IgE Receptor-Activated Calcium Permeability Pathway in Rat Basophilic Leukemia Cells: Measurement of the Unidirectional Influx of Calcium Using Quin2-Buffered Cells†

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ABSTRACT: The intracellular calcium indicator and buffer quin2 has been used to generate a large calcium buffering capacity in the cytoplasm of rat basophilic leukemia cells. Above 3 mM intracellular quin2, there is no further increase in the initial rate of antigen-induced ^{45}Ca uptake, suggesting that ^{45}Ca buffering by quin2 is now sufficient to prevent the immediate efflux of ^{45}Ca from the cells. Thus, the initial rate of ^{45}Ca uptake should reflect the true unidirectional influx of calcium that occurs when immunoglobulin E (IgE) receptors are aggregated by antigen. The antigen-induced calcium permeability pathway appears to be saturable, with a K_m of about 0.7 mM and a V_{max} of 0.9 nmol of calcium $(10^6 \text{ cells})^{-1}$ min^{-1}. Although net ^{45}Ca uptake reaches a plateau a few minutes after antigen stimulation, the increase in plasma membrane permeability is maintained for at least an hour, provided that receptors for IgE remain aggregated. The initial rate of ^{45}Ca influx correlates well with the subsequent secretion of [^{3}H]serotonin in response to different concentrations of antigen. Both ^{45}Ca uptake and [^{3}H]serotonin secretion are maximal when only 10% of the receptors are occupied with antigen-specific IgE. Thus, ^{45}Ca influx correlates more closely with secretion than with the number of IgE receptors aggregated by antigen.

When receptors for immunoglobulin E (IgE)[1] on the surface of mast cells and basophils are aggregated, for example, when receptor-associated IgE binds antigen, the cells are stimulated to secrete a variety of mediators of immediate hypersensitivity (Metzger & Ishizaka, 1982). Exocytosis of granules containing histamine, serotonin, acid hydrolases, and other preformed mediators requires metabolic energy and shows an absolute dependence on extracellular calcium concentration (Douglas, 1975). Furthermore, as is the case in many other secretory systems, direct introduction of calcium into the cytoplasm using a divalent cation ionophore such as A23187 is a sufficient, though not necessarily the only, stimulus for the initiation of secretion (Foreman et al., 1973).

Rat basophilic leukemia (RBL) cells (Eccleston et al., 1973), which were recently shown to be derived from mucosal mast cells (Seldin et al., 1985), share many of the properties of their normal counterparts (Fewtrell & Metzger, 1981). In the case of the secreting subline 2H3 (Barsumian et al., 1981) of RBL cells, this includes the ability to secrete in a calcium-dependent manner when receptors for IgE are aggregated (Fewtrell et al., 1979) or when the cells are exposed to noncytotoxic amounts of the divalent cation ionophores A23187 or ionomycin (Fewtrell et al., 1981). Secretion from RBL cells occurs relatively slowly, taking 30–60 min to reach completion, and since these cells are only sparsely granulated, secretion is not accompanied by the same profound morphological changes that are seen with normal mast cells. For these and a variety of other reasons [discussed more fully in Fewtrell and Metzger (1981)], RBL cells have become the model system of choice in a wide range of studies aimed at understanding the mechanism by which receptors for IgE induce secretion (Metzger & Ishizaka, 1982).

While release of calcium from intracellular stores in RBL cells may contribute to the increase in intracellular Ca^{2+} concentration seen in response to antigen (Mohr & Fewtrell, 1987a), it is generally agreed that the major source of calcium is extracellular (Beaven et al., 1984a). The exact nature of the IgE receptor-activated Ca^{2+} influx pathway has yet to be determined, but a number of observations are consistent with the idea that it may be a channel. However, it is clearly very different from the voltage-sensitive calcium channels seen in excitable cells (Hagiwara & Byerly, 1981) since it is not activated when RBL cells are depolarized (Kanner & Metzger, 1984; Mohr & Fewtrell, 1987a) and cannot be blocked by a variety of organic calcium antagonists (Middleton et al., 1981). A candidate for the Ca^{2+} channel from RBL cells has recently been isolated (Mazurek et al., 1982), reincorporated into cells (Mazurek et al., 1983), and reconstituted into lipid bilayers (Mazurek et al., 1984). This protein, which binds the antiallergic drug cromolyn, appears to have many of the properties that would be expected of an IgE receptor-activated channel; however, its unqualified acceptance as the calcium permeability pathway in mast cells and basophils awaits more detailed studies (Gomperts & Fewtrell, 1985; Metzger et al., 1986).

Whatever the nature of the calcium influx pathway, it is clear that aggregation of receptors for IgE on the surface of RBL cells leads to a rapid uptake of ^{45}Ca (Kanner & Metzger, 1984; Crews et al., 1981) and a very large increase in total cell calcium concentration (Mohr & Fewtrell, 1987a). The total calcium content of resting RBL cells is in the region of 0.5 nmol/10^6 cells, and this increases 5–6-fold on stimulation (Mohr & Fewtrell, 1987a). If this calcium were uniformly distributed throughout the cell, it would correspond to an intracellular calcium concentration of 3 mM. In marked

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[1] Abbreviations: AM, acetoxymethyl ester; DNP, dinitrophenyl; IgE, immunoglobulin E; RBL, rat basophilic leukemia; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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contrast to this, it has been shown, using the fluorescent indicator quin2 (Tsien et al., 1982), that the cytoplasmic free ionized calcium concentration increases from 0.1 μM to, at most, somewhere in the region of 1 μM in response to antigen (Beaven et al., 1984a). This means that the vast majority (>99.9%) of the calcium entering the cell upon stimulation is rapidly and efficiently buffered and/or removed from the cytoplasm to maintain a cytoplasmic free ionized Ca^{2+} concentration in the submicromolar range. While a small amount of this calcium may bind to sites such as cytoplasmic calcium binding proteins, the majority is presumably sequestered in membrane-bound stores such as endoplasmic reticulum and perhaps mitochondria. It is also likely that a significant fraction of the calcium entering the cell in response to antigen is expelled again since extrusion mechanisms in the plasma membrane must also be extremely active under these conditions. This means that measurements of the apparent unidirectional influx of calcium into cells are likely to be considerable underestimates of the true rates since they will not account for this rapid expulsion of calcium from the cells. We have therefore devised an approach in which the intracellular calcium buffer and indicator quin2 (Tsien et al., 1982) has been used to generate a large calcium buffering capacity or "sink" in the cytoplasm of RBL cells. Under these conditions, extrusion of incoming ^{45}Ca is prevented for at least 3 min, and so the initial rate of ^{45}Ca uptake should be a true reflection of the unidirectional influx of calcium into stimulated cells. Since similar underestimates are likely to occur in any system where calcium uptake is measured, this method for determining the unidirectional influx of calcium should have wide applicability. In this paper, we describe this approach and its use in characterizing the IgE receptor-activated calcium permeability pathway of RBL cells.

Materials and Methods

Reagents and Solutions. The standard isotonic saline solution was a modified Tyrode's solution of the following composition: 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ 5.6 mM glucose, 0.1% bovine serum albumin, and 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) adjusted to pH 7.4 with NaOH. The ice-cold quenching solution contained 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM NaHEPES, pH 7.4. [^{3}H]Serotonin (5-[1,2-^{3}H(N)]hydroxytryptamine binoxalate) and $^{45}CaCl_2$ were purchased from New England Nuclear (Boston, MA). Quin2 and quin2 acetoxymethyl ester (AM) were obtained from Sigma (St. Louis, MO). Purified rat IgE from myeloma IR 162 (Kulczycki & Metzger, 1974) was kindly provided by Dr. Henry Metzger (NIH, Bethesda, MD). Purified ^{125}I-labeled IgE and unlabeled monoclonal mouse IgE directed against dinitrophenyl (DNP) haptens (Holowka & Metzger, 1982; Liu et al., 1980) were gifts from Drs. Barbara Baird and David Holowka (Department of Chemistry, Cornell University). The antigen used was bovine γ-globulin to which an average of 15 dinitrophenyl groups per molecule had been coupled (Eisen et al., 1959).

Cells. All experiments were performed with a secreting subline (2H3) of rat basophilic leukemia cells (Barsumian et al., 1981) maintained in monolayer culture as described (Taurog et al., 1979). The day before an experiment, 0.4×10^6 cells in 0.25 mL of culture medium were seeded into each well of several 24-well plates (Falcon, Oxnard, CA) and incubated overnight at 37 °C in a humid atmosphere containing 5% CO_2. In experiments where secretion or total cell calcium was to be measured, [^{3}H]serotonin or ^{45}Ca was added to the cells immediately prior to plating (see below).

Sensitization with IgE. The day of an experiment, the cells in each well were sensitized by incubation for 1 h at 37 °C with 0.15 μg of mouse IgE anti-DNP in 0.25 mL of culture medium. In experiments where the amount of antigen-specific IgE on the cell surface was varied, the cells were exposed to mixtures of antigen-specific mouse IgE anti-DNP and rat myeloma IgE of unknown antigenic specificity (Fewtrell, 1985). The relative binding activities of the two IgE preparations were determined by measuring their ability to compete with ^{125}I-labeled IgE for binding to receptors for IgE on RBL cells (Kulczycki & Metzger, 1974). The unlabeled IgE preparations were mixed in the proportions necessary to ensure that 0, 1, 3, 10, 30, and 100% of the receptors would be occupied by antigen-specific IgE. The total amount of active IgE added to the cells (0.25 μg/well) corresponded to an approximately 5-fold excess over the number of receptors for IgE present.

Release of Incorporated [^{3}H]Serotonin. [^{3}H]Serotonin (2 μCi/mL of culture medium) was added to RBL cells immediately prior to seeding in multiwell plates. Incorporation of [^{3}H]serotonin was allowed to take place overnight (Baird et al., 1983). The monolayers were washed twice with warm isotonic saline solution, and the cells were exposed to antigen (1 μg/mL unless otherwise stated) for the requisite length of time before being quenched with 2 mL of ice-cold quenching solution. An aliquot (0.5 mL) of each supernatant was taken for counting, and the counts were expressed as a percentage of the total [^{3}H]serotonin content of the cells prior to stimulation.

Quin2 Loading. The cell monolayers were washed once with isotonic saline solution and incubated at 37 °C for 1 h with quin2/AM in 0.25 mL of isotonic saline solution. In experiments where the quin2 load was varied, cells were incubated with between 10 and 160 μM quin2/AM; otherwise, the standard loading concentration was 100 μM quin2/AM. At the end of the loading period, the unincorporated quin2/AM was removed, and 0.25 mL of isotonic saline solution was added per well. The cells were incubated for a further 20 min to ensure that all the incorporated quin2/AM was hydrolyzed to yield the calcium binding free acid quin2.

^{45}Ca Uptake. This was measured essentially as described (Kanner & Metzger, 1984; Crews et al., 1981). ^{45}Ca uptake was initiated by the addition of ^{45}Ca (10 μCi/mL) with or without antigen (1 μg/mL unless otherwise stated) in 0.25 mL of isotonic saline solution at 37 °C. At the indicated times, 2 mL of ice-cold quenching solution was added to each well. The cell monolayers were then washed twice with the same quenching solution. The cells were solubilized in 1 mL of 0.1% Triton X-100, and 0.5 mL from each well was counted for ^{45}Ca.

Determination of Total Cell Calcium in Quin2-Loaded RBL Cells. Cell monolayers in multiwell plates were loaded to isotopic equilibrium with ^{45}Ca by overnight culture with ^{45}Ca (5–10 μCi/mL of culture medium containing 1.8 mM $CaCl_2$). Growing the cells with ^{45}Ca for longer periods (up to 2 days) did not increase the amount of cell-associated ^{45}Ca, indicating that isotopic equilibrium with ^{45}Ca had been reached. ^{45}Ca was maintained at the same specific activity during loading and hydrolysis of quin2/AM and until the final quenching and washing with ice-cold quenching solution (see above). A few experiments were carried out with cells in suspension rather than in monolayer plates. In these instances, ^{45}Ca was added to flasks of RBL cells growing in monolayer culture the day before the experiment. After trypsinization, ^{45}Ca was maintained at the same specific activity throughout the experiment.

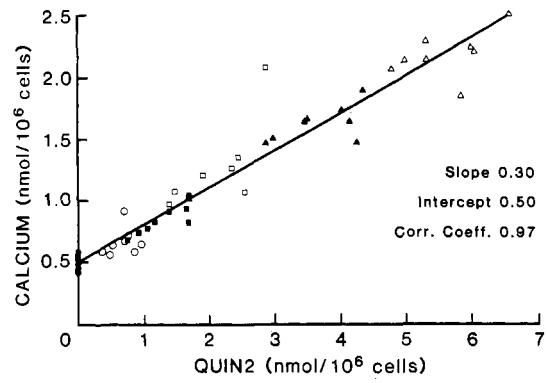

FIGURE 1: Effect of intracellular quin2 load on total cell calcium in resting RBL cells. The quin2/AM loading concentrations used to obtain the measured amounts of intracellular quin2 were as follows: (●) 0 μM; (○) 10 μM; (■) 20 μM; (□) 40 μM; (▲) 80 μM; (△) 160 μM. The results from a single representative experiment are shown. Almost identical results were obtained in three other separate experiments.

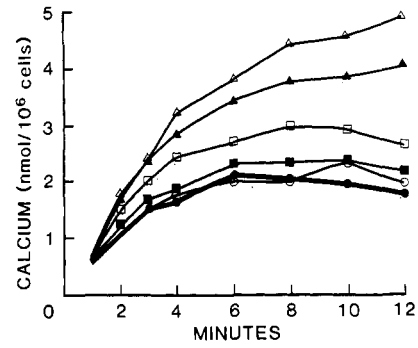

FIGURE 2: Initial rate of antigen-stimulated calcium influx was markedly increased in quin2-loaded cells. ^{45}Ca was added to the cells at zero time, with or without antigen (1 μg/mL). The quin2/AM loading concentrations and the symbols used to depict the results were the same as those in Figure 1. The actual intracellular quin2 concentrations are indicated below. Antigen-induced secretion was monitored by using the same population of quin2-loaded cells and was as follows: (●) control (no quin2), 50% ± 5%; (○) 0.4 mM quin2, 50% ± 2%; (■) 0.9 mM quin2, 49% ± 2%; (□) 2.0 mM quin2, 55% ± 3%; (▲) 3.9 mM quin2, 52% ± 3%; (△) 6.6 mM quin2, 43% ± 6%.

Aliquots (100 μL) of the quin2-loaded cells were spun through a phthalate oil mixture [dibutyl phthalate/bis(2-ethylhexyl) phthalate, 6:4] in 400-μL microfuge tubes. The tips of the tubes containing the cell pellets were incubated overnight in 10% Triton X-100 to solubilize the cells prior to counting. Counts due to ^{45}Ca in the entrapped extracellular water space (0.2 μL/10^6 cells; C. Fewtrell, unpublished observation) were deducted.

Determination of Intracellular Quin2 Load. At the end of the experiment, 1.5 mL of 0.1% Triton X-100 was added to the remaining 0.5 mL of solubilized cells per well and the quin2 fluorescence (excitation 339 nm, emission 492 nm) compared with that of freshly prepared standard solutions of quin2 free acid (Tsien et al., 1982) in a Perkin-Elmer LS-5 fluorescence spectrophotometer. A cell volume of 1 μL/10^6 cells (C. Fewtrell, unpublished observation) was used to convert the amount of quin2 incorporated by the cells to an intracellular quin2 concentration.

Cell Counting and Viability. The cells in each of four wells were dislodged by trypsinization and counted. In general, each well contained 0.5 × 10^6 cells at the time of the experiment, and cell viability was greater than 98%. Release of the cytoplasmic enzyme lactate dehydrogenase was assayed spectrophotometrically (Taurog et al., 1979). Cell viability was also assessed by trypan blue exclusion. There was no significant decrease in viability in any of the experiments.

ATP. Intracellular ATP levels were measured by using a modification of the firefly luciferase assay (Ko & Lagunoff, 1976).

Scintillation Counting. ^{45}Ca and [^{3}H]serotonin were measured in a Beckman LS 1800 scintillation counter using Amersham ACS scintillation cocktail (Amersham, Arlington Heights, IL).

RESULTS

Effect of Quin2 Loading on Cell Calcium Levels. When RBL cells were loaded with increasing amounts of the calcium buffer quin2, they accumulated calcium to compensate for the binding of cytoplasmic calcium by quin2 (Figure 1). The total cell calcium in resting RBL cells in the absence of quin2 was routinely found to be 0.5 nmol/10^6 cells, but this may increase severalfold in quin2-loaded cells (Figure 1). When measured by fluorescence, the fraction of quin2 complexed to calcium is generally around 0.44–0.46 in resting cells (corresponding to an intracellular Ca^{2+} concentration of 90–100 mM), and a similar stoichiometry would thus have been predicted for the increase in total cell calcium as a function of intracellular quin2 concentration. The slope of the line in Figure 1 was 0.3 which would correspond to a free Ca^{2+} concentration of 50 nM, and similar results were obtained in a number of additional experiments. The most likely explanation for these somewhat low values is that quin2/AM was incompletely cleaved to yield the calcium binding free acid quin2. This was clearly a problem in a few early experiments, and so an additional 20-min incubation after 1 h of loading with quin2 was routinely carried out. Under these conditions, the emission spectrum (excitation 339 nm) of quin2/AM-loaded RBL cells after detergent lysis corresponded to that of quin2 free acid, indicating more complete hydrolysis of the acetoxymethyl ester. However, we cannot exclude the possibility that some partially hydrolyzed molecules were still present.

Our finding that total cell calcium increased linearly with intracellular quin2 load (Figure 1) showed that the calcium homeostatic mechanisms of the cells compensate for the increased calcium buffering capacity by allowing the cells to accumulate calcium from the extracellular solution. Calcium homeostasis remained effective even at high intracellular quin2 concentrations when the total cell calcium concentration was more than 5 times the normal level. In agreement with this, we found that the cells appeared able to maintain a constant resting cytoplasmic free ionized Ca^{2+} concentration with intracellular quin2 loads up to 10 mM (not shown).

We (see below) and others (Beaven et al., 1984a) have found that antigen-induced secretion from RBL cells is not affected until the intracellular quin2 concentration is greater than 7 mM. High levels of intracellular quin2 were not cytotoxic to RBL cells, and ATP levels were unaffected over a wide range of intracellular quin2 concentrations (0–20 mM). This is in contrast to thymocytes where quin2 caused a reduction in cell ATP (Hesketh et al., 1983).

From these results, it was clear that the cells were not adversely affected and that their calcium homeostatic mechanisms function efficiently even at high intracellular quin2 loads. We therefore felt justified in using quin2-loaded cells to measure the initial rate of ^{45}Ca influx into antigen-stimulated RBL cells.

Effect of Quin2 Load on Antigen-Stimulated ^{45}Ca Uptake. When IgE receptors on the surface of RBL cells were aggregated by antigen, there was an immediate influx of ^{45}Ca into the cells (Figure 2). In the absence of quin2, the initial

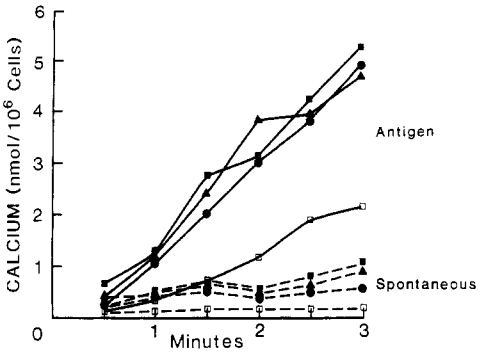

FIGURE 3: Above 3 mM intracellular quin2, the initial rate of calcium influx in response to antigen remained constant. Antigen (1 μg/mL) and ^{45}Ca were added to the cells at zero time. Dashed lines, spontaneous uptake; solid lines, antigen-stimulated calcium uptake. Open symbols, control cells without quin2. Closed symbols, cells containing the indicated concentrations of quin2: (●) 2.9 mM quin2; (▲) 4.7 mM quin2; (■) 7.3 mM quin2.

rate of ^{45}Ca uptake was relatively slow and reached a plateau within a few minutes. As the intracellular quin2 load was increased, there was an increase in both the initial rate of ^{45}Ca uptake and the time taken to reach a plateau. Furthermore, the steady-state level of cell-associated ^{45}Ca in antigen-stimulated cells increased with quin2 load. The greater capacity of the cells to accumulate ^{45}Ca in response to antigen and the increased time taken to reach a plateau clearly demonstrated that quin2 was substantially increasing the ability of the cells to buffer incoming ^{45}Ca. More importantly, our finding that the initial rate of ^{45}Ca uptake was also significantly increased confirmed that in the absence of quin2, the unidirectional influx of calcium was considerably underestimated even though the initial rate of uptake appeared to be linear for the first minute or two. At low intracellular quin2 concentrations, the initial rate of ^{45}Ca uptake increased with the quin2 load (Figure 2), but above 3 mM intracellular quin2, there was no further increase in the initial rate of uptake (Figure 3). This suggested that ^{45}Ca buffering by quin2 was now sufficient to prevent the immediate extrusion of ^{45}Ca from the cells, and so the initial rate of ^{45}Ca uptake should reflect the true unidirectional influx of calcium into the cells. ^{45}Ca uptake in quin2-loaded RBL cells was therefore used in all subsequent experiments to examine the changes in calcium permeability that occur when IgE receptors are aggregated by antigen. Provided that the intracellular quin2 load was below 7 mM, antigen-induced secretion was unaffected [see Figure 2 legend and Beaven et al. (1984a)].

Calcium Dependence of ^{45}Ca Influx. Antigen-stimulated ^{45}Ca uptake is dependent on the concentration of extracellular calcium (Figure 4). At low extracellular calcium concentrations, ^{45}Ca influx was almost completely inhibited. As the extracellular calcium concentration was raised, antigen-induced ^{45}Ca uptake increased and reached a maximum when extracellular calcium was in the millimolar range. The apparent K_m for calcium influx was 0.7 mM (Figure 4 inset) which is similar to, but a little higher than, the usual calcium concentrations for half-maximal histamine (Beaven et al., 1984a) or [^3H]serotonin (Mohr & Fewtrell, 1987a) release. Inositol phosphate production (Beaven et al., 1984b) and the peak increase in cytoplasmic Ca^{2+} concentration (Beaven et al., 1984a) are also half-maximal at a similar extracellular calcium concentration (approximately 0.4 mM). The V_{max} for antigen-stimulated calcium uptake was 0.9 nmol of calcium (10^6 cells)$^{-1}$ min^{-1} which is almost identical with the value of 1 nmol (10^6 cells)$^{-1}$ min^{-1} calculated by Beaven and his colleagues (Beaven et al., 1984a). This corresponds to an enormous

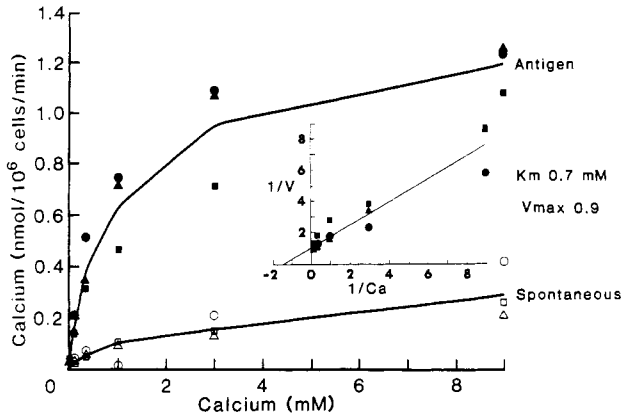

FIGURE 4: Effect of extracellular calcium concentration on the initial rate of antigen-stimulated calcium influx. Uptake was measured 3 min after the addition of ^{45}Ca ± antigen (1 μg/mL) to the cells. Inset: Lineweaver–Burk plot of the data for antigen-stimulated calcium influx. Open symbols, spontaneous uptake; closed symbols, antigen-stimulated calcium influx. The different symbols represent the results obtained in three separate experiments in which the quin2 loads were 2.7 (△, ▲), 3.9 (○, ●), and 7.1 mM (□, ■).

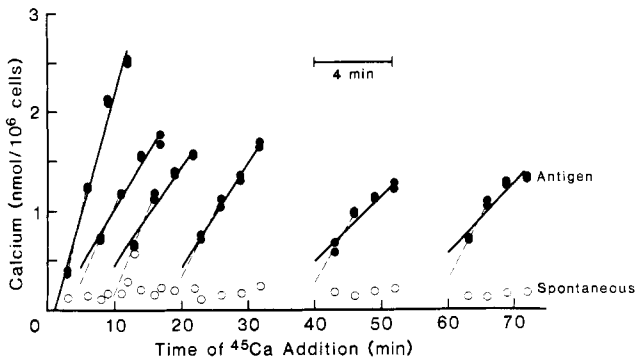

FIGURE 5: Increase in plasma membrane permeability to calcium was maintained for at least an hour after antigen stimulation. RBL cells were stimulated with antigen (1 μg/mL) in the presence of extracellular calcium. ^{45}Ca was added 0, 5, 10, 20, 40, or 60 min later, and its uptake by the cells was measured every minute for the next 4 min (solid symbols). The open symbols show spontaneous ^{45}Ca uptake by unstimulated cells over the same time periods. Linear regression lines were drawn through all four time points (solid lines) and through the first two points only (dashed lines). The intracellular quin2 load was 7.4 mM.

calcium influx at the physiological extracellular calcium concentration (1.8 mM).

Calcium Permeability Pathway Persists until Receptor Aggregates Are Disrupted. Although ^{45}Ca uptake appeared to plateau a few minutes after antigen stimulation, even in quin2-loaded cells (see Figure 2), the increase in plasma membrane permeability to calcium was maintained for at least an hour after the cells had been stimulated (Figure 5). Quin2-loaded cells were stimulated with antigen in the presence of extracellular calcium. Trace amounts of ^{45}Ca were then added at various times after antigen, and the rate of ^{45}Ca uptake was measured over the next 4 min. When ^{45}Ca was added together with antigen, there was a short lag followed by a rapid influx of ^{45}Ca (Figure 3 and Figure 5, left-most curve) that remained linear for at least 3 min. When ^{45}Ca was added at various times after antigen, the tracer was immediately taken up by the cells without any discernible lag (Figure 5). Although the initial rate of uptake was not as great as it was at the time of antigen addition, it was still many times greater than that seen in unstimulated cells (open symbols) even when ^{45}Ca was added as much as 1 h after antigen. It is also clear that the initial rate determined over the first 4 min of uptake (solid lines) considerably underestimated the

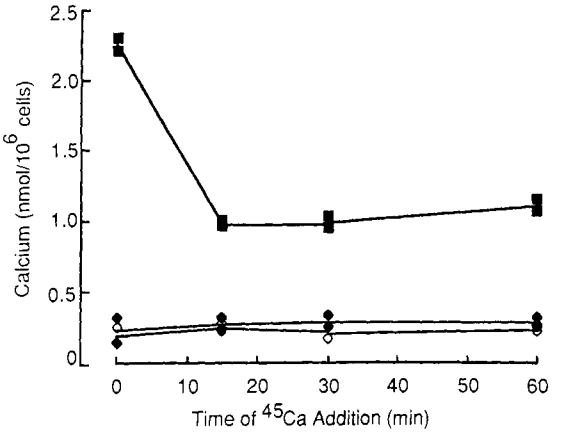

FIGURE 6: Disruption of IgE receptor aggregates immediately halted calcium influx. RBL cells were stimulated with antigen at zero time, and at the times indicated, ^{45}Ca was added to the cells, and its uptake by the cells was determined 3 min later. The monovalent hapten DNP-lysine (10^{-4} M) was added simultaneously (♦) or 5 min before (◇) ^{45}Ca to displace antigen and disaggregate receptors. DNP-lysine was not added to the control cells (■).

true unidirectional influx since the apparent rate of uptake decreased 2–3 min after the addition of ^{45}Ca even though the cells contained a high concentration of quin2. This suggests that the prior influx of nonradioactive ^{40}Ca in response to antigen reduced the ability of quin2 to buffer ^{45}Ca subsequently entering the cells. It is also probable that the increase in cytoplasmic Ca^{2+} had activated Ca^{2+} extrusion mechanisms, thus allowing ^{45}Ca to be removed almost immediately from the cell. If the initial rate is measured over the first 2 min of ^{45}Ca uptake (dashed lines), a better fit is obtained, and the apparent rates of unidirectional influx are now only slightly lower than that seen when ^{45}Ca was added at the same time as antigen. This somewhat reduced rate of uptake may be due to the fact that the cells rapidly depolarize in response to antigen (Mohr & Fewtrell, 1987b) which will reduce the electrical driving force for calcium entry into the cells (Mohr & Fewtrell, 1987a).

Although the IgE receptor-activated increase in plasma membrane permeability to calcium is extremely long-lived, IgE receptors must remain aggregated for this pathway to be maintained. When the monovalent hapten dinitrophenyllysine was added to cells that had been stimulated with multivalent antigen (dinitrophenylated bovine γ-globulin), further receptor aggregation was prevented and/or receptor aggregates were disrupted, and ^{45}Ca uptake was immediately reduced to basal levels (Figure 6).

^{45}Ca Influx and [^{3}H]Serotonin Secretion at Different Antigen Concentrations. The effect of antigen concentration on the initial rate of calcium influx and [^{3}H]serotonin secretion is shown in Figure 7. At antigen concentrations below 10^{-1} μg/mL, [^{3}H]serotonin secretion was reduced (Figure 7b), but above this concentration, there was little further increase in the secretory response even at a hundredfold higher antigen concentration. The initial rate of ^{45}Ca influx followed a similar pattern (Figure 7a), with little further increase in uptake occurring at antigen concentrations above 10^{-1} μg/mL. The relationship between ^{45}Ca influx and [^{3}H]serotonin release at 4 min is shown as a function of antigen concentration in figure 7c. It is clear that there is a good correlation between the two although it seems that a somewhat higher rate of calcium influx is necessary to elicit a secretory response at low antigen concentrations.

Antigen-Stimulated ^{45}Ca Influx Correlates with Secretion Rather than with IgE Receptor Number. We have previously

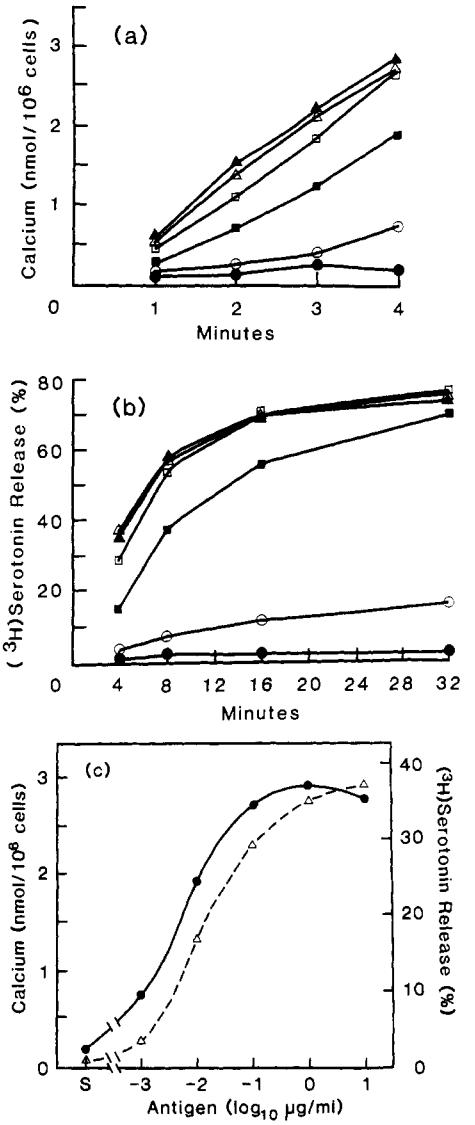

FIGURE 7: Calcium influx and [^{3}H]serotonin secretion induced by various concentrations of antigen. (a) ^{45}Ca uptake by cells containing 3.7 mM intracellular quin2: spontaneous (●); antigen concentration of 0.001 (○), 0.01 (■), 0.1 (□), 1 (▲), or 10 μg/mL (△). (b) [^{3}H]Serotonin secretion [symbols as in (a)]. (c) ^{45}Ca uptake (●) and [^{3}H]serotonin secretion (△) 4 min after stimulation, plotted as a function of antigen concentration. "S" denotes spontaneous uptake or secretion in the absence of antigen [data from (a) and (b)].

shown that the number of receptors occupied with antigen-specific IgE does not have to be very great in order to elicit a maximal secretory response in RBL cells (Fewtrell, 1985). It was therefore of interest to see whether activation of the calcium permeability pathway by an optimal concentration of antigen was related to the number of receptors occupied with antigen-specific IgE or whether it correlated more closely with the subsequent secretory response of the cells. Our results (Figure 8b) confirmed that a maximal secretory response can be obtained when as few as 10% of the receptors are occupied with antigen-specific IgE. Furthermore, the same was true for ^{45}Ca influx (Figure 8a). Although influx was reduced significantly when less than 10% of the IgE receptors were able to bind antigen, the initial rate of ^{45}Ca influx increased only very slightly when 30% or 100% of the receptors were available to interact with antigen. If calcium influx correlated with IgE receptor number, then it should follow the thin dashed line in Figure 8c, but it is clear that it correlated much more closely with [^{3}H]serotonin secretion (Figure 8c). This suggested that the extent of secretion was limited by the rate of calcium influx

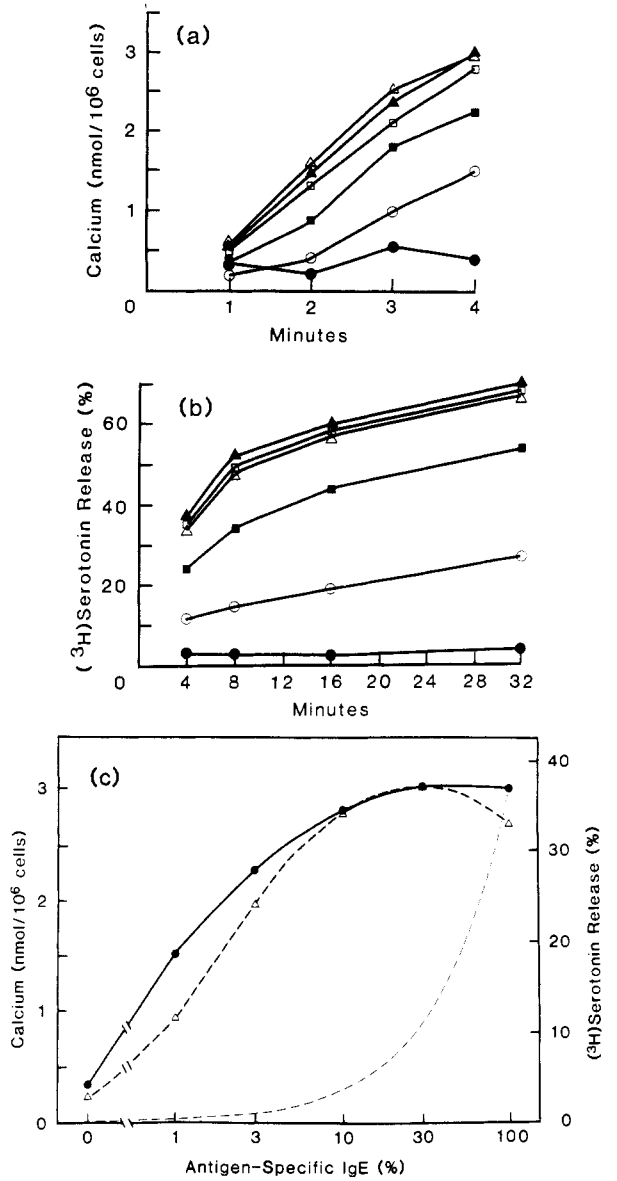

FIGURE 8: Calcium influx and [^{3}H]serotonin secretion were not reduced until less than 10% of the receptors were occupied with antigen-specific IgE. (a) ^{45}Ca uptake by cells containing 4.6 mM intracellular quin2. The symbols indicate the percent of the receptors occupied with antigen-specific IgE: 0% (●); 1% (○); 3% (■); 10% (□); 30% (▲); 100% (△). (b) [^{3}H]Serotonin secretion [symbols as in (a)]. (c) ^{45}Ca uptake (●) and [^{3}H]serotonin secretion (△) 4 min after stimulation with antigen (1 μg/μL) plotted as a function of the percent of receptors occupied with antigen-specific IgE [data from (a) and (b)]. The thin dashed line shows the expected relationship if the responses were directly proportional to the antigen-specific IgE receptor number.

into the cells and that there is a substantial pool of "spare" receptors.

Discussion

In order to understand the relationship between IgE receptor activation and the resulting increase in cytoplasmic free Ca^{2+} concentration in RBL cells, it is important to be able to distinguish between the various calcium regulatory components that contribute to this response. It is known that receptor activation leads to a substantial increase in total cell calcium, and it is generally accepted that this is due to the activation of a calcium permeability pathway or channel in the plasma membrane (Kanner & Metzger, 1984). It is also likely that release of calcium from intracellular stores contributes to the rise in cytoplasmic Ca^{2+} concentration (Mohr & Fewtrell, 1987a,c) although the relative contribution of this source of calcium has yet to be defined. Furthermore, since the antigen-induced increase in total cell calcium concentration (from 0.5 to 2.5 mM; Mohr & Fewtrell, 1987a) is several thousandfold greater than the increase in cytoplasmic free Ca^{2+} concentration (from 0.1 μM to between 0.3 and 1 μM; Mohr & Fewtrell, 1987a; Beaven et al., 1984a), the calcium sequestering and extrusion mechanisms of the cells must be extremely active. From this, it is clear that while measurements of free ionized Ca^{2+} may provide a good indication of the calcium levels necessary for the initiation of secretion, they are likely to be a rather indirect measure of permeability changes that occur in the plasma membrane when receptors for IgE are activated. Traditionally, ^{45}Ca uptake has been used to measure changes in calcium permeability in small cells, and it has generally been assumed that the initial rate of ^{45}Ca uptake, provided that it is linear, represents the true unidirectional influx of calcium into the cells (Wollheim & Sharp, 1981) and should be unaffected by the intracellular Ca^{2+} concentration [see p 231 in Hille (1984)]. However, it is clear from the above discussion that the calcium load on the cells when IgE receptors are activated is very large and that calcium binding and sequestering in intracellular stores and also reextrusion of calcium across the plasma membrane must be occurring at an extremely rapid rate. Thus, it is highly probable, even at very early time points after the addition of ^{45}Ca, that reextrusion of ^{45}Ca will significantly reduce the measured influx of ^{45}Ca into the cells.

We have therefore used the calcium buffer quin2 to generate a substantial calcium buffering capacity or "sink" in the cytoplasm of RBL cells to ensure that for at least the first few minutes after antigen stimulation, ^{45}Ca entering the cells is bound by quin2 and is not immediately reextruded. We have shown (Figure 1) that as the quin2 load increases, RBL cells compensate by accumulating calcium from the extracellular solution. At intracellular quin2 concentrations above 3 mM, the total cell calcium is more that 3 times higher than that of normal resting cells and continues to increase with increasing quin2 load. As predicted, the initial rate of antigen-induced ^{45}Ca influx was significantly higher in quin2-loaded cells (Figures 2 and 3). Above 3 mM intracellular quin2, the initial rate of uptake was constant, suggesting that ^{45}Ca buffering by quin2 was now sufficient to prevent the immediate efflux of ^{45}Ca from the cells. Although ^{40}Ca will be extruded during this time, ^{45}Ca entering the cells should be rapidly buffered by quin2 and will also be significantly diluted in the large reservoir of ^{40}Ca that is generated in the cytoplasm of quin2-loaded cells. We therefore conclude that measurements of ^{45}Ca uptake in RBL cells loaded with greater than 3 mM quin2 provide a reasonable estimate of the unidirectional influx of calcium and that ^{45}Ca efflux is negligible during the first 2–3 min after addition of ^{45}Ca. Using this approach, we have examined a number of properties of the calcium permeability pathway in RBL cells and its relationship to IgE receptor activation by antigen. The long-term aim of these studies is to determine whether this pathway is indeed a channel or whether its properties more closely resemble those expected for a Ca^{2+} carrier or exchange mechanism.

In vivo, mast cells and basophils bind IgE molecules with a wide variety of antigenic specificities. It is likely, therefore, that a significant excess of receptors is necessary to ensure that the cells are capable of responding independently to any of the antigens to which they have been sensitized. Our finding that ^{45}Ca influx and [^{3}H]serotonin secretion were almost maximal when as few as 10% of the receptors were occupied

with antigen-specific IgE (Figure 8) confirms that there is indeed a considerable number of "spare" receptors (Fewtrell, 1985; Stephenson, 1956). This would be consistent with a model such as the mobile receptor theory, which was first proposed to explain the ability of a number of different receptors to activate adenylate cyclase (Cuatrecasas, 1974). Thus, IgE receptors, when aggregated by antigen, might become associated with, and activate, the calcium permeability pathway. If the number of pathways is small, full activation will be seen when only a few receptors are aggregated. In agreement with this, we found that ^{45}Ca influx reached a plateau at relatively low concentrations of antigen (Figure 7) and that even though more receptors were aggregated, further increases in antigen concentration did not increase ^{45}Ca influx. These results are again consistent with the mobile receptor theory, although other models could also account for our results.

Our findings would, on first sight, appear to be at odds with the results of Maeyama and his colleagues (Maeyama et al., 1986). These workers have shown, using oligomers of IgE, that although secretion reaches a plateau, inositol phosphate production and the peak level of cytoplasmic Ca^{2+} continue to rise with increasing receptor aggregation. It is likely that a significant fraction of the initial rise in cytoplasmic Ca^{2+} concentration in RBL cells is due to the release of calcium from intracellular stores (Mohr & Fewtrell, 1987a; P. Millard, D. Gross, W. Webb, and C. Fewtrell, unpublished observations) as has been shown to be the case in many other cell types. The close correlation between inositol phospholipid breakdown and the peak increase in cytoplasmic Ca^{2+} concentration is consistent with this since release of stored calcium is believed to be mediated by inositol 1,4,5-trisphosphate (Streb et al., 1983) and certain other inositol phosphates (Burges et al., 1984).

An attractive explanation for the apparent discrepancy between our results and those of Maeyama et al. (1986) might be that inositol phosphate production and the subsequent, relatively transient, release of calcium from intracellular stores do indeed correlate closely with IgE receptor aggregation. In contrast, the increase in plasma membrane permeability to calcium is maximal when only a small fraction of the receptors are activated and is maintained as long as receptors are aggregated. Since secretion does not occur unless there is a sustained increase in cytoplasmic Ca^{2+} concentration (Mohr & Fewtrell, 1987a), and this requires extracellular calcium (Fewtrell, 1985), secretion should correlate more closely with calcium influx, as we have shown, rather than with the initial peak of cytoplasmic Ca^{2+}. Further experiments will be necessary to confirm this interpretation, and other explanations such as differences in the nature and rate of formation of receptor aggregates in response to oligomers of IgE and antigen are equally plausible. Indeed, when the rise in cytoplasmic Ca^{2+} concentration in response to increasing concentrations of antigen (aggregated ovalbumin) is compared with secretion (Figure 2 in Beaven et al. (1984a)], the data are very reminiscent of our results (Figure 7). This is also consistent with the idea that aggregated ovalbumin causes little or no release of Ca^{2+} from intracellular stores (Beaven et al., 1984a).

When peritoneal mast cells are stimulated with antigen in the absence of calcium, they rapidly lose their ability to secrete when calcium is subsequently restored (Foreman & Garland, 1974), and it has been suggested that this desensitization is due to the inactivation or closure of calcium channels. Significant desensitization does not occur in RBL cells unless the number of antigen-specific IgE receptors is very low (Fewtrell, 1985). Provided that more than 10% of the receptors have been activated, a secretory response can still be elicited whenever calcium is added back to the cells. Consistent with this, we found that the increase in plasma membrane permeability to calcium persisted for at least an hour after the addition of antigen (Figure 5). Activated IgE receptors were required to maintain the permeability pathway since prevention of further receptor aggregation and/or displacement of antigen with monovalent hapten immediately reduced ^{45}Ca influx to the level seen in unstimulated cells (Figure 6).

What is the nature of the Ca^{2+} permeability pathway that we are measuring in RBL cells? For many years, it has been assumed that mast cells have an IgE receptor-activated calcium channel (Foreman & Mongar, 1975). Strong support for this idea came from the studies of Mazurek, Pecht, and their collegues, who showed that a protein that bound the antiallergic drug cromolyn had many of the properties expected of such a channel (Mazurek et al., 1982, 1983, 1984). When reconstituted into planar lipid bilayers, together with receptors for IgE, the purified protein behaved as a voltage-independent, IgE receptor-activated channel that was selective for calcium (Mazurek et al., 1984). Complete acceptance of these provocative findings will require independent confirmation and a more detailed description of the reagents and methods used in these studies. It will also be important to relate these results to other studies of the effects of cromolyn on different types of mast cell [see Gomperts and Fewtrell (1985) and Metzger et al. (1986) for a more detailed discussion]. Attempts to measure antigen-induced Ca^{2+} currents in mast cells and RBL cells using patch-clamping techniques have so far been unsuccessful (Ikeda & Weight, 1984; Lindau & Fernandez, 1986a,b), and the possibility that calcium is entering the cells by mechanism(s) other than a channel cannot be excluded. For example, it was recently proposed that reversal of Na^+–Ca^{2+} exchange might be one such pathway (Snowdowne & Borle, 1985; Stump et al., 1986).

A common property of channels is that they only reach saturation at very high ion concentrations (usually >0.1 M) whereas carriers or exchangers saturate at much lower ion concentrations (Hille, 1984). A calcium channel that appears to saturate at millimolar concentrations of calcium has, however, been described in *Paramecium* (Satow & Kung, 1979). Furthermore, the channel conductance of the reconstituted cromolyn binding protein is saturated in a physiological saline solution containing 2 mM $CaCl_2$ (Mazurek et al., 1984). In agreement with this, we found (Figure 4) that the IgE receptor-activated Ca^{2+} permeability pathway had an apparent K_m of about 0.7 mM and was fully saturated at Ca^{2+} concentrations in the millimolar range.

The open state of the cromolyn binding protein has a conductance of 2 pS for calcium when reconstituted into planar lipid bilayers (Mazurek et al., 1984). This corresponds to a calcium flux of about 3×10^5 ions/s at a membrane potential of −55 mV (Sagi-Eisenberg & Pecht, 1983). The rate of ion transport through a pore or channel is usually in this range, or higher, whereas the turnover numbers for enzymes or carriers are generally at least 1 or more orders of magnitude lower (Hille, 1984). The apparent V_{max} for IgE receptor-activated calcium influx was 0.9 nmol $(10^6 \text{ cells})^{-1}$ min^{-1} (Figure 4). An RBL (2H3) cell has about 3×10^5 IgE receptors (Fewtrell & Metzger, 1980), so if we assume that a cluster of three receptors (Fewtrell & Metzger, 1980) activates one calcium permeability pathway and that only 10% of the receptors are required to activate all of the pathways (Figure 8), there should be about 10^4 pathways per cell. If this is the case, then each will allow an average of 10^3 Ca^{2+} ions/s to

pass into the cell. It should be pointed out that Mazurek and his collegues imply that an RBL cell has as many as 8×10^5 cromolyn binding protein molecules and suggest that two of these molecules may be required to form a single channel (Mazurek et al., 1983). If this is the case and if all the channels are activated by antigen, then calcium influx would be as low as 23 ions channel^{-1} s^{-1}. Clearly, both these values are several orders of magnitude slower than the expected rate of ion movement through an open channel. However, channels are not open all the time, and so it was of interest to compare our values for ^{45}Ca influx with those obtained under conditions where influx was known to be occurring through a well-characterized channel. Tan and Tashjian (1984) measured ^{45}Ca uptake in a clonal pituitary cell line (GH_4C_1) when voltage-sensitive calcium channels were opened in response to depolarization with high potassium. Under these conditions, they measured a ^{45}Ca uptake of 1.8 nmol (10^6 cells)$^{-1}$ min^{-1}. Assuming that there are about 2000 calcium channels per cell (Dubinsky & Oxford, 1984) and that all these channels are activated, this would correspond to about 10^4 Ca^{2+} ions channel^{-1} s^{-1}. This is only an order of magnitude higher than the upper value we estimated for the permeability pathway in RBL cells, and these calculations required assumptions that could be off by at least 1 order of magnitude. Thus, it is possible that we are measuring the movement of Ca^{2+} through a channel but that even when it is activated the probability of the channel being open is very low.

While many properties of the calcium permeability pathway in RBL cells are those expected of a channel such as the putative cromolyn binding Ca^{2+} channel, other properties are more reminiscent of a carrier or exchange mechanism. Further experiments using a variety of different approaches will be necessary to distinguish between these possibilities. Nevertheless, our results emphasize the importance of studying the calcium permeability pathway in intact cells as well as in reconstituted systems and attempting to reconcile results obtained from flux experiments with those obtained from single-channel and other electrophysiological measurements. The method that we have developed for estimating the true unidirectional influx of calcium using quin2-buffered cells has been particularly valuable in these studies and should be widely applicable to other cell types.

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Propeptide of Human Protein C Is Necessary for γ-Carboxylation†

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ABSTRACT: Protein C is one of a family of vitamin K dependent proteins, including blood coagulation factors and bone proteins, that contains γ-carboxyglutamic acid. Sequence analysis of the cDNAs for these proteins has revealed the presence of a prepro leader sequence that contains a pre sequence or hydrophobic signal sequence and a propeptide containing a number of highly conserved amino acids. The pre region is removed from the growing polypeptide chain by signal peptidase, while the pro region is subsequently removed from the protein prior to secretion. In the present study, deletion mutants have been constructed in the propeptide region of the cDNA for human protein C, and the cDNAs were then expressed in mammalian cell culture. These deletions included the removal of 4, 9, 12, 15, 16, or 17 amino acids comprising the carboxyl end of the leader sequence of 42 amino acids. The mutant proteins were then examined by Western blotting, barium citrate adsorption and precipitation, amino acid sequence analysis, and biological activity and compared with the native protein present in normal plasma. These experiments have shown that protein C is readily synthesized in mammalian cell cultures, processed, and secreted as a two-chain molecule with biological activity. Furthermore, the pre portion or signal sequence in human protein C is 18 amino acids in length, and the pro portion of the leader sequence is 24 amino acids in length. Also, during biosynthesis and secretion, the amino-terminal region of the propeptide (residues from about −12 through −17) is important for γ-carboxylation of protein C, while the present data and those of others indicate that the carboxyl-terminal portion of the propeptide (residues −1 through −4) is important for the removal of the pro leader sequence by proteolytic processing.

Protein C is a precursor to a serine protease present in plasma and plays an important physiological role in the regulation of blood coagulation (Esmon, 1987). Protein C is a vitamin K dependent glycoprotein (M_r 62 000) composed of a heavy chain (M_r 41 000) and a light chain (M_r 21 000), and these two chains are held together by a disulfide bond (Stenflo, 1976; Kisiel et al., 1976). The light chain of human protein C contains nine residues of γ-carboxyglutamic acid (DiScipio & Davie, 1979; Fernlund & Stenflo, 1982) and one β-hydroxyaspartic acid residue (Drakenberg et al., 1983; McMullen et al., 1983). Protein C shows considerable amino acid sequence homology with the other vitamin K dependent plasma proteins involved in blood coagulation, including prothrombin, factor VII, factor IX, factor X, and protein S. Analyses of the cDNA (Foster & Davie, 1984; Beckman et al., 1985) and

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