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Intracellular Free Calcium Concentrations in Isolated Pancreatic Acini; Effects of Secretagogues

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Isolated pancreatic acini were loaded with the calcium selective fluorescent indicator, quin-2. Measurements of cellular K^+ content and lactic dehydrogenase release indicated that cell viability was not affected by quin-2 loading. The concentration of intracellular free calcium of unstimulated acinar cells was calculated to be 180 $\pm$ 4 nM. When cells suspended in media containing millimolar calcium were exposed to the secretagogues carbachol and cholecystokinin a rapid increase in $[Ca^{2+}]_i$ occurred. Both the amplitude and rate of rise of the concentration increase were dose dependent with $[Ca^{2+}]_i$ reaching a maximum of 860 $\pm$ 41 nM. The dose-response relationship coincides with the known concentration dependence of the stimulation of amylase release by these agents. In the absence of extracellular calcium, carbachol was still able to elicit a rise in $[Ca^{2+}]_i$. These studies indicate that pancreatic secretagogues induce an increase in $[Ca^{2+}]_i$ of acinar cells, both in the presence or absence of extracellular calcium.

It has been proposed that calcium plays a role as a secondary messenger in stimulus-secretion coupling of pancreatic acinar cells (1,2). The interaction of secretagogues with their membrane receptors is thought to stimulate an elevation of $[Ca^{2+}]_i$, which in turn mediates an increase in the rate of enzyme secretion. This hypothesis is supported by the following observations (3-5): (1) secretagogues cause a rapid efflux of ^{45}calcium from acinar cells; (2) artifically induced increases in $[Ca^{2+}]_i$ stimulate enzyme secretion; and (3) depletion of cell calcium stores inhibits enzyme secretion. Acinar cells suspended in calcium-free media can initially be stimulated to release enzymes; but sustained, stimulated release occurs only in the presence of extracellular calcium (5). Calcium efflux studies indicate that this initial enzyme release is mediated via the release of calcium from intracellular

Abbreviations: quin-2, 2-[[2-[bis[(carbonyl)methyl]amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis[(carbonyl)methyl]amino]quinoline; quin-2AM, the acetoxymethyl derivative of quin-2; $[Ca^{2+}]_i$, concentration of intracellular free calcium; CCh, Carbachol; CCK, Cholecystokinin; dbcGMP, Dibutyryl cyclic GMP

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stores (5). Intracellular calcium microelectrodes, however, have failed to demonstrate any change in $[Ca^{2+}]_i$ of cells in calcium-free media following stimulation by acetylcholine, although in the presence of extracellular calcium an increase in $[Ca^{2+}]_i$ was observed (6). To further investigate calcium's role in stimulus-secretion coupling, we have monitored the $[Ca^{2+}]_i$ of quin-2 loaded pancreatic acinar cells at rest and following stimulation by secretagogues. Quin-2 (compound 3b of reference 7) is a calcium selective fluorescent indicator which can be loaded into cell cytoplasm through the use of its hydrophobic acetoxymethyl ester (8).

METHODS AND MATERIALS

Intact, isolated pancreatic acini were prepared from 6-8 unfasted, male white Swiss mice by collagenase digestion and suspended in 10-15 ml of HEPES buffered Ringers solution (HR) containing 1.25 mM $CaCl_2$, an essential amino acid supplement, 0.1% bovine serum albumin, and 0.01% soybean trypsin inhibitor (9,10). The cell suspension contained approximately $5x10^6$ cells/ml. 20 µM quin-2AM was added and the cells were incubated for 20 min at 37°C, shaken at 60 cycles per min, and gassed continuously with O_2. The cells were then centrifuged at 50 x g for 3 min, resuspended in fresh HR media at 8 times the original volume, and maintained at room temperature without gassing or shaking. Fluorescence measurements were made within the following 70 min. Immediately before a fluorescence recording, 14 ml of the cell suspension were centrifuged and the cells resuspended in 2.5 ml fresh HR media at 37°C. Fluorescence measurements were made from samples continuously stirred and maintained at 37°C using an Aminco-Bowman spectrofluorometer. The excitation and emission wavelengths were 340 nm with 5.5 nm slits and 480 nm with 22 nm slits, respectively. All additions were made through a light tight guide without interrupting monitoring. Unloaded cells showed a small amount of fluorescence, which was not affected by the addition of any of the compounds tested here. All experimental tracings shown were repeated at least three times on different batches of acini. All data is reported as the mean ± standard error.

The intracellular concentration of free calcium was calculated according to the following equation (11):

$$[Ca^{2+}]_i = Kd\ (F-Fmin)/(Fmax-F)$$

Kd is the apparent dissociation constant of quin-2 for calcium in the presence of 1mM Mg (151nM); F is the fluorescence signal in arbitrary units of intact, quin-2 loaded cells; Fmax is the fluorescence signal of quin-2 in the presence of saturating concentrations of calcium; and Fmin is the fluorescence signal of quin-2 when it is not bound to calcium (12). Fmax and Fmin were determined following solubilization of the cells with 50 µM digitonin which released all of the intracellular quin-2 (11). The intracellular concentration of quin-2 was determined by comparing the value of Fmax to the fluorescence intensity of known concentrations of quin-2 added to the same media containing lysed, unloaded cells.

Lactic dehydrogenase was measured as described by Neilands (13). Potassium measurements on acinar pellets were made using an IL model 343 flame photometer with correction for trapped extracellular K^+ using [^{14}C] sucrose (14). Protein was determined according the the method of Bradford (15).

Atropine sulfate, dbcGMP, CCh, digitonin, HEPES, and Type 1S soybean trypsin inhibitor were from Sigma Chemical Co. Bovine serum albumin and [^{14}C] sucrose (673 mCi/mmol) were from Miles Laboratories, Inc. and New England

Nuclear, respectively. Quin-2 and quin-2AM were synthesized by J.I.K. using slight modifications of the procedure of Tsien (7,11) (details to be published elsewhere). CCK and its desulfated analog were gifts from Squibb Institute.

RESULTS AND DISCUSSION

Incubation of pancreatic acini with 20μM quin-2AM for 20 min resulted in the intracellular accumulation of 860 ± 60 μM quin-2 (n=10). According to two criteria, which are sensitive indicators of cell integrity, this level of quin-2 loading did not significantly alter the acinar cell's viability. First, the cellular potassium level of quin-2 loaded cells was the same as that of unloaded cells maintained under similar conditions (Fig. 1). Second, the release of lactic dehydrogenase from quin-2 loaded cells, both during loading and up to 70 min after loading, did not differ from control (Fig. 1). The physiology of quin-2 loaded cells was not totally unaffected, however, as the amount of amylase released in response to an optimal concentration of carbachol was approximately 70% of that released by control cells (data not shown). This effect is probably due to intracellular calcium buffering by quin-2.

The $[Ca^{2+}]_i$ of unstimulated pancreatic acinar cells was calculated from the fluorescence of quin-2 to be 180 ± 4 nM (n=13). This value is in close

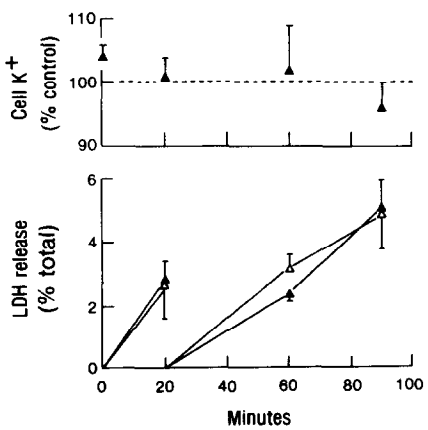

Figure 1. Cell viability of pancreatic acini following quin-2 loading. Cellular potassium was measured on quin-2 loaded cell samples and compared to identically treated control samples. Zero time indicates the point at which 20μM quin-2AM was added. Lactic dehydrogenase (LDH) release was measured prior to and following washing at 20 min to remove extracellular quin-2AM. Total LDH was measured following cell lysis by sonication. Each point represents the mean ± SE of three experiments carried out in duplicate. (▲) quin-2 loaded cells, (Δ) Control cells.

agreement with reported values for $[Ca^{2+}]_i$ measured using quin-2 in lymphocytes (11), platelets (16), hepatocytes (17), and neutrophils (18). Using intracellular calcium sensitive microelectrodes, the $[Ca^{2+}]_i$ of pancreatic acinar cells was recently reported to be 430 ± 30 nM (6).

To determine the effects of secretagogues on $[Ca^{2+}]_i$ of pancreatic acinar cells, we monitored the fluorescence of quin-2 loaded cells following the addition of CCK or the acetylcholine analog, CCh. Figure 2A shows that 10^{-6}M CCh caused a rapid increase in fluorescence, followed by a slow decay to a new steady value higher than that of unstimulated cells. The maximal $[Ca^{2+}]_i$ was 860 ± 41 nM (n=7). This value represents the average rise in $[Ca^{2+}]_i$, assuming that all cells in the suspension responded similarly. Calcium reached its highest level approximately 6 sec after the addition of CCh. This value is an upper limit, as the instrumental time constant of response (approximately 2 sec) could have kinetically distorted the fluorescent signal. Figure 2A also illustrates that the extent and rate of rise in $[Ca^{2+}]_i$ was proportional to agonist concentrations over the range of 10^{-8} to 10^{-6}M. This

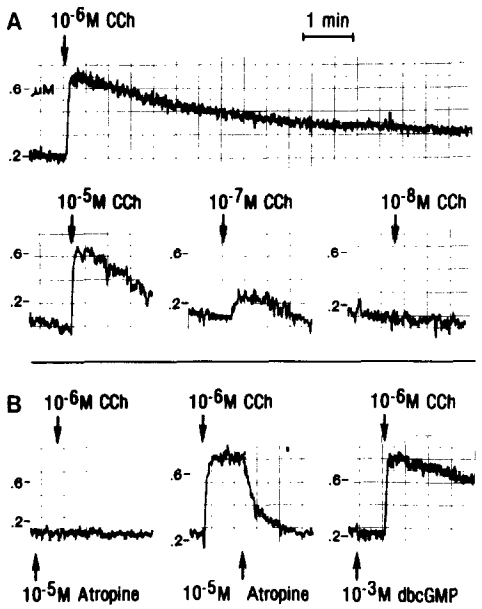

Figure 2. Effects of CCh on the fluorescence of intracellular quin-2 in pancreatic acini. (A) Various concentrations of CCh were added at the times indicated by the arrows. (B) The receptor blockers atropine and dbcGMP were added at the times indicated by the arrows. Calculated $[Ca^{2+}]_i$ (µM) is indicated at left of tracing.

dose-dependence closely coincides with the known concentration dependence of the stimulation of amylase release by CCh (9). The change in fluorescence observed in response to 10^{-5}M CCh (Figure 2A) was similar to that of 10^{-6}M CCh. This may indicate that these levels of CCh stimulate a maximal increase in $[Ca^{2+}]_i$. If, however, significant amounts of the intracellular quin-2 are sequestered within intracellular organelles, or if the cytoplasmic release of calcium is not spatially uniform, the observed limit could be the result of dye saturation.

The stimulatory effect of CCh arises from interaction with its specific receptors, since it did not enhance fluorescence when 10^{-5}M atropine was present in the incubation medium (Fig. 2B). Furthermore, if atropine was added after $[Ca^{2+}]_i$ was increased by CCh, there was a rapid return, within 1 min, of $[Ca^{2+}]_i$ to basal levels (Fig. 2B). Figure 2B also shows that dbcGMP, a specific blocker of the CCK receptor, did not inhibit the action of CCh.

Figure 3A shows that 10^{-10}M CCK, an optimal concentration for stimulating amylase release (19), caused an increase in the fluorescence of quin-2 loaded cells resembling that elicited by 10^{-6}M CCh. This effect was completely blocked by dbcGMP, but not by atropine (Figs. 3B and 3C). By contrast, the

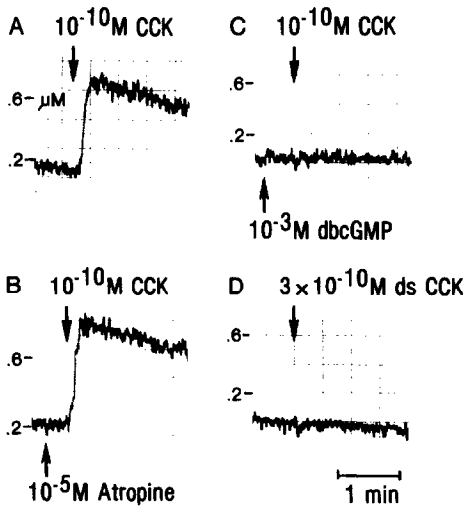

Figure 3. Effects of CCK on the fluorescence of intracellular quin-2 in pancreatic acini. CCK, dbcGMP, atropine, and desulfated cholecystokinin octapeptide (dsCCK) were added at the times indicated by the arrows.

desulfated form of CCK, at a 3-fold higher concentration, did not alter $[Ca^{2+}]_i$ (Fig. 3D). Desulfated CCK binds to the CCK receptor, but its affinity and resultant biological activity is only about one two-hundreth that of CCK (20).

To determine whether the rise in $[Ca^{2+}]_i$ observed following stimulation by secretagogues is dependent on extracellular calcium, we monitored the fluorescence of quin-2 loaded cells that were suspended in calcium-free media. Figure 4 shows that 10^{-6}M CCh stimulated an increase in $[Ca^{2+}]_i$, although the extent and rate of rise for this response was less than that observed in media containing calcium. Following the addition of 10^{-5}M CCh, however, the increase in $[Ca^{2+}]_i$ was similar to that observed in the presence of extracellular calcium, but the $[Ca^{2+}]_i$ decayed more rapidly and returned to near its starting value despite the continued presence of secretagogue (Fig. 4). These data indicate that intracellular calcium pools are sufficient to account for the rise in $[Ca^{2+}]_i$ observed following stimulation of pancreatic acinar cells by CCh, but a higher concentration of CCh appear to be required to elicit changes similar to those observed in the presence of extracellular calcium. Furthermore, the change in $[Ca^{2+}]_i$ observed in calcium-free media decays more rapidly than in normal calcium.

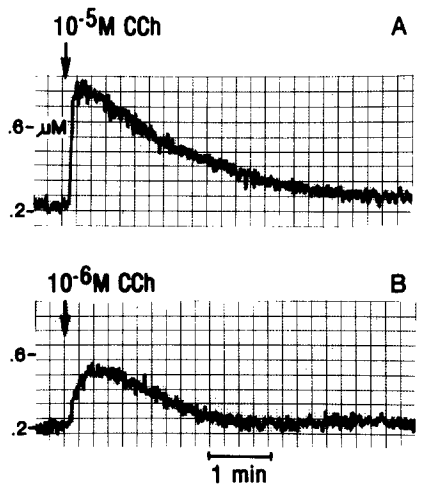

Figure 4. Effects of CCh on the fluorescence of intracellular quin-2 in pancreatic acini suspended in media containing 10^{-4}M EGTA and no added calcium.

In summary, we demonstrated that the $[Ca^{2+}]_i$ of pancreatic acinar cells was 180 ± 4 nM and that stimulation by CCh or CCK resulted in a rapid, transient five-fold enhancement of this value. Furthermore, the ability of CCh to induce this increase was not dependent upon the presence of extracellular calcium.

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