{2+}]_i$ from the prestimulation level was dose-dependent. CCK at 1, 10, 20, 30, and 100 p*M* and 1 n*M* elevated $[Ca^{2+}]_i$ from 94 ± 4 n*M* (n = 119) to 138 ± 28 n*M* (n = 3), to 250 ± 66 n*M* (n = 10), to 275 ± 13 n*M* (n = 32), to 281 ± 24 n*M* (n = 5), to 326 ± 36 n*M* (n = 10), and to 660 ± 100 n*M* (n = 11), respectively. The maximum oscillation frequency was obtained when 10 p*M* CCK was applied (1.57 ± 0.12 times/min). Frequency was almost constant from 1 to 30 p*M* CCK. At 100 p*M* CCK, both oscillation frequency and amplitude were reduced, resulting in an initial global rise in $[Ca^{2+}]_i$ followed by the sustained $[Ca^{2+}]_i$ plateau above the prestimulation level. CCK (1 n*M*) has essentially caused the oscillation frequency to disappear. When 1 n*M* CCK was used, only 18% (2/11) of cells showed oscillation which occurred after $[Ca^{2+}]_i$ had almost reached baseline. Application of 1–30 p*M* CCK failed to cause the initial global rise in $[Ca^{2+}]_i$ but induced repetitive and regular-sized oscillation in most cases (50/56 cells). Six cells were silent to 1–30 p*M* CCK stimulation. The data suggest that the threshold of peak $[Ca^{2+}]_i$ for generating synchronized Ca^{2+} oscillation is below 300 n*M*.

In addition, there was no significant difference in spike frequency from 1 to 30 p*M* CCK (Fig. 3, inset). The amplitude of the increased $[Ca^{2+}]_i$ seems to give a threshold for generating synchronized Ca^{2+} oscillation. When the amplitude of the increased $[Ca^{2+}]_i$ was above 300 n*M*, which was usually induced by high concentrations of CCK (0.1–1 n*M*), the signaling mode of Ca^{2+} consisted of an initial global rise in $[Ca^{2+}]_i$ followed by a sustained plateau of $[Ca^{2+}]_i$ without synchronized oscillation (Fig. 3, inset, and Fig. 6). Thus, the frequency and the mode of Ca^{2+} spikes were closely related to $[Ca^{2+}]_i$ amplitude. Although basal $[Ca^{2+}]_i$ levels remained low during 20 p*M* CCK-induced oscillation, the total integrated area of the increased $[Ca^{2+}]_i$ in the cytosol over a 30-min period was the same or similar to that evoked by 1 n*M* CCK (not shown). Both concentrations of CCK caused an almost identical release of amylase over a 30-min period [11]. This suggests an efficient signaling transmission of the oscillation with maintenance of low levels of $[Ca^{2+}]_i$ during cell stimulation.

In addition, there was considerable individual variation among cells exhibiting a unique pattern of oscillations with respect to amplitude, shape, frequency, and latency at the onset of the initial response. The lowest and highest values before and after stimulation of cells with 20 p*M* CCK were as follows, respectively: resting $[Ca^{2+}]_i$, 29 and 138 n*M;* peak $[Ca^{2+}]_i$ level, 95 and 370 n*M;* percentage increase in $[Ca^{2+}]_i$ from the rest, 162 and 881%; frequency, 1 pulse/24-100 s; time latency, 30 and 120 s (n = 32). This suggests that individual cells exhibited an ansynchronous response pattern to hormonal stimulation.

Relationship between Cytosolic Free Calcium Oscillation and Intracellular pH

The molecular mechanism and biological significance of the Ca^{2+} oscillation are still unknown. Since the close relationship between Ca^{2+} mobilization and pH_i has been extensively discussed [12–14], the effect of pH_i on

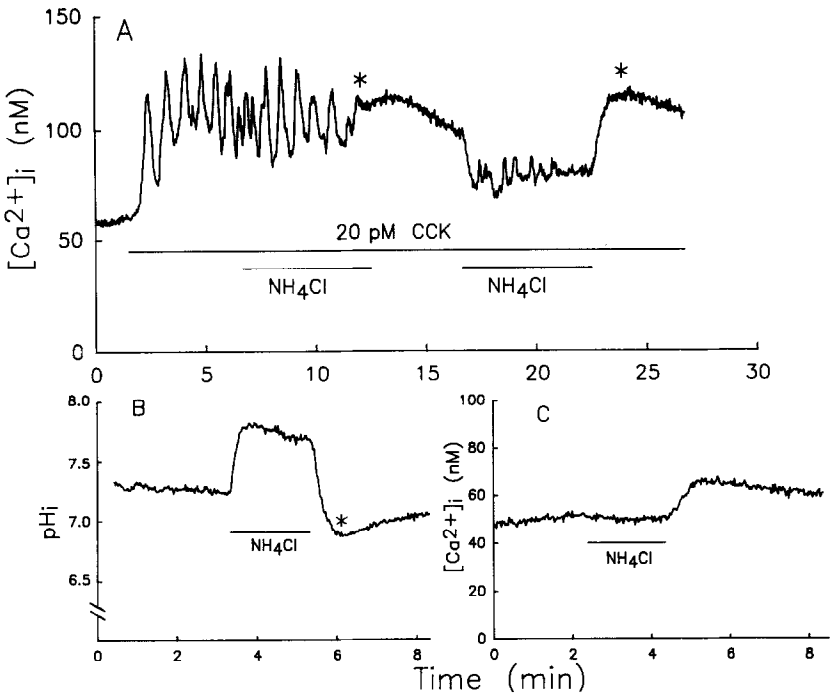

FIG. 4. Effects of pH_i on CCK-induced Ca^{2+} oscillation. Increasing basal $[Ca^{2+}]_i$ with inhibition of oscillations was observed when NH_4Cl was removed by changing the superfusion medium (*). The concentration of NH_4Cl was 20 m*M*. The data are representative of eight separate (A) and three to six separate (B, C) experiments.

Ca^{2+} oscillation was examined. To examine the relationship between oscillation and pH_i, the effects of NH_4Cl were studied as NH_4Cl addition initially induces an intracellular alkalinization which after its removal is followed by acidification resulting from binding and dissociation of H^+ to NH_3 in the cytosol [15]. Conspicuous changes in oscillation were observed when pH_i was switched by withdrawing and reapplying NH_4Cl (Fig. 4A). The first application of NH_4Cl (20 m*M*) during CCK-induced repetitive Ca^{2+} spikes affected the oscillation less. However, the acidification process, changing from the alkalinization phase with removal of NH_4Cl, caused an increase in basal $[Ca^{2+}]_i$ up to the spike peak level, inhibiting Ca^{2+} oscillation. Reapplication of NH_4Cl, which caused an alkalinization from subresting pH_i levels, immediately decreased the $[Ca^{2+}]_i$ level to the prestimulation level. It seems that this decrease in $[Ca^{2+}]_i$ is due to reuptake of Ca^{2+} into the stores since brisk spikes appeared again after a decrease in basal $[Ca^{2+}]_i$. Figure 4B is evidence that NH_4Cl changes pH_i. When BCECF-loaded single cells were treated with 20 m*M* NH_4Cl, the increase in pH_i was 0.73 unit (from resting pH_i 7.18 ± 0.06 (n = 6) to 7.91 ± 0.27 (n = 3)). Withdrawal of NH_4Cl resulted in a large acid shift (to 6.96 ± 0.18 (n = 3)) followed by the recovery phase of pH_i toward the prestimulation level. Application of NH_4Cl did not produce any significant changes in $[Ca^{2+}]_i$ and its removal (acidification) caused a small increase in basal $[Ca^{2+}]_i$ (20 ± 1 n*M* increase above the prestimulation level) (Fig. 4C). It was evident that an increase and/or decrease in pH_i alone did not produce any significant changes in $[Ca^{2+}]_i$. On the other hand, CCK alone did not produce any significant changes in pH_i (not shown). Therefore, both inhibition of oscillation and basal increase or decrease in $[Ca^{2+}]_i$ are required for the combined effects of CCK plus pH_i change. Similar oscillatory change during cell stimulation with CCK and a varied pH_i was observed when propionate was applied, producing acidification followed by alkalinization after removal of propionate [15]. Application of propionate (20 m*M*) during cell stimulation with 20 p*M* CCK, which resulted in acidification from the resting pH_i, affected the oscillation less (Fig. 5A). Withdrawal of propionate, which resulted in alkalinization changing from the acidification phase, caused a decrease in basal $[Ca^{2+}]_i$ to the prestimulation level, inhibiting Ca^{2+} oscillation. After pH_i returned to near the prestimulation level the oscillation recurred, possibly due to Ca^{2+} reuptake into the stores. The evidence that propionate leads to pH_i change is shown in Fig. 5B. Application of propionate (20 m*M*) decreased pH_i to 6.99 ± 0.16 (n = 3) (0.19 unit) followed by the slow recovery phase toward the subresting pH_i level. Withdrawal of propionate resulted in alkalinization, changing from the acid phase (pH_i 7.32 ± 0.17, n = 3), followed by a recovery phase near the prestimulation level. As well as NH_4Cl, application of propionate (acidification) resulted in a small increase in $[Ca^{2+}]_i$ (18 ± 2 n*M* above basal, n = 4), suggesting again that pH_i

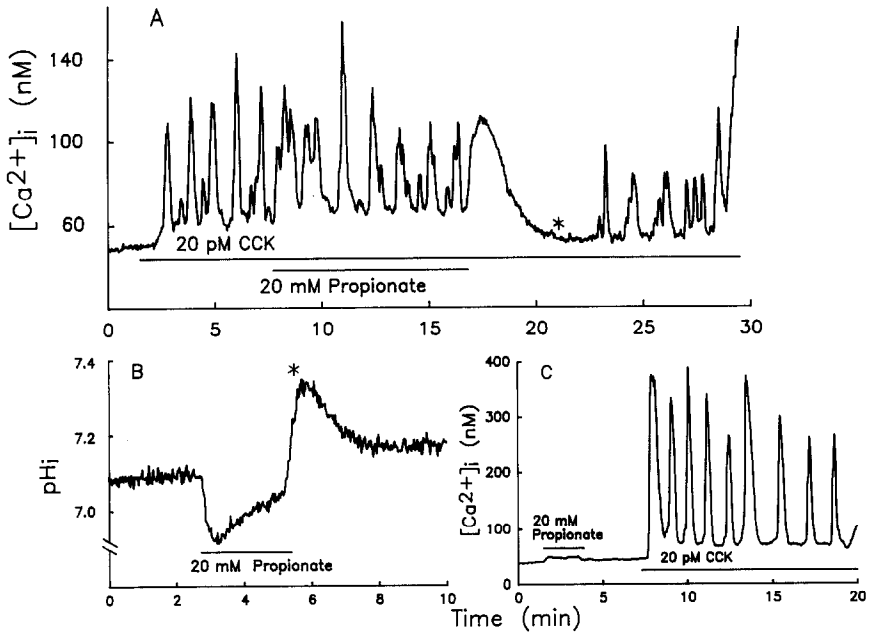

FIG. 5. Effects of pH_i change on CCK-induced Ca^{2+} oscillation. Decreasing basal $[Ca^{2+}]_i$ with inhibition of oscillations was observed when propionate was removed by changing the superfusion medium (*). The concentration of propionate was 20 m*M*. The data are representative of eight separate (A) and three to six separate experiments (B, C).

change alone in the absence of CCK is not sufficient for changing Ca^{2+} spikes (Fig. 5C).

Since intracellular pH change affects Ca^{2+} oscillation, the effect of altering extracellular pH (pH_o) was examined next. A number of ion channels have a pH-dependent conductivity [16]. With respect to Ca^{2+} channels, H^+ can compete with Ca^{2+} for a binding site, thereby preventing channel opening [17]. Also, in pancreatic acinar cells, it has been shown that H^+ can inhibit Ca^{2+} entry at the outer plasma membrane and that decreasing extracellular H^+ concentration results in an increase in Ca^{2+} flux rate and a shortening of the Ca^{2+} refilling period into the stores [18]. As shown in Fig. 6A, the higher concentration of CCK-induced Ca^{2+} mobilization was biphasic comprising the initial large Ca^{2+} transient ($[Ca^{2+}]_i$ 660 ± 100 n*M*, $n = 11$) and the sustained plateau peak in $[Ca^{2+}]_i$ (29 ± 5 n*M* above the prestimulation level). The initial phase and the sustained plateau originate from intracellular and extracellular Ca^{2+}, respectively [7, 8]. Decreasing pH_o from 7.4 to 6.5 during cell stimulation resulted in an elimination of the sustained $[Ca^{2+}]_i$ plateau. This sustained plateau recovered when pH_o was returned to its initial level. Decreasing pH_o alone neither affected the resting level in $[Ca^{2+}]_i$ nor inhibited the CCK-induced initial range Ca^{2+} transient, thus suggesting that low pH_o inhibition of the sustained $[Ca^{2+}]_i$ plateau is not due to inhibition of CCK-receptor binding. The amplitude of the sustained $[Ca^{2+}]_i$ plateau, which was dependent on extracellular Ca^{2+}, was more remarkable when cells were stimulated by 10 μM carbamylcholine (47 ± 6 n*M* above the prestimulation level, $n = 16$) (Fig. 6C). Also, in this case, reduction of pH_o resulted in an elimination of the plateau in a reversible manner. Thus, extracellular H^+ regulates the Ca^{2+} entry process at the outer plasma membrane. Decreasing pH_o, on the other hand, failed to totally block the CCK-induced Ca^{2+} oscillation, although both the amplitude and the frequency decreased (Fig. 6D). The results suggest again that the major regulating sites by which H^+ affects the Ca^{2+} oscillation are accessible from the intracellular space.

DISCUSSION

The present study revealed that in single rat pancreatic acinar cells physiological concentrations of CCK caused a same-sized periodic Ca^{2+} spike. This oscillatory frequency (1 pulse/49 s) is included in the range of that which occurred in various cell types, ranging from 5 to 60 s [1]. Whereas a higher concentration of CCK (1 n*M*) caused a large initial Ca^{2+} transient up to 660 n*M* followed by a sustained elevation in $[Ca^{2+}]_i$ above the prestimulation level, the amplitude of repetitive Ca^{2+} spikes, which were induced by 20 p*M* CCK, was limited below 300 n*M*. Although the basic source for Ca^{2+} oscillation was intracellular, Ca^{2+} entry from the extracellular space was complementary to and necessary for maintenance of high frequency and amplitude. The increase in basal $[Ca^{2+}]_i$ with superimposed Ca^{2+} oscillation was totally dependent on extracellular Ca^{2+}. This digitized form of Ca^{2+} signaling seems to be important for cellular

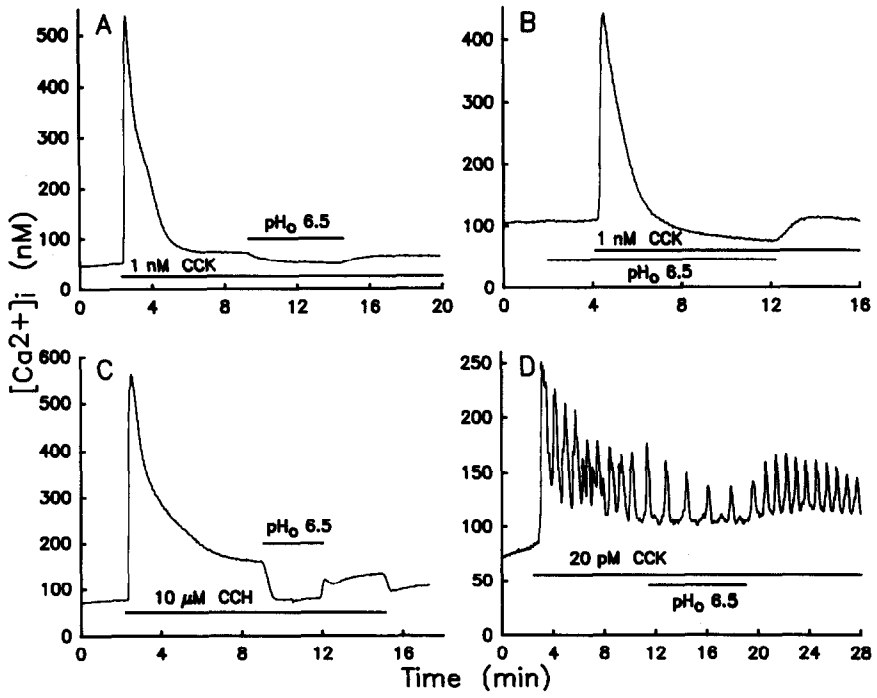

FIG. 6. The effect of changing pH_o on the CCK- or the carbachol(CCH)-induced Ca^{2+} mobilization. PSS-A with changing pH was used for perfusion medium. Each panel is representative of four to six separate determinations.

response. The precise mechanism of Ca^{2+} oscillation is, however, still unknown.

Recently two models of cytosolic free calcium oscillation have been proposed [1]: the first is IP_3-induced calcium release (IICR) and the second is calcium-induced calcium release (CICR). In the first model the oscillation is dependent on a periodic release of Ca^{2+} from an IP_3-sensitive pool driven by fluctuations in the level of IP_3. IICR is modulated by protein kinase C through a negative feedback regulation [8, 19, 20]. The role of the Ca^{2+} released from the stores is more complex. One aspect is that the Ca^{2+} released by IP_3 activates phosphatidylinositol breakdown to amplify the production of IP_3 (positive feedback regulation). Another is that the increased $[Ca^{2+}]_i$ inhibits the IP_3-induced Ca^{2+} release (negative feedback regulation). Sequestration of Ca^{2+} into the stores, on the other hand, results in an IP_3-operated Ca^{2+} channel opening. Recent study with respect to detergent-solubilized and membrane-associated IP_3 receptors from rat cerebellum has suggested that IP_3 binding to membrane (three molecules bind to the receptor to open channels, cooperatively [21]) is inhibited by 300 n*M* Ca^{2+} [22]. This value closely corresponds to the Ca^{2+} spike amplitude obtained in this study, suggesting that this is the $[Ca^{2+}]_i$ threshold for causing synchronized Ca^{2+} oscillation. The amplitude of the increased $[Ca^{2+}]_i$ being close to 300 n*M* also seems to affect the activity of the Ca^{2+} pump which is located in the stores or plasma membrane. Energy-dependent Ca^{2+} sequestration in mitochondrial poisoned permeabilized pancreatic acinar cells is stimulated by submicromolar Ca^{2+} ($K_{0.5}$ = 156 n*M*), which has a higher affinity to Ca^{2+} than that in the plasma membrane [23]. It is, therefore, likely that the small increase in $[Ca^{2+}]_i$ could be taken up by the stores rather than by the plasma membrane, resulting in a replenishment of Ca^{2+} in the stores for maintenance of the Ca^{2+} spike. The CCCR model suggests that Ca^{2+} released from the IP_3-sensitive pool is taken up by the IP_3-insensitive pool, resulting in an overloading of this pool. This is then triggered to release Ca^{2+}, which is then recycled back to the IP_3-sensitive pool. This theory is based on the fact that IP_3 cannot fully release Ca^{2+} from the stores in various cell types. Also, in plasmalemmal permeabilized pancreatic acinar cells, up to 55% of the amount of Ca^{2+} stored is IP_3-releasable with an EC_{50} of 1 μM [24]. Thus, the Ca^{2+} oscillation seems to be regulated by autocatalytic action between IP_3 and Ca^{2+}.

However, there is a still controversy between the IICR (or CCCR) model and the Ca^{2+} oscillation mechanism. In pancreatic acinar cells, the time course of the hormone-induced IP_3 generation is biphasic [25]. The first global generation of IP_3 is followed by sustained levels of IP_3, lasting at least 20 min into the cell stimulation. The controversy, however, is that the production of IP_3 in this cell type requires higher concentrations of agonists (carbachol, 0.1–1 m*M;* CCK analogue caerulein 1–100 n*M*) [25–27] (these results cannot be tested in individual cells because the IP_3 level cannot be measured in a single cell). These high concentrations of agonists fail to generate the symmetrical repetitive Ca^{2+} spikes with

increase in initial large Ca^{2+} transient followed by the sustained elevation in $[Ca^{2+}]_i$ above the prestimulation level [8]. When IP_3 is added successively several times the subsequent effects on Ca^{2+} release decrease with time in permeabilized insulin-secreting cells [28], possibly due to desensitization of the IP_3 receptor or to Ca^{2+} uptake into other Ca^{2+} stores. In guinea pig hepatocytes and mouse pancreatic acinar cells, continuous Ca^{2+} oscillation could be induced by injection of IP_3 or nonmetabolizable IP_3 analogue, which suggests that periodic changes in the IP_3 production and/or concentration are not required [6, 29]. In any case, there is a discrepancy between the affinity of the receptor and the potency of IP_3 in releasing Ca^{2+} [30]. Much of the pathway and driving force for continuous Ca^{2+} spikes might be therefore, at least in part, independent of IP_3.

This study examined the effect of pH_i on cytosolic free calcium oscillation during 20 p*M* CCK stimulation. The major results obtained are as follows: (i) pH_i change from alkalinization to acidification caused a basal increase in $[Ca^{2+}]_i$ to the spike peak level with inhibition of oscillations; (ii) pH_i change from acidification to alkalinization caused a basal decrease in $[Ca^{2+}]_i$ to the prestimulation level with inhibition of oscillations. The easiest explanation for these results is that the pH gradient inside-to-outside of Ca^{2+} stores is, at least in part, involved in the Ca^{2+} oscillation mechanism. It has been suggested that in rat parotid gland both a Ca^{2+} pump and a proton pump are located in an ATP-dependent and IP_3-sensitive pool [31]. Ca^{2+} mobilization is coupled to a pH gradient inside-to-outside of stores [31]. It is, therefore, likely that the release and uptake of Ca^{2+} by the stores requires a compensating flow of protons in the opposite direction to maintain electroneutrality. In the cytosolic acidification phase, H^+ flows from the cytosol to the stores, resulting in a Ca^{2+} leak from the stores. In the cytosolic alkalinization phase, H^+ flows from the stores to the cytosol, resulting in a sequestration of Ca^{2+} into the stores. The large shifts of both pH and Ca^{2+} gradients may result in an inhibition of Ca^{2+} oscillations with changing basal $[Ca^{2+}]_i$. It has not yet been determined why the pH_i effect on oscillations needs a large shift of pH_i from alkalinization to acidification or vice versa. Twenty picomolar CCK alone, which was able to cause Ca^{2+} oscillations, failed to affect pH_i in the bulk of cytosol. pH_i change during cell stimulation might be too localized to detect with the pH_i-sensitive dye BCECF. Since pH_i change alone is a minor factor in Ca^{2+} mobilization, pH_i effect on oscillations must be associated with receptor activation.

This study also showed that extracellular H^+ is inhibitory for Ca^{2+} entry. Since the enhancement of Ca^{2+} entry by increasing pH_o is abolished by lanthanum and pH_i change is not involved in the Ca^{2+} entry process, at least in pancreatic acinar cells [18], modification of H^+ on Ca^{2+} entry does not occur through a Ca^{2+}–H^+ antiport but does occur through channels at the outer plasma membrane. Considered from this point of view, pH change in the stores but not in the cytosol may be a regulatory factor in Ca^{2+} oscillations (and/or Ca^{2+} release) rather than the Ca^{2+}–H^+ antiport across the stores' membrane. However, it is difficult to predict the effect of the stores' pH in intact cells. The pH-sensitive Ca^{2+} mobilization pathway is the same or similar to that by which IP_3-induced Ca^{2+} release occurs and also remains to be determined. The ambient pH markedly affects IP_3 binding and IP_3-releasable Ca^{2+} [32–35]. An increase in IP_3 binding or in IP_3-releasable Ca^{2+} at slightly alkaline pH has been observed. However, the study reported here suggests that alkaline pH_i led to Ca^{2+} reuptake into the stores rather than to Ca^{2+} release. Concerning this, it has been suggested that the endoplasmic reticulum (ER) pump of human platelets increases Ca^{2+} uptake by 20% when pH is increased from 7.0 to 7.4 [36]. But in pancreatic acinar cells, increasing pH from 7.0 to 7.4 results in 40–60% inhibition of Ca^{2+} uptake in both ER vesicles and permeabilized cells [23, 37, 38]. Similar increases in pH also inhibit Ca^{2+}–Mg^{2+} ATPase activity associated with the ER Ca^{2+} pump by 40% [39]. Thus, at least in pancreatic acinar cells, it seems unlikely that the effect of pH_i on Ca^{2+} oscillations occurs through the ER Ca^{2+} pump or IP_3. Although it is difficult to predict the effect of pH on the agonist-stimulated Ca^{2+} signaling pathway, this study suggests that the pH_i change might be, at least in part, involved in the Ca^{2+} oscillation mechanism. Ca^{2+} oscillation seems to be regulated by multiple factors such as IP_3, Ca^{2+} ($[Ca^{2+}]_i$ and Ca^{2+} pump), and pH_i change. Further investigation is needed to determine the exact mechanism by which pH_i changes Ca^{2+} oscillation.

REFERENCES

1. Berridge, M. J., and Galione, A. (1988) *FASEB J.* **2,** 3074–3082.
2. Jacob, R., Merrit, J. E., Hallam, T. J., and Rink, T. J. (1988) *Nature (London)* **335,** 40–45.
3. Meyer, T., and Stryer, L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5051–5055.
4. Pralong, W. F., Wollheim, C. B., and Bruzzone, R. (1988) *FEBS Lett.* **242,** 79–84.
5. Yule, D. I., and Gallacher, D. V. (1988) *FEBS Lett.* **239,** 358–362.
6. Wakui, M., Potter, B. V. L., and Petersen, O. H. (1989) *Nature (London)* **339,** 317–320.
7. Stuenkel, E. L., Tsunoda, Y., and Williams, J. A. (1989) *Biochem. Biophys. Res. Commun.* **158,** 863–869.
8. Tsunoda, Y., Stuenkel, E. L., and Williams, J. A. (1990) *Amer. J. Physiol.* **258,** [*Cell Physiol.* **27**], C147–C155.
9. Williams, J. A., Kroc, M., and Dormer, R. L. (1978) *Amer. J. Physiol.* **253,** E517–E524.
10. Thomas, J. A., Buschbaum, R. N., Zimniak, A., and Racker, E. (1979) *Biochemistry* **18,** 2210–2218.
11. Bruzzone, R., Regazzi, R., and Wollheim, C. B. (1988) *Amer. J. Physiol.* **255,** G33–G39.

12. Grienstein, S., and Cohen, S. (1987) *J. Gen. Physiol.* **89,** 185–213.
13. Siffert, W., and Akkerman, W. N. (1987) *Nature (London)* **325,** 456–458.
14. Simpson, A. W. M., and Rink, T. J. (1987) *FEBS Lett.* **222,** 144–148.
15. Boron, W. F., and De Weer, P. (1976) *J. Gen. Physiol.* **67,** 91–112.
16. Moody, W. J., Jr. (1984) *Annu. Rev. Neurosci.* **7,** 257–278.
17. Christensen, O., and Zeuthen, T. (1987) *Pflugers Arch.* **408,** 249–259.
18. Muallem, S., Pandol, S. J., and Beeker, T. G. (1989) *Amer. J. Physiol.* **257,** G917–G924.
19. Woods, N. M., Cuthbertson, S. R., and Cobbold, P. H. (1987) *Biochem. J.* **246,** 619–623.
20. Sage, S. O., Adams, D. J., and Breemen, C. V. (1989) *J. Biol. Chem.* **264,** 6–9.
21. Meyer, T., Holowka, D., and Stryer, L. (1988) *Science* **240,** 653–656.
22. Supattapone, S., Worley, P. F., Baraban, J. M., and Snyder, S. H. (1988) *J. Biol. Chem.* **263,** 1530–1534.
23. Hurley, T. W. (1988) *Amer. J. Physiol.* **254,** C621–C627.
24. Wille, P. H. G. M., Broek, B. A. M. V. D., Os, C. H. V., and De Pont, J. J. H. H. M. (1989) *J. Biol. Chem.* **264,** 9762–9767.
25. Dixon, J. F., and Hokin, L. E. (1987) *J. Biol. Chem.* **262,** 13892–13895.
26. Merrit, J. E., Taylor, C. W., Rubin, R. P., and Putney, J. W., Jr. (1986) *Biochem. J.* **238,** 825–829.
27. Trimble, H. R., Bruzzone, R., Meehan, C. J., and Biden, T. J. (1987) *Biochem. J.* **242,** 289–292.
28. Biden, T. J., Prentki, M., Irvine, R. F., and Wollheim, C. B. (1984) *Biochem. J.* **223,** 467–473.
29. Capid, T., Field, A. C., Dgden, D. C., and Sanford, C. A. (1987) *FEBS Lett.* **217,** 247–252.
30. Kojima, I., Kojima, K., Kreutler, D., and Rasmussen, H. (1984) *J. Biol. Chem.* **259,** 14448–14457.
31. Thévendo, F., and Schulz, I. (1988) *Amer. J. Physiol.* **255,** G429–G440.
32. Brass, L. F., and Joseph, K. (1985) *J. Biol. Chem.* **260,** 15172–15179.
33. Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S., and Snyder, S. H. (1987) *J. Biol. Chem.* **262,** 12132–12136.
34. Guillemette, G., and Segui, J. A. (1988) *Mol. Endocrinol.* **2,** 1249–1255.
35. Rossier, M. F., Capponi, A. M., and Vallotton, M. B. (1989) *J. Biol. Chem.* **264,** 14078–14084.
36. Adunyan, S. A., and Dean, W. L. (1986) *J. Biol. Chem.* **261,** 3122–3127.
37. Bayerdorffer, E., Streb, H., Eckhardt, L., Haase, W., and Schulz, I. (1984) *J. Membr. Biol.* **81,** 69–82.
38. Richardson, A. E., and Dormer, R. L. (1984) *Biochem. J.* **219,** 679–685.
39. Imamura, T., and Schulz, I. (1985) *J. Biol. Chem.* **260,** 11339–11347.

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