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Release of Nonmitochondrial Sequestered Ca^{2+} from Permeabilized Muscle Cells in Culture

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SUMMARY

Activation of α_1-adrenergic receptors in BC3H-1 muscle cells results in the rapid elevation of intracellular Ca^{2+}, accompanied by an unusually slow and small increase in inositol 1,4,5-trisphosphate (IP_3) formation [*J. Biol. Chem.* **263**:1952–1959 (1988); *Mol. Pharmacol.* **32**:376–383 (1987)]. To further assess the role of IP_3 in receptor-stimulated Ca^{2+} mobilization, we have examined Ca^{2+} disposition in saponin-permeabilized BC3H-1 cells. Permeabilized cells loaded with tracer $^{45}Ca^{2+}$ in a buffer containing 100 nM free Ca^{2+} accumulated >75% of their Ca^{2+} into an ATP-sensitive compartment and were insensitive to inhibitors of mitochondrial Ca^{2+} uptake. Application of IP_3 resulted in a rapid increase in $^{45}Ca^{2+}$ efflux. Under isotopic equilibrium, approximately 90% of the total membrane-enclosed $^{45}Ca^{2+}$ was released by 10 μM IP_3 within 30 sec. Maximally and half-maximally effective concentrations of IP_3 were 22 μM and 0.9 μM, respectively. Application of 10 μM GTP, but not guanine triphosphate-γ-sulfate, resulted in a slight increase in $^{45}Ca^{2+}$ efflux, which reflected a loss in total cellular Ca^{2+}. The GTP-mediated response was slower and of far smaller magnitude than that mediated by IP_3. A Ca^{2+}-triggered Ca^{2+} release mechanism appears not to amplify the receptor response in BC3H-1 cells, inasmuch as $^{45}Ca^{2+}$ efflux was not appreciably increased by elevated concentrations of free Ca^{2+}. Furthermore, caffeine and ryanodine had no effect on basal, IP_3-mediated, or α_1-adrenergic-stimulated Ca^{2+} release from intact or permeabilized cells. In conclusion, BC3H-1 cells, although showing small and slow increases in IP_3 formation upon agonist stimulation, exhibit normal sensitivity to IP_3-elicited release of Ca^{2+} and low sensitivity to other candidate Ca^{2+}-mobilizing agents. The IP_3-sensitive Ca^{2+} stores may be localized within specialized compartments and may play a greater role in the maintenance of elevated cytosolic Ca^{2+} than in the initial response to receptor activation.

The BC3H-1 cell line has been used as a cell culture model for receptor activation of intracellular responses in smooth muscle (1–4). Agonist occupation of the α_1-adrenergic receptor in BC3H-1 cells results in a 10-fold increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) within 3–5 sec of agonist addition (3). The source of the vast majority of the Ca^{2+} entering the cytoplasm of BC3H-1 cells is an intracellular membrane-bound store. Currently, the most favored candidate for the mediation of receptor activation of Ca^{2+} mobilization is 1,4,5-IP_3 (see Ref. 5 for review). Activation of receptors in a variety of cell systems stimulates the hydrolysis of phosphatidylinositol bisphosphate by phospholipase C, thereby rapidly causing an increase in 1,4,5-IP_3 accumulation (5–8).

The correlation of receptor-mediated 1,4,5-IP_3 accumulation with Ca^{2+} mobilization does not appear to be as strong in BC3H-1 cells as it is in other cell systems, inasmuch as 1,4,5-IP_3 does not significantly accumulate above basal concentrations before 30 sec after agonist addition (2). Accordingly, it is conceivable that, in cells with more highly organized architectures, such as smooth muscle, the elevation of 1,4,5-IP_3 concentration need only occur in small localized compartments. Alternatively, additional mediators may be responsible for releasing Ca^{2+} from intracellular stores, either independently of or synergistically with 1,4,5-IP_3. To investigate the effectiveness of 1,4,5-IP_3 as well as other agents in releasing Ca^{2+} from sequestered stores within BC3H-1 cells, we examined Ca^{2+} disposition and release in plasma membrane-permeabilized BC3H-1 cells. Selective permeabilization of the plasma membrane maintains the integrity of the intracellular organelles while exposing the sites of Ca^{2+} release to defined buffer conditions. Using this technique, 1,4,5-IP_3 has been shown to release sequestered Ca^{2+} from other cell systems (see Ref. 5 for review).

Experimental Procedures

Materials. 1,4,5-IP_3, saponin, and ryanodine were obtained from Calbiochem (La Jolla, CA). Stock solutions of 5 mM 1,4,5-IP_3 were stored at −20° in deionized H_2O. $^{45}Ca^{2+}$ was purchased from New England Nuclear (Boston, MA). Incubation buffer components (see

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ABBREVIATIONS: 1,4,5-IP_3, inositol 1,4,5-trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; GTP-γ-S, guanine triphosphate-γ-sulfate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

below), GTP, GTP-γ-S, caffeine, and hexokinase were obtained from Sigma Chemical Co. (St. Louis, MO). All other compounds were of reagent grade.

Experimental conditions. Experiments were carried out on confluent BC3H-1 cells that had been maintained in culture in 35-mm dishes for 13–16 days (1). The cells were washed free of culture medium with three 2-ml aliquots of wash buffer designed to mimic cytosolic ionic conditions. The wash buffer consisted of (in millimolar): KCl, 140; NaCl, 10; $MgCl_2$, 1.0; $CaCl_2$, 0.24; K_2HPO_4, 1.0; EGTA, 1.0; and HEPES, 25.0; containing 1 mg/ml bovine serum albumin at pH 7.05, 37°. Unless specified, all incubations were carried out in cytosolic-like buffer, which consisted of the wash buffer (without NaCl) plus the following cellular substrates (in millimolar): Na_2·ATP, 3; Na_2·creatine·PO_4, 8; malate, 2.5; pyruvate, 2.5; succinate, 2.5; and $MgCl_2$, 2.9; plus 6 units/ml creatine kinase at pH 7.05, 37°. The final free Ca^{2+} and Mg^{2+} concentrations were calculated to be 100 nM and 1 mM, respectively, using the dissociation constants for Ca^{2+} and Mg^{2+} binding to EGTA, ATP, and phosphate derived by Fabiato and Fabiato (9, 10).

Plasma membranes of BC3H-1 cells were permeabilized by a 3-min incubation in 1 ml of buffer plus 50 μg/ml saponin. This protocol produced cells that retained the majority of their sequestered Ca^{2+} and were maximally responsive to 1,4,5-IP_3 (data not shown). The saponin was removed from the cells by two 2-ml washes with the wash buffer. Unless otherwise indicated, the cells were further incubated in 1 ml of buffer plus 0.65 μCi/ml $^{45}Ca^{2+}$ for between 30 and 60 min. This interval enabled tracer $^{45}Ca^{2+}$ to reach isotopic equilibrium with $^{45}Ca^{2+}$.

Measurement of $^{45}Ca^{2+}$ fluxes and total retained Ca^{2+} in permeabilized BC3H-1 cells. Efflux of $^{45}Ca^{2+}$ from permeabilized BC3H-1 cells was monitored by determining the amount of $^{45}Ca^{2+}$ remaining associated with the cells after termination of a specified incubation interval in the absence of extracellular $^{45}Ca^{2+}$. Cells were washed free of $^{45}Ca^{2+}$ with three 2-ml aliquots of wash buffer. Further incubations were carried out in 1 ml of buffer containing only $^{45}Ca^{2+}$ and any added experimental compounds.

To determine the effects of various agents on total cell-associated Ca^{2+}, permeabilized BC3H-1 cells were maintained at isotopic equilibrium with $^{45}Ca^{2+}$. Instead of removing the extracellular $^{45}Ca^{2+}$, experimental compounds were applied to the cells in a fresh 1-ml aliquot of buffer plus 0.65 μCi/ml $^{45}Ca^{2+}$, without intervening washes.

The incubation period was terminated by washing the cells with three 3-ml aliquots of stop buffer at 4°. The stop buffer consisted of (in millimolar): KCl, 140; NaCl, 10; $CaCl_2$, 0.1; HEPES, 25; and $LaCl_3$, 5; at pH 7.05. The cells were removed from the dishes in two 0.5-ml aliquots of 3% Triton X-100 containing 5 mM EGTA. $^{45}Ca^{2+}$ radioactivity was determined by scintillation counting.

Results

Effect of 1,4,5-IP_3 on $^{45}Ca^{2+}$ efflux from permeabilized BC3H-1 cells. Unidirectional efflux measurements were obtained from cells that had been loaded with $^{45}Ca^{2+}$ to isotopic equilibrium. Average total cellular Ca^{2+} content was calculated from the specific activity to be 6.93 ± 0.68 nmol of Ca^{2+}/mg of cell protein, which was very similar to that of intact cells (1), indicating that sequestration and binding of Ca^{2+} was not greatly disturbed by the saponin treatment. The kinetics of $^{45}Ca^{2+}$ efflux from permeabilized cells are shown in Fig. 1. Basal efflux consisted of at least two kinetic components (k_1 = 3.07 ± 0.41 min^{-1}; k_2 = 0.08 ± 0.01 min^{-1}). The application of 1,4,5-IP_3 resulted in a rapid increase in the initial rate of $^{45}Ca^{2+}$ efflux (k_1 = 11.1 ± 1.2 min^{-1} at 10 μM 1,4,5-IP_3) from the permeabilized cells (Fig. 1). The rate of efflux from the more slowly exchanging compartments of $^{45}Ca^{2+}$ did not appear to be significantly altered by any added concentration of 1,4,5-IP_3 (k_2 = 0.14 ± 0.04 min^{-1} at 10 μM 1,4,5-IP_3). Exposure of 1,4,5-IP_3 to intact BC3H-1 cells had no effect on Ca^{2+} mobilization (data not shown).

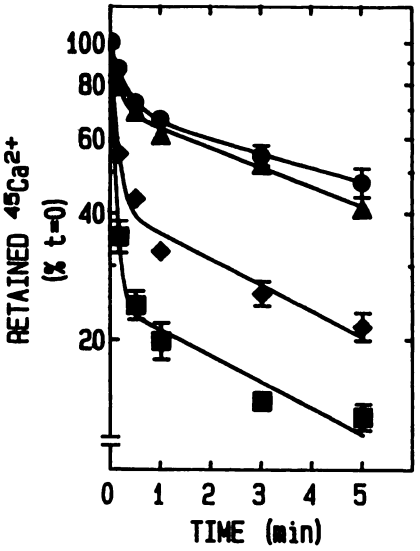

Fig. 1. Efflux of $^{45}Ca^{2+}$ from permeabilized BC3H-1 cells in response to varying 1,4,5-IP_3 concentrations. BC3H-1 cells were permeabilized and loaded with $^{45}Ca^{2+}$ as outlined in Experimental Procedures. Unidirectional efflux was monitored over the indicated time intervals in the presence of the following concentrations of 1,4,5-IP_3: 0, (●); 0.1 μM (▲); 1.0 μM (◆); and 10.0 μM (■). The data are expressed as the percentage of $^{45}Ca^{2+}$ retained by the cells relative to that retained at time zero. Each *point* represents an average ± standard error of values from three experiments, each consisting of triplicate culture dishes.

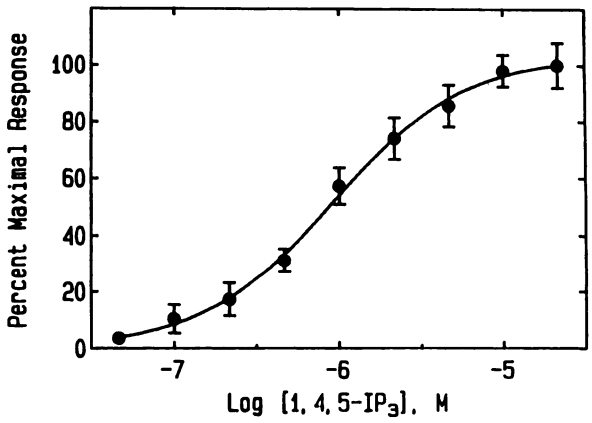

Fig. 2. Concentration dependence for 1,4,5-IP_3-mediated $^{45}Ca^{2+}$ efflux. The extent of $^{45}Ca^{2+}$ release during a 10-sec incubation interval with different concentrations of 1,4,5-IP_3 was determined as in Fig. 1. The data are expressed as the percentage of the maximal efflux response obtained with 22 μM 1,4,5-IP_3. The concentration of 1,4,5-IP_3 that elicited a half-maximal response was 0.90 ± 0.03 μM, with a Hill slope of 1.07 ± 0.09. The data were obtained from four experiments of triplicate culture dishes.

To determine the dependence of $^{45}Ca^{2+}$ efflux on 1,4,5-IP_3 concentrations, the efflux response was measured over 10 sec in order to achieve an approximation of initial rate conditions. Maximal and half-maximal $^{45}Ca^{2+}$ release over a 10-sec incubation period were obtained with 22 μM and 0.90 ± 0.03 μM 1,4,5,-IP_3, respectively (Fig. 2). The Hill slope of the response was 1.07 ± 0.09, revealing little or no cooperativity in 1,4,5-IP_3 induced $^{45}Ca^{2+}$ release.

Calcium compartments within permeabilized BC3H-1 cells. In order to differentiate between the possible stores of

Ca^{2+} within permeabilized BC3H-1 cells, the cells were incubated under conditions that would selectively inhibit Ca^{2+} uptake into the mitochondria or into the endoplasmic reticulum and other intracellular organelles. Uptake of $^{45}Ca^{2+}$ by the mitochondria was inhibited by removing the mitochondrial substrates, malate, pyruvate, and succinate, from the buffer and adding 2 μM ruthenium red (at low concentrations, a specific inhibitor of mitochondrial Ca^{2+} uptake) (11) and 5 μM CCCP (a mitochondrial uncoupler) (12) to the buffer. Inhibition of the accumulation of $^{45}Ca^{2+}$ by the endoplasmic reticulum was achieved by removing ATP from the incubation medium plus adding 25 units/ml hexokinase and 10 mM glucose to the buffer to hydrolyze any ATP synthesized by the cells. The uptake of $^{45}Ca^{2+}$ into permeabilized BC3H-1 cells reached isotopic equilibrium within 30 min of incubation with $^{45}Ca^{2+}$ (data not shown). Inhibition of mitochondrial Ca^{2+} uptake reduced the total amount of $^{45}Ca^{2+}$ accumulated by the cells from 6.45 ± 0.41 nmol of Ca^{2+}/mg of cell protein to 6.05 ± 0.07 nmol of Ca^{2+}/mg of cell protein. Incubation of permeabilized BC3H-1 cells in the absence of ATP greatly reduced Ca^{2+} accumulation to 1.76 ± 0.21 nmol of Ca^{2+}/mg of cell protein, which was less than 5% above the amount of $^{45}Ca^{2+}$ bound to cells during an instantaneous exposure to buffer plus $^{45}Ca^{2+}$. In conclusion, at 100 nM free Ca^{2+}, the majority of $^{45}Ca^{2+}$ associated with permeabilized BC3H-1 cells appeared to be in an ATP-dependent, nonmitochondrial compartment, which is presumably the endoplasmic reticulum.

The 1,4,5-IP_3-sensitive pool of Ca^{2+} was determined by monitoring $^{45}Ca^{2+}$ efflux from permeabilized BC3H-1 cells incubated in the compartment-selective buffers (Fig. 3). The relative amount of $^{45}Ca^{2+}$ released from mitochondrial-inhibited cells was equivalent to the amount released from control cells, both under basal conditions and in response to 10 μM 1,4,5-IP_3. Conversely, 1,4,5-IP_3 was unable to enhance the rate of $^{45}Ca^{2+}$ efflux from ATP-depleted cells. Additionally, 1 μM ionomycin had no effect on $^{45}Ca^{2+}$ efflux from the ATP-dependent cells, indicating that most of the Ca^{2+} bound to the cells in the absence of ATP was not in a membrane-enclosed compartment. Ionomycin did enhance $^{45}Ca^{2+}$ efflux from the ATP-loaded compartments, although the initial rate of efflux stimulated by ionomycin appeared slower in comparison with the rate of $^{45}Ca^{2+}$ efflux stimulated by 1,4,5-IP_3. The extent of efflux over 3 min was the same for both ionomycin and 1,4,5-IP_3, indicating that 1,4,5-IP_3 releases the majority of Ca^{2+} sequestered within membrane-enclosed organelles.

Effect of 1,4,5-IP_3 on total cellular Ca^{2+} in permeabilized BC3H-1 cells. In order to determine whether the 1,4,5-IP_3 mediated response desensitizes, Ca^{2+} release from permeabilized BC3H-1 cells was monitored at isotopic equilibrium with tracer $^{45}Ca^{2+}$. The amount of $^{45}Ca^{2+}$ associated with cells at isotopic equilibrium is a direct reflection of the total cellular Ca^{2+}, which has been confirmed in other studies using atomic absorption measurement of Ca^{2+} (1). Under these conditions, any reduction, or desensitization, in the 1,4,5-IP_3-mediated Ca^{2+} release could be detected by the reaccumulation of $^{45}Ca^{2+}$ by the cells. As illustrated in Fig. 4, application of 10 μM 1,4,5-IP_3 resulted in a decrement in total cell Ca^{2+} within 10 sec. The cell-associated Ca^{2+} reached a new steady state of ~55% of control levels within 30 sec of 1,4,5-IP_3 addition. Under these same conditions, 1 μM ionomycin released approximately 50% of the total cellular Ca^{2+}. Thus, 1,4,5-IP_3 was able to release

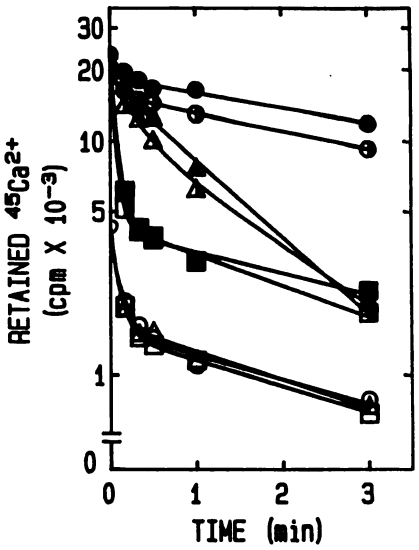

Fig. 3. 1,4,5-IP_3-sensitive compartments within permeabilized BC3H-1 cells. Permeabilized BC3H-1 cells were incubated for 60 min in different $^{45}Ca^{2+}$-containing buffers that allowed Ca^{2+} accumulation into the different cellular compartments (see text). After the extracellular $^{45}Ca^{2+}$ was washed away, the cells were incubated in the appropriate buffer for the indicated times. The cpm values at $t = 0$ for each of the respective buffers were: 23,270 cpm for control buffer (*solid symbols*); 20,180 cpm for endoplasmic reticulum-selective buffer (no malate, succinate, or pyruvate; plus 2 μM ruthenium red and 5 μM CCCP) (*half-solid symbols*); and 4,290 cpm for mitochondrial-selective buffer (no ATP; plus 25 units/ml hexokinase and 10 mM glucose) (*open symbols*). The symbols are: *triangles*, basal efflux; *squares*, efflux in response to 10 μM 1,4,5-IP_3; *triangles*, ionomycin-mediated $^{45}Ca^{2+}$ efflux. The data are from two experiments of duplicate culture dishes.

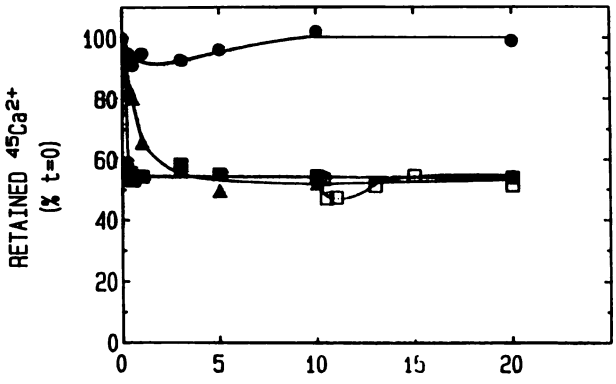

Fig. 4. Effect of 1,4,5-IP_3 on total cellular Ca^{2+} retained in permeabilized BC3H-1 cells. Total cellular Ca^{2+} was determined by measuring the amount of cell-associated $^{45}Ca^{2+}$ under isotopic equilibrium, as described in Experimental Procedures. The *symbols* represent incubations in buffer containing $^{45}Ca^{2+}$ plus the following additions: ●, no addition; ■, 10 μM 1,4,5-IP_3; □, replacement of the buffer with a fresh aliquot of buffer plus $^{45}Ca^{2+}$ plus 10 μM 1,4,5-IP_3; ▲, 1 μM ionomycin. The data were calculated from two experiments of duplicate culture dishes and are expressed as the amount of $^{45}Ca^{2+}$ retained relative to t = 0.

~90% of the membrane-enclosed Ca^{2+}. The 1,4,5-IP_3-mediated response did not appear to diminish or desensitize, inasmuch as the total cellular Ca^{2+} did not return to control levels but rather was maintained at a decreased steady state level for at least 20 min in the continued presence of 1,4,5-IP_3. Application of a fresh aliquot of 10 μM 1,4,5-IP_3 did not further decrease total cell Ca^{2+} beyond the decrement achieved by replacing the buffer alone. Thus, it appears that little breakdown of 1,4,5-IP_3

occurred during the 20-min incubation period with BC3H-1 cells because the fresh aliquot of 1,4,5-IP_3 would have elicited a response if the effective concentration of 1,4,5-IP_3 had decreased due to its metabolism. Furthermore, specific inhibition of 1,4,5-IP_3 breakdown by 2,3-bisphosphoglycerate (13) did not enhance the 1,4,5-IP_3-mediated $^{45}Ca^{2+}$ efflux response (data not shown).

Additional mediators of Ca^{2+} release from intracellular organelles. A variety of compounds other than 1,4,5-IP_3 have also been shown to be capable of releasing Ca^{2+} from the intracellular organelles of muscle and nonmuscle cells. It has been demonstrated that micromolar concentrations of the nucleotide GTP are also capable of releasing Ca^{2+} from intracellular compartments of hepatocytes, N1E-115 neuronal cells, and DDT-1 smooth muscle cells (14, 15). Application of 10 μM GTP to permeabilized BC3H-1 cells also resulted in an increase in the rate of $^{45}Ca^{2+}$ efflux (Fig. 5), although the response was markedly slower and smaller than that in response to 1,4,5-IP_3. The GTP-mediated $^{45}Ca^{2+}$ efflux was slightly enhanced by 3% polyethylene glycol (data not shown), in a manner analogous to that reported by Gill and collaborators (15). The nonhydrolyzable GTP analogue GTP-γ-S did not mimic the effects of GTP but in fact inhibited the actions of GTP. Efflux of $^{45}Ca^{2+}$ was not increased further by the addition of both GTP and 1,4,5-IP_3 simultaneously, relative to that released by 1,4,5-IP_3 alone, indicating that GTP released Ca^{2+} from the same pool as that which is sensitive to 1,4,5-IP_3. Additionally, the concentration dependency for 1,4,5-IP_3-mediated $^{45}Ca^{2+}$ efflux was not altered by pretreatment of the permeabilized cells with GTP (data not shown). Application of 10 μM GTP to cells at isotopic equilibrium resulted in the slow loss of approximately 20% of the total cellular Ca^{2+} (data not shown). Cell Ca^{2+} was maintained at this new level for at least 10 min in the continuous presence of GTP.

Recent evidence from platelets (16) suggests that the alkalinization of the cytoplasm is necessary, although insufficient itself, to allow for receptor-mediated mobilization of sequestered Ca^{2+}. Additionally, Brass and Joseph (17) have demonstrated that the elevation of the pH from 7.1 to 7.4 slightly reduced the K_m of 1,4,5-IP_3-mediated Ca^{2+} release from permeabilized platelets. In contrast, we observed that varying the pH from 6.5 to 7.5 did not affect either the basal rate of $^{45}Ca^{2+}$ efflux or the rate of efflux mediated by a 10-sec exposure of BC3H-1 cells to 10 μM 1,4,5-IP_3 (Table 1). Furthermore, the 1,4,5-IP_3 concentration dependency for $^{45}Ca^{2+}$ release was not altered when the pH of the buffer was varied from pH 6.3 to pH 7.5 (0.71 ± 0.09 μM versus 0.62 ± 0.04 μM).

Recently, it has been shown that a Ca^{2+}-sensitive Ca^{2+} channel in the sarcoplasmic reticulum of skeletal and cardiac muscle may be the same protein that binds the Ca^{2+}-release inhibitor ryanodine and also the Ca^{2+}-releasing agent caffeine (18–21). We found that caffeine had no effect on $^{45}Ca^{2+}$ efflux from permeabilized or intact BC3H-1 cells (Table 1). Varying the $[Ca^{2+}]_{free}$ did not alter the rate of $^{45}Ca^{2+}$ efflux in the absence or presence of 10 μM 1,4,5-IP_3. Furthermore, ryanodine was unable to inhibit either 1,4,5-IP_3-mediated efflux in permeabilized cells (Table 1) or phenylephrine-stimulated efflux in intact cells (data not shown). Together with the small effect of increased Ca^{2+}_{free} concentrations on $^{45}Ca^{2+}$ efflux, these results indicate that the release of sequestered Ca^{2+} in BC3H-1 cells does not proceed via the ryanodine-binding protein.

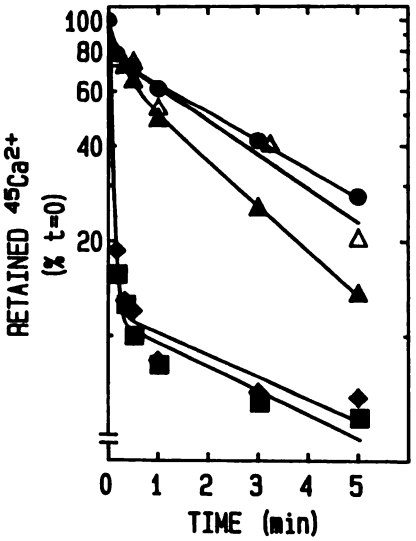

Fig. 5. Efflux of $^{45}Ca^{2+}$ from permeabilized BC3H-1 cells in the presence of guanine nucleotides. The efflux of $^{45}Ca^{2+}$ was monitored as described in Fig. 1. The cells were treated with 3% polyethylene glycol for 20 min at isotopic equilibrium before the efflux interval. The efflux buffers (containing 3% polyethylene glycol) were added at time 0. The data are expressed as the percentage of $^{45}Ca^{2+}$ retained by the cells relative to that retained at the time of efflux buffer addition (t = 0). The different conditions are represented by the following symbols: ●, buffer alone; ■, plus 10 μM 1,4,5-IP_3; ▲, plus 10 μM GTP; △, plus 10 μM GTP plus 100 μM GTP-γ-S; ◆, plus 10 μM 1,4,5-IP_3 plus 10 μM GTP. Each *point* represents the average of two experiments consisting of duplicate culture dishes.

Discussion

An increase in the concentration of cytosolic 1,4,5-IP_3 is thought to be the major signal mediating receptor activation of the mobilization of intracellularly sequestered Ca^{2+} (5). However, the interdependence of 1,4,5-IP_3 accumulation and the mobilization of intracellular Ca^{2+} is not as definitive in the BC3H-1 muscle cell line (2), or GH_4C_1 cells (22), as for other cell types that have been examined. In BC3H-1 cells, receptor-activated 1,4,5-IP_3 accumulation exhibits a lag of approximately 30 sec (2), which is well behind the half-time for the elevation of intracellular $[Ca^{2+}]$ (~2 3 sec) (3). Moreover, BC3H-1 cells show rapid oscillations in $[Ca^{2+}]_i$ after α_1-adrenergic or H_1-

TABLE 1

Release of $^{45}Ca^{2+}$ from permeabilized BC3H-1 cells

Permeabilized cells were loaded with $^{45}Ca^{2+}$ in buffer containing 100 nM free Ca^{2+}. The efflux interval was 10 sec. Free Ca^{2+} concentrations were varied in the efflux buffers by increasing the total $[Ca^{2+}]$ in buffer containing 7.88 mM EGTA, as determined from the dissociation constants from Refs. 9 and 10. The pH was 7.05, unless indicated. Each value is the average of two experiments consisting of duplicate culture dishes.

Conditions	Ca^{2+} Released	
	−1,4,5-IP_3	+10 μM 1,4,5-IP_3
	%	
$[Ca^{2+}]_{free} = 10^{-7}$ M	20.5	78.4
+10 μM ryanodine	18.4	76.7
+10 mM caffeine	18.0	ND*
pH = 7.5	27.6	75.6
pH = 6.5	24.2	79.9
$[Ca^{2+}]_{free} = 10^{-6}$ M	21.4	76.7
$[Ca^{2+}]_{free} = 10^{-5}$ M	24.1	76.1
$[Ca^{2+}]_{free} = 10^{-4}$ M	35.6	77.4

* ND, not determined.

histaminergic receptor activation (3). If 1,4,5-IP_3 is the sole mediator of Ca^{2+} release, its concentration may also oscillate in individual cells. However, the technology is not presently available that could test for this possibility within single cells.

Given this unusual behavior of the BC3H-1 cells, saponin-permeabilized cells were used in order to determine whether appreciable quantities of 1,4,5-IP_3-sensitive Ca^{2+} gates or channels do exist within the intracellular organelles of BC3H-1 cells. Application of 1,4,5-IP_3 to permeabilized, $^{45}Ca^{2+}$-loaded BC3H-1 cells did result in the rapid release of sequestered Ca^{2+} (Fig. 1). The concentrations of 1,4,5-IP_3 that induced Ca^{2+} release were within the range of values reported for 1,4,5-IP_3-mediated release of Ca^{2+} from other cell types (5). The half-maximally effective 1,4,5-IP_3 concentration was 0.9 μM, and maximal Ca^{2+} release was achieved with 22 μM 1,4,5-IP_3 (Fig. 2). The source of the Ca^{2+} was a nonmitochondrial, ATP-dependent pool (Fig. 3), presumably the endoplasmic reticulum. Thus, 1,4,5-IP_3 appeared to act on a store of Ca^{2+} that is similar to the Ca^{2+} stores characterized in other systems (5).

A Hill slope of 1.07 ± 0.09 for the 1,4,5-IP_3-induced Ca^{2+} release (Fig. 2) indicates that no cooperativity in the response occurred. This observation is difficult to reconcile with the small 2-fold change in total cellular 1,4,5-IP_3 levels measured in intact BC3H-1 cells following α_1-adrenergic receptor activation (2). This again suggests that changes in 1,4,5-IP_3 concentration would have to occur primarily within localized compartments of the cell if 1,4,5-IP_3 is the sole mediator of initial Ca^{2+} release *in vivo* and, hence, would be of far greater magnitude in these local regions than the increase in total cellular 1,4,5-IP_3 accumulation. Alternatively, increased affinity or sensitization of the 1,4,5-IP_3 receptor may occur, but cytoplasmic alkalinization does not appear to play a role (Table 1), as suggested by other investigators (17).

A recent proposal for agonist-elicited oscillations in intracellular Ca^{2+} is based on the cooperativity of the 1,4,5-IP_3-elicited Ca^{2+} release and the buffering capacity of he mitochondrial compartment (23). However, BC3H-1 cells, despite exhibiting prolonged oscillations (3), do not exhibit such cooperativity (Fig. 2) or appreciable mitochondrial buffering (Fig. 3; Table 1). Hence, this proposed mechanism for oscillations of $[Ca^{2+}]_i$ upon receptor activation would not appear to be universal. However, we cannot completely exclude the possibility that saponin selectively alters the cooperativity parameters and mitochondrial capacities.

Analysis of the effects of 1,4,5-IP_3 on Ca^{2+} release from suspended preparations of permeabilized cells has prompted some investigators (24) to propose that desensitization of the 1,4,5-IP_3 response occurs, inasmuch as the ability of 1,4,5-IP_3 to maintain an elevated extraorganellular $[Ca^{2+}]$ diminishes with time. However, these investigators observed that subsequent additions of 1,4,5-IP_3 still elicit the mobilization of sequestered Ca^{2+} in their preparations. In other preparations (25), the continuous infusion of 1,4,5-IP_3 prevents the reaccumulation of Ca^{2+} by the endoplasmic reticulum. In addition, incubation of the permeabilized cells with 2,3-bisphosphoglycerate (an inhibitor of 1,4,5-IP_3 breakdown) concomitant with 1,4,5-IP_3 results in the continued release of organellular Ca^{2+} (25). Our own studies on permeabilized BC3H-1 cells maintained at isotopic equilibrium with tracer $^{45}Ca^{2+}$ showed that 1,4,5-IP_3 released approximately 90% of the membrane-enclosed Ca^{2+}, resulting in the total cellular Ca^{2+} reaching a new steady state level, which was maintained for at least 20 min (Fig. 4). Because the volume of buffer applied to the permeabilized cells was approximately 3 orders of magnitude greater than that of the BC3H-1 intracellular organelles, a decrease in the concentration of 1,4,5-IP_3 due to its breakdown would be expected to be quite slow and calculations based on the IP_3 hydrolysis capacity of BC3H-1 cells (2) indicate that 10 μM IP_3 should be preserved in the incubation interval. Thus, it appears that releasable Ca^{2+} pools remain sensitive to 1,4,5-IP_3 for extended lengths of time and the reaccumulation of Ca^{2+} into these pools depends upon the removal of 1,4,5-IP_3 and not upon the desensitization of the response.

It is of interest to note that the level of 1,4,5-IP_3 accumulated within intact BC3H-1 cells remains constant for at least 30 min during exposure to adrenergic agonists (2). Because the 1,4,5-IP_3-induced release of Ca^{2+} from permeabilized cells did not appear to diminish as long as 1,4,5-IP_3 was present, it is expected that the effects of 1,4,5-IP_3 on Ca^{2+} disposition in intact cells would continue as long as agonist is present. Indeed, the total cellular Ca^{2+} content of intact BC3H-1 cells exposed to α_1-adrenergic agonists for prolonged periods is maintained at the new steady state level of 60–70% of control values as long as agonist is present (1). Thus, 1,4,5-IP_3 may be primarily responsible for preventing the refilling of Ca^{2+} stores during long term receptor activation. Other mediators may play a critical role in the initial increases in $[Ca^{2+}]_i$, either directly or through facilitation of the 1,4,5-IP_3-mediated response.

A variety of other compounds have been shown to induce the release of Ca^{2+} from the intracellular organelles of different cell types. For example, it has been known for some time that micromolar concentrations of Ca^{2+} will "trigger" the release of Ca^{2+} sequestered within skeletal sarcoplasmic reticulum (21). Recently, a sarcoplasmic reticulum-associated protein has been isolated from skeletal muscle, which appears to exhibit Ca^{2+} channel properties in artificial bilayers (18–20). The Ca^{2+} permeability regulated by this protein is sensitive to the $[Ca^{2+}]_{free}$, inhibited by ryanodine, and increased by caffeine. Incubation of permeabilized BC3H-1 cells in buffer containing an elevated $[Ca^{2+}]_{free}$ did not greatly elevate the rate of $^{45}Ca^{2+}$ efflux (Table 1). Additionally, neither caffeine nor ryanodine had an effect on 1,4,5-IP_3-mediated or adrenergic-stimulated $^{45}Ca^{2+}$ efflux (Table 1). These results indicate that the ryanodine receptor is a distinct entity from the IP_3 receptor and that Ca^{2+}-triggered Ca^{2+} release is probably of minor importance in BC3H-1 cells.

The nucleotide GTP also slightly increased BC3H-1 $^{45}Ca^{2+}$ efflux from permeabilized BC3H-1 cells (Fig. 5), which resulted in the loss of total cellular Ca^{2+}, although the response was far smaller and slower than that activated by 1,4,5-IP_3. The GTP-sensitive Ca^{2+} pool in BC3H-1 cells appears to be completely encompassed within the 1,4,5-IP_3-sensitive Ca^{2+} compartment, whereas the other cells (14, 15) appear to have a GTP-sensitive pool that is insensitive to 1,4,5-IP_3. Thus, the mechanism(s) by which GTP releases Ca^{2+} from intracellular organelles may not be ubiquitous in nature but may depend upon the particular function and type of cell.

This study has shown that BC3H-1 cells exhibit a sensitivity to 1,4,5-IP_3 similar to that displayed by other cells, despite the small increases 1,4,5-IP_3 levels observed after receptor activation in BC3H-1 cells. These data provide further evidence that the initial action of 1,4,5-IP_3 may be confined to defined regions

or compartments within the cell or may be facilitated by other mediators in the intact cell. Under prolonged agonist exposure, 1,4,5-IP_3 may also operate throughout the entire cytoplasm to maintain a new steady state of total cellular Ca^{2+}, in which very little Ca^{2+} resides in the agonist-sensitive pools.

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