Cytosolic Calcium During Contraction of Isolated Mammalian Gastric Muscle Cells

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During activation of visceral smooth muscle there is an increase in cytosolic-free calcium, but the source (intracellular calcium release or calcium influx), kinetics, and stoichiometry of this increase have not been determined. Here, the fluorescent indicator, quin2-acetoxymethyl ester, was used to measure directly cytosolic-free calcium during contraction of isolated stomach muscle cells induced by the two neuropeptides cholecystokinin-octapeptide and Met-enkephalin as well as acetylcholine. An increase in cytosolic-free calcium was seen that was (i) dependent on the concentration of contractile agonist, (ii) derived from intracellular sources (that is, not significantly affected by removal of ambient calcium or addition of a calcium channel blocker), and (iii) kinetically and stoichiometrically related to net calcium efflux and contraction. In contrast, the increase in cytosolic-free calcium induced by depolarizing concentrations of potassium was caused by influx of calcium through voltage-dependent calcium channels.

VISCERAL SMOOTH MUSCLE CAN BE activated to contract pharmacochemically (pharmacomechanical coupling) or electrically by membrane depolarization and action potentials (electromechanical coupling) (*1*). During both types of activation there is an increase in the concentration of cytosolic-free calcium ($[Ca^{2+}]_i$) caused by release from intracellular stores or influx into the cell. When the luminescent Ca^{2+} indicator, aequorin, is injected into isolated muscle cells (*2*) or intact muscle strips (*3*), a correlation between light emission and electrically induced or K^+-induced contraction is observed. However, these experiments cannot identify the source, kinetics, or stoichiometry of the increase in $[Ca^{2+}]_i$ during contraction.

With smooth muscle cells isolated from human and guinea pig stomach we showed recently that contraction induced by the neuropeptides cholecystokinin-octapeptide (CCK-8) and Met-enkephalin as well as acetylcholine is accompanied by a sixfold increase in net efflux of $^{45}Ca^{2+}$ without a change in Ca^{2+} influx; this increase in efflux reflects a release of Ca^{2+} from a depletable, nonmitochondrial store that is capable of sustaining at least one initial maximal contraction (*4*). Contraction and net Ca^{2+} efflux can be correlated and depend on the concentration of agonist. Here quin2-acetoxymethyl ester (quin2-AM) (*5, 6*), the permeant derivative of the fluorescent Ca^{2+} indicator quin2, was used to measure directly the increase in $[Ca^{2+}]_i$ during agonist-induced contraction of isolated human and guinea pig gastric muscle cells. The increase in $[Ca^{2+}]_i$ depended on the concentration of agonist, was not significantly affected by removal of Ca^{2+} from the medium or addition of the Ca^{2+} channel blocker methoxyverapamil, and was kinetically and stoichiometrically related to net Ca^{2+} efflux and contraction. In contrast, contraction and the increase in $[Ca^{2+}]_i$, which were induced by depolarizing concentrations of K^+ and mediated by influx of Ca^{2+} via voltage-dependent Ca^{2+} channels, were blocked by removal of Ca^{2+} from the medium or addition of methoxyverapamil.

Smooth muscle cells were isolated from the circular muscle layer of guinea pig stomach and human gastric antrum by enzymatic digestion and filtration (*7*). Cells were loaded with quin2 by incubation at 31°C in 20 μM quin2-AM (Fig. 1). The acetoxymethyl ester permeated muscle cells readily where it was hydrolyzed and trapped as the impermeant tetracarboxylate anion quin2. Uptake of quin2-AM and its hydrolysis to quin2 were monitored by the gradual shift of the emission spectrum from that of the ester (430 nm) to that of the final indicator (492 nm). The spectral shift was complete in less than 90 minutes. The concentration of $[Ca^{2+}]_i$ was calculated from the equation relating $[Ca^{2+}]_i$ and observed fluorescence (F).

$$[Ca^{2+}]_i = K_d\,(F - F_{min})/(F_{max} - F)$$

where K_d is the dissociation constant and F_{min} and F_{max} are minimal and maximal fluorescence at very low and very high Ca^{2+} concentrations, respectively. F_{min} was determined by setting $[Ca^{2+}]$ to <1 nM by addition of 2 mM EGTA and tris buffer to raise the pH above 8.3, and F_{max} determined by permeabilizing the muscle cells with Triton X-100 to release the indicator into a medium containing 2 mM Ca^{2+} (*5*).

The resting $[Ca^{2+}]_i$ was similar in guinea pig and human gastric muscle cells (Table 1) and similar to that in other cell types (*8–11*). Exposure of the cells to CCK-8 caused an instantaneous increase in $[Ca^{2+}]_i$ that

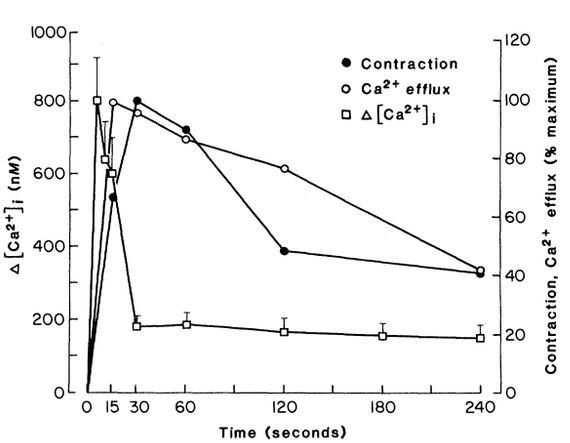

Fig. 1. Time course of increase in cytosolic-free Ca^{2+}, net Ca^{2+} efflux, and contraction in isolated guinea pig gastric muscle cells in response to $10^{-9}M$ CCK-8. Data on Ca^{2+} efflux and contraction were derived from previous studies (*4, 7, 12*) and plotted here for comparison with the time course of $[Ca^{2+}]_i$. Data for $[Ca^{2+}]_i$ are means ± SEM of four to nine experiments. Smooth muscle cells were isolated from circular muscle layer of guinea pig or human stomach by digestion of muscle strips in 0.1 percent collagenase followed by repeated washing in enzyme-free medium. Spontaneously dissociated cells were harvested by filtration as described (*7*). The cells were suspended in Hepes medium containing 115 mM NaCl, 5.8 mM KCl, 2.2 mM KH_2PO_4, 2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 25 mM Hepes, and 12 mM glucose. Ca^{2+}-free medium contained no added Ca^{2+} and 2 mM EGTA. Cells were loaded with quin2-AM dissolved in dimethyl sulfoxide (DMSO) (*5*). Cell suspension (2 ml) (5×10^6 cells per ml) were incubated in 20 μM quin2-AM (final concentration of DMSO < 0.5 percent) for 20 minutes; the suspension was diluted fivefold and incubation was continued for another 60 minutes. The suspension was filtered through 10-μm Nitex mesh; cells trapped on the mesh were washed four times to minimize carry-over of extracellular quin2-AM and resuspended in Hepes medium. Fluorescence was measured with a Perkin-Elmer fluorescence spectrophotometer and recorded on an Ominiscribe recorder. Standard monochromator settings were 339 nm for excitation (4 nm slits) and 492 nm for emission (10 nm slits). Samples were placed in temperature-controlled 1-cm quartz cuvettes and stirred with rotating micromagnets. The suspension was gassed and reagents were added through side tubes leading into the cuvettes.

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Table 1. Resting and CCK-stimulated cytosolic-free calcium in isolated human and guinea pig smooth muscle cells. Data are means ± SEM obtained 5 seconds after addition of CCK-8. Number of experiments in parentheses.

Treatment	Cytosolic-free calcium (n*M*)	
	Guinea pig	Human
Resting	176 ± 12 (25)	171 ± 4 (5)
CCK-8 ($10^{-13}M$)	310 ± 53 (4)	—
CCK-8 ($10^{-12}M$)	406 ± 70 (3)	428 ± 42 (4)
CCK-8 ($10^{-11}M$)	432 ± 55 (3)	—
CCK-8 ($10^{-10}M$)	723 ± 76 (4)	—
CCK-8 ($10^{-9}M$)	1006 ± 175 (15)	1071 ± 105 (5)
CCK-8 ($10^{-8}M$)	1187 ± 450 (15)	—

reached a peak in 5 seconds, declined rapidly in 30 seconds, and then fell gradually over the next 4 to 8 minutes without completely reverting to base line (Fig. 1). The peak in $[Ca^{2+}]_i$ occurred before the peak in Ca^{2+} efflux at 15 seconds (*4*) or in muscle contraction (at 30 seconds) (*7*) (Fig. 1). The increase in $[Ca^{2+}]_i$ induced by CCK-8 was concentration-dependent (Table 1 and Fig. 2). The concentration of CCK-8 that induced maximal contraction ($10^{-9}M$) caused a fivefold increase above resting levels in cells from both species (Tables 1 and 2). Similar increases were elicited by equipotent, maximally effective concentrations of Met-enkephalin ($10^{-6}M$) and acetylcholine ($10^{-7}M$), and maximally effective depolarizing (that is, contractile) concentrations of K^+ (30 m*M*) (Table 2). A supramaximal concentration of CCK-8 ($10^{-8}M$) did not cause a further increase in $[Ca^{2+}]_i$, although it did cause a 30 percent decrease in contraction (Table 1 and Fig. 2).

The Ca^{2+} responsible for the agonist- and K^+-induced increases in $[Ca^{2+}]_i$ could have been from intracellular stores of Ca^{2+} or from influx of extracellular Ca^{2+}. To test which of these possibilities was more likely we incubated cells in Ca^{2+}-free medium (2 m*M* EGTA) or with the Ca^{2+} channel blocker methoxyverapamil ($10^{-6}M$). This concentration of methoxyverapamil is optimally effective in blocking contraction and Ca^{2+} influx in isolated gastric smooth muscle cells (*4*). Neither incubation in Ca^{2+}-free medium nor exposure to methoxyverapamil had any significant effect (Student's *t* test, $P = 0.05$) on resting $[Ca^{2+}]_i$ or on the increase in $[Ca^{2+}]_i$ induced by maximal concentrations of CCK-8, Met-enkephalin, or acetylcholine in guinea pig muscle cells (Table 2). Similar results were obtained in human gastric muscle cells ($[Ca^{2+}]_i$ = 1071 ± 105 (SEM) n*M* with $10^{-9}M$ CCK-8 in control medium, 1016 ± 182 n*M* in control medium containing $10^{-6}M$ methoxyverapamil, and 990 ± 104 n*M* in Ca^{2+}-free medium containing 2 m*M* EGTA). In contrast, the increase in $[Ca^{2+}]_i$ induced by depolarizing concentrations of K^+ was virtually abolished by these treatments (Table 2). A minor residual increase in $[Ca^{2+}]_i$ appeared to be the result of K^+-induced intracellular release of Ca^{2+}; this was consistent with our previous finding of a small residual contraction in Ca^{2+}-free medium or in the presence of methoxyverapamil (*4*).

The trapping of the Ca^{2+} buffer, quin2, in muscle cells provided an opportunity to test the extent to which the increase in cytosolic-free Ca^{2+} was coupled to contraction. Muscle cells from guinea pig stomach were incubated in 20 μ*M* quin2-AM for 90 minutes and examined at intervals for their contractile response to CCK-8 ($10^{-9}M$). Contraction was measured by image-splitting micrometry and expressed as the mean

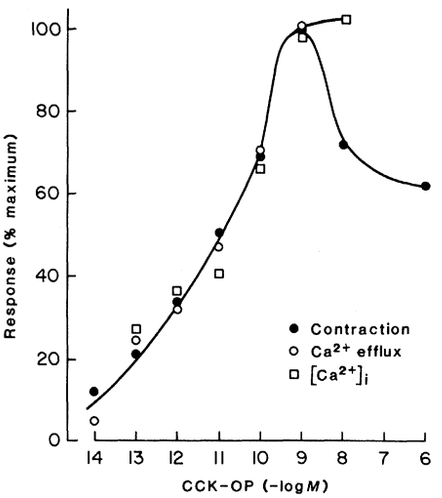

Fig. 2. Cytosolic-free Ca^{2+} in guinea pig gastric muscle cells in response to CCK-octapeptide. Data for $[Ca^{2+}]_i$ from Table 1. Data for contraction and net $^{45}Ca^{2+}$ efflux derived from (*2*) and (*5*) for comparison with data for $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured 5 seconds after the addition of CCK-8, the net $^{45}Ca^{2+}$ efflux was measured 15 seconds after, and peak contraction was measured 30 seconds after (see Fig. 1).

decrease in cell length from control (*7*). The contractile response of cells containing quin2 to a maximal concentration of CCK-8 decreased in proportion to the duration of incubation [19 ± 2 percent inhibition at 30 minutes ($P < 0.01$) and 29 ± 4 percent inhibition at 90 minutes ($P < 0.01$)]. The decrease suggests that quin2 blunted the $[Ca^{2+}]_i$ transient induced by CCK-8 and implies that the maximal fivefold increase in $[Ca^{2+}]_i$ may be an underestimate.

There was a close correlation between peak $[Ca^{2+}]_i$, peak net $^{45}Ca^{2+}$ efflux, and peak contraction in guinea pig gastric muscle cells (Fig. 2). The data on Ca^{2+} efflux and muscle contraction were derived from earlier studies (*4, 7, 12*) and those on $[Ca^{2+}]_i$ from Table 1. The concentration-response curves for the three measurements could be superposed, suggesting a close functional coupling between these events. The binding of CCK-8 (or other agonists, for example, acetylcholine) to its receptors on gastric muscle cells leads to release of Ca^{2+} from an intracellular store into the cytosol. The Ca^{2+} is rapidly extruded from the cell and so results in net Ca^{2+} efflux.

Agonist-induced Ca^{2+} release has been measured in other cell types [for example, pancreatic acinar cells (*8, 9*) and liver cells (*10*)] with quin2-AM. Resting and maximally stimulated levels of $[Ca^{2+}]_i$ in these and other cell types (*5, 6, 11*) are similar to those reported here for human and guinea pig gastric muscle cells. Maximally effective concentrations of muscarinic agonists in-

Table 2. Cytosolic-free calcium (mean ± SEM) and percent increase above resting $[Ca^{2+}]_i$ obtained 5 seconds after addition of maximally effective contractile concentrations of CCK-8, Met-enkephalin, acetylcholine, and K^+. The increase elicited by K^+ only was blocked in Ca^{2+}-free medium or in the presence of methoxyverapamil. Number of experiments in parentheses.

Treatment	Cytosolic-free calcium					
	Control medium				Ca^{2+}-free medium + 2 m*M* EGTA	
	2 m*M* Ca^{2+}		$10^{-6}M$ Methoxyverapamil			
	n*M*	Increase (%)	n*M*	Increase (%)	m*M*	Increase (%)
CCK-8 ($10^{-9}M$)	1006 ± 175 (15)	478 ± 78	919 ± 135 (5)	430 ± 82	990 ± 104 (5)	459 ± 51
Met-enkephalin ($10^{-6}M$)	929 ± 129 (5)	450 ± 69	919 ± 38 (4)	445 ± 75	1123 ± 270 (5)	644 ± 90
Acetylcholine ($10^{-7}M$)	959 ± 173 (5)	435 ± 110	883 ± 142 (4)	403 ± 110	806 ± 188 (7)	390 ± 104
KCl (30 m*M*)	1077 ± 77 (3)	603 ± 94	219 ± 25 (4)	36 ± 10	218 ± 17 (6)	25 ± 4

duce similar fivefold increases of $[Ca^{2+}]_i$ in pancreatic acinar cells and gastric smooth muscle cells of the guinea pig, and preloading with quin2 inhibits maximal response in both cell types to the same extent (29 to 32 percent) (*8*). However, agonist-induced secretory and metabolic responses in pancreatic acinar and liver cells have yet to be measured with the speed necessary to establish the kinetic sequence or the stoichiometric coupling of $[Ca^{2+}]_i$ and cell response. These relations are more easily demonstrated in isolated muscle cells as shown in Fig. 1 for the kinetic sequence and in Fig. 2 for the stoichiometric coupling of peak $[Ca^{2+}]_i$, peak net Ca^{2+} efflux, and peak contraction.

Studies with saponin-permeabilized gastric muscle cells suggest that release of intracellular Ca^{2+} is mediated by inositol 1,4,5-trisphosphate ($InsP_3$) (*12, 13*). The water-soluble derivative of phosphatidylinositol 4,5-bisphosphate releases Ca^{2+} from the same pool as that shown to be the source of the intracellular Ca^{2+} released by CCK-8 and acetylcholine. The maximal increase in $[Ca^{2+}]_i$, net Ca^{2+} efflux, and contraction elicited by $InsP_3$ are similar to those elicited by CCK-8 and acetylcholine, consistent with a role for $InsP_3$ as the membrane-derived messenger for mobilization of intracellular Ca^{2+}.

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Gene Interaction at HLA-DQ Enhances Autoantibody Production in Primary Sjögren's Syndrome

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Primary Sjögren's syndrome is an autoimmune disorder characterized by dryness of the mouth and eyes. The human leukocyte antigen (HLA) locus DQ is related to the primary Sjögren's syndrome autoantibodies that bind the RNA proteins Ro/SSA and La/SSB. Both DQ1 and DQ2 alleles are associated with high concentrations of these autoantibodies. An analysis of all possible combinations at DQ has shown that the entire effect was due to heterozygotes expressing the DQ1 and DQ2 alleles. These data suggest that gene interaction between DQ1 and DQ2 (or alleles at associated loci), possibly from gene complementation of *trans*-associated surface molecules, influences the autoimmune response in primary Sjögren's syndrome.

THE MAJOR HISTOCOMPATIBILITY complex, which is known in humans as the HLA system, has an important role in controlling immunologic defense mechanisms. Recent advances in the model for the genetic organization of the HLA system have resulted in a redefinition of the class II loci as HLA-DP (previously SB), HLA-DQ (previously DS, DC, and MB), and HLA-DR (unchanged) (*1*). The cell surface molecules encoded by class II genes are composed of pairs of transmembrane peptides termed α and β. The HLA-DR polymorphisms defined between individuals are a result of allelic variations in β chain structure. No polymorphism has been recognized in the HLA-DR α chain. Both the α and the β HLA-DQ chains are polymorphic, and *trans*-associations as well as *cis*-associations of these peptides have been described (*2*).

Diseases associated with an HLA specificity in which an antigen is known and in which the response to the antigen clearly causes the disease are rare. Myasthenia gravis is perhaps the best example. This disorder is associated with the B8,DR3 haplotype in caucasoids, and all available evidence supports the hypothesis that it is caused by autoantibody to the acetylcholine receptor (*3*).

The primary form of Sjögren's syndrome or the sicca complex is another autoimmune disease associated with B8,DR3 (*4*). It is characterized by lymphocytic infiltration of exocrine glands, particularly the salivary and lacrimal. Patients with this disorder complain of dry mouth and eyes. Extraglandular manifestations including vasculitis and purpura as well as central nervous system, muscle, hematologic, kidney, and lung involvement may be present. When this disorder occurs in the absence of another diagnosis, it is referred to as primary Sjögren's syndrome. The sicca complex is commonly found in patients with other rheumatic diseases including rheumatoid arthritis, systemic lupus erythematosus, progressive systemic sclerosis, and polymyositis. These patients are referred to as having secondary Sjögren's syndrome. Sicca complex is very common; estimates of prevalence in populations over 55 years old exceed 1 percent (*5*).

Antibodies to Ro/SSA and La/SSB are found in nearly all Sjögren's syndrome patients, and many of the extraglandular features are associated with high concentrations of these autoantibodies (*6*). The Ro/SSA and La/SSB antigens are RNA-protein complexes, and the RNA's are RNA polymerase III products. La/SSB binds to terminal uridine-rich regions of RNA polymerase III transcripts (*7*).

An evaluation of these autoantibodies and the DQ alleles in Sjögren's syndrome was undertaken when it was discovered that both DQ1 and DQ2 might be associated with elevated binding of antibodies to Ro/SSA and La/SSB ($0.08 > P > 0.03$) (*8*). Further analysis of the HLA-DQ relations to Ro/SSA and La/SSB antibodies showed that the entire effect of DQ was found in the patients with primary Sjögren's syndrome (Table 1). The associations with both DQ1 and DQ2 raised the possibility that particular combinations of HLA-DQ alleles could account for this effect. Indeed, when all possible combinations of the HLA-DQ alleles were evaluated for Ro/SSA and La/SSB antibodies, only patients heterozy-

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