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Store Depletion and Calcium Influx

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Parekh, Anant B., and Reinhold Penner. Store Depletion and Calcium Influx. *Physiol. Rev.* 77: 901–930, 1997.—Calcium influx in nonexcitable cells regulates such diverse processes as exocytosis, contraction, enzyme control, gene regulation, cell proliferation, and apoptosis. The dominant Ca^{2+} entry pathway in these cells is the store-operated one, in which Ca^{2+} entry is governed by the Ca^{2+} content of the agonist-sensitive intracellular Ca^{2+} stores. Only recently has a Ca^{2+} current been described that is activated by store depletion. The properties of this new current, called Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}), have been investigated in detail using the patch-clamp technique. Despite intense research, the nature of the signal that couples Ca^{2+} store content to the Ca^{2+} channels in the plasma membrane has remained elusive. Although I_{CRAC} appears to be the most effective and widespread influx pathway, other store-operated currents have also been observed. Although the Ca^{2+} release-activated Ca^{2+} channel has not yet been cloned, evidence continues to accumulate that the *Drosophila trp* gene might encode a store-operated Ca^{2+} channel. In this review, we describe the historical development of the field of Ca^{2+} signaling and the discovery of store-operated Ca^{2+} currents. We focus on the electrophysiological properties of the prototype store-operated current I_{CRAC}, discuss the regulatory mechanisms that control it, and finally consider recent advances toward the identification of molecular mechanisms involved in this ubiquitous and important Ca^{2+} entry pathway.

I. INTRODUCTION

One of the most important properties of any cell is its ability to interact with other cells within the organism. Such communication is mediated by a variety of primary signals received at the cell surface. These signals affect intracellular behavior (cell division, secretion, and motility) via specific second messengers that are elicited in response to the primary signal. Of the known second messengers, cytosolic Ca^{2+} is a major one in virtually every cell throughout the phylogenetic tree, where it regulates key processes like gene expression, secretion, contraction, and cellular metabolism (8). Eukaryotic cells can increase cytosolic free Ca^{2+} in one of two ways: either by releasing compartmentalized Ca^{2+} from intracellular stores or by evoking Ca^{2+} influx into the cell from the extracellular solution. The Ca^{2+} release phase is usually transient, fully deactivating within a few to a few tens of seconds. However, many processes require a sustained increase in cytosolic Ca^{2+} (e.g., release of hormones, contraction of blood vessels, gene transcription), and therefore, Ca^{2+} influx into the cell is crucial.

In excitable cells like neurons, muscle, and endocrine cells, Ca^{2+} influx is generally accomplished by voltage-operated Ca^{2+} channels. These are Ca^{2+}-selective pores that become briefly activated during action potentials and thereby generate inward Ca^{2+} currents. These currents are usually quite large, being in the range of several hundred picoamperes to a few nanoamperes, and can therefore easily be measured in patch-clamp recordings. On the basis of single-channel conductance, voltage dependence, pharmacological profile, and molecular biology, several different classes of Ca^{2+} channel have been characterized and termed L-, T-, N-, P-, and Q-type channels (229).

In nonexcitable cells like cells of the immune system, endothelial cells lining the blood vessels, epithelial cells in the respiratory and digestive tracts, and hepatocytes that constitute the liver, voltage-operated Ca^{2+} channels are not expressed. Until relatively recently, the Ca^{2+} entry pathways present in such cells have been a contentious issue. Although numerous Ca^{2+} entry mechanisms have been postulated, it has now been firmly established that store-operated Ca^{2+} entry, where Ca^{2+} influx is specifically controlled by the filling state of intracellular Ca^{2+} stores, is the predominant pathway.

The importance of this signaling pathway has been recognized in numerous investigations and received a great deal of attention as witnessed by a large number of reviews on the topic (10, 32, 55, 163). In the present review, we attempt to cover most of the classical and more recent aspects of store-operated Ca^{2+} entry, well aware that the rapid pace at which the field advances may render some of the ideas emphasized here obsolete or substantiated by the time this review leaves the press.

II. STORE-OPERATED CALCIUM INFLUX: HISTORY

A. Capacitative Refilling of Calcium Stores

The concept of store-operated Ca^{2+} influx took its roots in a series of pioneering experiments conducted independently in parotid acinar and smooth muscle cells. Taking advantage of the fact that parotid acinar cells express Ca^{2+}-dependent K^+ channels, Putney (182) monitored submembranous cytosolic Ca^{2+} through the efflux rates of $^{42}K^+$ and $^{86}Rb^+$ from the cells. Application of the muscarinic receptor agonist carbachol triggered a biphasic release of the radioisotope from $^{86}Rb^+$-preloaded cells (182). The rapid transient efflux phase was unaffected by the removal of extracellular Ca^{2+}, whereas the smaller sustained phase was abolished after either chelation of external Ca^{2+} or application of La^{3+}. Unidirectional flux studies then demonstrated that carbachol increased $^{45}Ca^{2+}$ uptake into the parotid gland (5, 183). Hence, receptor stimulation evoked a biphasic increase in cytosolic Ca^{2+}, due to release from an internal store and subsequent Ca^{2+} influx into the cell.

A clever series of "cross inactivation" experiments by Putney's group demonstrated that the receptor agonists carbachol, phenylephrine, and substance P all mobilized the same intracellular Ca^{2+} store and that this store could be depleted by repetitive stimulation in Ca^{2+}-free solution (184). Refilling of the stores (monitored through receptor-evoked $^{86}Rb^+$ efflux in Ca^{2+}-free external solution) required the presence of extracellular Ca^{2+} and was fast, being complete within 4 min. This was in marked contrast to the depletion of the stores, which could take more than 20 min when cells were bathed in Ca^{2+}-free ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)-containing solution. Hence, the stores depleted slowly but refilled quickly. A critical observation was that the stores refilled in the absence of an increase in $^{86}Rb^+$ efflux. In these experiments, Ca^{2+} was readmitted to parotid gland cells whose internal Ca^{2+} stores had been depleted by repetitive stimulation with substance P in Ca^{2+}-free solution. Calcium readmission per se did not trigger any $^{86}Rb^+$ efflux, but the subsequent challenge with substance P in Ca^{2+}-free solution resulted in large $^{86}Rb^+$ efflux.

Putney (184) suggested that receptor-evoked Ca^{2+} influx directly passed into the internal store without first traversing the cytosol. Further experiments utilizing a different protocol were consistent with this idea (177). After carbachol stimulation, application of the receptor antagonist atropine transiently increased $^{45}Ca^{2+}$ uptake, but this was associated with a decrease in $^{86}Rb^+$ efflux rather than an increase, which one would have expected if the Ca^{2+} first passed into the cytosol. Thus Putney's work on pa-

rotid gland suggested that receptor stimulation evoked a biphasic increase in Ca^{2+} that was due to Ca^{2+} release and then Ca^{2+} influx. The internal Ca^{2+} store filled rapidly from the external solution, and Ca^{2+} moved from the extracellular solution directly into the stores without first entering the cytosol.

Independent experiments on smooth muscle, an excitable cell, drew similar conclusions. Casteels and Droogmans (26) examined the properties of the norepinephrine-sensitive Ca^{2+} store in smooth muscle cells from rabbit ear artery. They found that the store could be depleted by repetitive stimulation in Ca^{2+}-free solution and that the store refilled very quickly when external Ca^{2+} was readmitted. The refilling process did not evoke a muscle contraction, despite significant Ca^{2+} entry. This led Casteels and Droogmans (26) to suggest that the stores refilled through a pathway that communicated directly with the extracellular solution. Unidirectional $^{45}Ca^{2+}$ flux experiments then demonstrated that Ca^{2+} uptake was substantially larger in muscle strips whose stores had been depleted by a challenge with norepinephrine in Ca^{2+}-free solution than in control strips, despite both having been exposed to Ca^{2+}-free solution for the same period of time. This crucial result demonstrated that the Ca^{2+} content of the agonist-sensitive store was capable of regulating a Ca^{2+} influx pathway in the plasma membrane.

B. Store-Operated Calcium Entry Across the Plasma Membrane

The experiments described above led Putney (185) to propose a model for receptor-evoked Ca^{2+} influx in 1986, which he termed capacitative Ca^{2+} influx. In this early formulation, Ca^{2+} entry would be determined by the concentration gradient for Ca^{2+} between the external solution and the stores, since a direct communication was envisaged. Emptying stores would automatically cause Ca^{2+} influx which, if the stores retained a high permeability to Ca^{2+} [through elevated inositol 1,4,5-trisphosphate ($InsP_3$)], would pass directly through the stores into the cytosol. Because of this direct communication, there was no requirement for any signal other than the depletion of the stores.

Despite its simplicity, store-operated Ca^{2+} influx did not gain wide acceptance as a general mechanism for Ca^{2+} entry at the time. Contemporary reviews instead focused on another mechanism for Ca^{2+} influx involving inositol 1,3,4,5-tetrakisphosphate ($InsP_4$), a more long-lived metabolite derived from $InsP_3$ (81, 82, 172; see sect. IV*B*). Indeed, some features of Ca^{2+} entry found in other cells were not compatible with a direct refilling pathway. For example, experiments employing the patch-clamp technique in mast cells demonstrated that Ca^{2+} influx occurred after a substantial delay following Ca^{2+} release induced by $InsP_3$ (164). Moreover, this influx was exquisitely sensitive to membrane potential changes. This is difficult to reconcile with a direct passage of Ca^{2+} from the external solution into the cytosol via the stores, because this would be expected to occur quickly and not be sensitive to voltage.

Around the time Putney proposed the model of store-operated Ca^{2+} influx, Tsien and colleagues (68) developed a series of new fluorescent probes for monitoring cytosolic Ca^{2+}. These molecules, epitomized by quin 2 and more importantly fura 2, were available in membrane-permeable forms that readily crossed the plasma membranes of a variety of different cells. The introduction of these molecules heralded a revolution in the field of Ca^{2+} signaling. For the first time, cytosolic Ca^{2+} could be measured in a noninvasive manner using a very sensitive assay. A second important tool was the introduction of thapsigargin as a means to deplete intracellular Ca^{2+} stores without a concomitant rise in $InsP_3$ (223). When combined with microfluorimetry it revealed that depletion of stores alone was a sufficient stimulus to initiate Ca^{2+} entry (219). This study also showed that agonists and thapsigargin activated the same Ca^{2+} entry pathway in a nonadditive manner. One important difference in the fura 2 experiments was that Ca^{2+} influx was detected as a rise in cytosolic Ca^{2+}. This led Takemura et al. (218) to conclude that Ca^{2+} influx passed first into the cytosol rather than directly into the stores, and the capacitative model was revised to account for the new insight (186). Since then, the presence of store-operated Ca^{2+} entry has been documented in a large variety of cells, in most cases based on measurements of intracellular Ca^{2+} levels after store depletion by thapsigargin (Table 1).

C. Store-Operated Calcium Current, I_{CRAC}

If depletion of stores activates Ca^{2+} influx, one would expect to measure this as a current flowing across the plasma membrane. Such currents can only be demonstrated using the patch-clamp technique. The first unambiguous demonstration of a store-operated Ca^{2+} current was shown in mast cells by Hoth and Penner (77), and the current was termed Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}). As of now, I_{CRAC} is the best-characterized store-operated Ca^{2+} current and has the highest selectivity for Ca^{2+} over other cations.

With the advantage of hindsight, two papers described currents that were subsequently shown to be store-operated Ca^{2+} currents. In 1988, Penner et al. (164) observed a small inward current in rat mast cells after elevation of $InsP_3$, and this current was tightly correlated with the development of Ca^{2+} plateaus arising from Ca^{2+} entry (164). In 1989, Lewis and Cahalan (105) described in more detail a similar Ca^{2+} current in Jurkat T cells that

TABLE 1. *Store-operated influx in various cell types*

Cell Type	Activation	Reference No.
Pancreatic acinar cells	$InsP_3$, thapsigargin, TBHQ, acetylcholine, cholecystokinin	6, 63, 153, 174
Pancreatic β-cells	Thapsigargin	12, 22
Insulinoma cells (RINm5F)	Thapsigargin	21
Hepatocytes	Thapsigargin, vasopressin, TBHQ, $InsP_3$	13, 108, 217
Lacrimal acinar cells	Thapsigargin, methacholine	17, 100
Colonic epithelial cells (HT-29)	CPA, carbachol, ATP, EGTA, BAPTA	19, 91
Smooth muscle	Caffeine, carbachol	111
Smooth muscle (A7r5)	Vasopressin	20, 25
Adrenal glomerulosa cells	Thapsigargin, TBHQ, angiotensin II	24, 193
Platelets	Thapsigargin, TBHQ, ionomycin, A-23187, PAF, thrombin	27, 46, 88, 195, 206, 241
Renal proximal cells	ATP	28
Glioma (C6-2B)	Thapsigargin, ionomycin, ATP, UTP	29, 144
HeLa	Thapsigargin, histamine	34, 181
Embryonic kidney (HEK 293)	Carbachol	37
Oocytes	$InsP_3$, serotonin, acethylcholine, thapsigargin	40, 161, 168, 169
Neutrophils	FMLP, thapsigargin	43, 139
Lymphocytes (Jurkat T cells)	Thapsigargin, TBHQ, CPA, ionomycin, mitogen, EGTA, BAPTA, $InsP_3$	44, 106, 119, 162, 179
Endothelial cells	Thapsigargin, TBHQ, CPA, $InsP_3$, bradykinin, substance P	61, 67, 151, 200, 205
Neuroblastoma (NG115-401L, SH-SY5Y, N1E-115)	Thapsigargin, carbachol	84, 101, 124
Fibroblasts	Thapsigargin, bradykinin	102
Basophilic leukemia cells (RBL-2H3, RBL-1)	Antigen, thapsigargin, $InsP_3$, EGTA, BAPTA, thimerosal	75, 103, 158, 248
Parotid acinar cells	Carbachol	109
Macrophages	Thapsigargin, $InsP_3$, EGTA, BAPTA	118
Sublingual mucosa cells	Thapsigargin, carbachol	129
Thyrocytes	Thapsigargin, ATP, UTP	201
Thyroid cells (PC C13)	Norepinephrine	132
Human leukemia (HL-60)	Thapsigargin	139
Gastric mucosa cells	Thapsigargin, ATP, UTP	202
Pinealocytes	Thapsigargin, norepinephrine, acetylcholine	203
Osteoclasts	Ionomycin	204
Nasal gland cells	Carbachol	209
Cerebellar granule cells	Thapsigargin	211
Megakaryocytes	Ionomycin	214
Parotid acinar cells	Methacholine	219
Smooth muscle (DDT1MF-2)	Thapsigargin, caffeine	230
Insect fat body cells	Adipokinetic hormone	236
Pituitary cells (GII_3)	Thapsigargin, ionomycin, TRH	239
Mast cells	$InsP_3$, ionomycin, EGTA, BAPTA	52–54, 77, 78

$InsP_3$, inositol 1,4,5-trisphosphate; PAF, platelet-activating factor; TRH, thyrotropin-releasing hormone; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; CPA, cyclopiazonic acid; TBHQ, di-*tert*-butylhydroquinone.

was activated by either high concentrations of the fast Ca^{2+} chelator 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) or receptor stimulation. Again, the presence of this Ca^{2+} current correlated well with Ca^{2+} influx monitored using indo 1. However, at the time, neither study realized that the current (now identified and characterized as I_{CRAC}) was in fact activated by store depletion.

The fact that Ca^{2+} does not directly pass into the stores is of major significance, because it means that an active signal is required to couple the Ca^{2+} content of the stores to Ca^{2+} channels in the plasma membrane, and it raises the key question as to the nature of the signal providing this link. Despite intense effort, this signal has remained elusive (see sect. III*B*). Because of this less direct coupling, the term *capacitative* is not strictly applicable to describe the influx mechanism. The participants of a recent meeting suggested to rename "capacitative" Ca^{2+} influx as store-operated Ca^{2+} influx (33), and we have therefore adopted this terminology in the review.

III. STORE-OPERATED CALCIUM CURRENTS: CALCIUM RELEASE-ACTIVATED CALCIUM CURRENT

Direct evidence in support of store-operated Ca^{2+} influx is provided by the electrophysiological demonstration of Ca^{2+} currents activated by depleting intracellular Ca^{2+} stores. Recent studies employing the patch-clamp technique have now clearly established the existence of store-operated Ca^{2+} currents in a variety of nonexcitable cells (Table 2). The currently best-characterized cells are mast cells, rat basophilic leukemia (RBL) cells, and Jurkat T cells, in which I_{CRAC} appears to be the crucial influx pathway.

TABLE 2. *Calcium influx channels and currents in various cell types*

Current	Conductance	Selectivity	Activation	Cell Type	Reference No.
		Store-operated channels			
I_{CRAC}	0.02 pS (110 Ca^{2+})	$Ca^{2+} > Ba^{2+} > Mn^{2+}$	Agonists	Mast cell	77
			$InsP_3$	RBL-1, RBL-2H3	53, 248
			Ionomycin	Jurkat T cells	128, 252
			EGTA/BAPTA	Macrophages	118
			Thapsigargin	Megakaryocytes	214
				MDCK cells	42
				3T3 fibroblasts	76
				HL-60 cells	76
				Hepatocytes	76
				Pancreatic acinar	6
				Endothelial cells	231, 232
				Oocytes	161
I_{DC}	11 pS (10 Ca^{2+})	$Ca^{2+} > Na^{+}$	Agonists	Endothelial cells	233
			TBHQ		
I_{DC}	2 pS (160 Ca^{2+}) 20 pS (160 Ba^{2+})	$Ba^{2+} > Ca^{2+} = Mn^{2+}$	BAPTA/0 Ca^{2+} Thapsigargin	A 431 cells	114
		Second messenger-operated channels			
$I_{Ca/InsP_4}$	2 pS (100 Mn^{2+})	$Ca^{2+} = Ba^{2+} = Mn^{2+} > Na^{+}$	Ca^{2+}, $InsP_4$	Endothelial cells	113
$I_{InsCa^{2+}}$	5–20 pS (90 Ca^{2+})	$Ca^{2+} = Na^{+} = K^{+}$	Ca^{2+}	Neutrophils	240
I_{InsP_3}	8 pS (100 Ca^{2+})	$Ca^{2+} > Ba^{2+} > Na^{+}$	$InsP_3$	Endothelial cells	234
I_{InsP_3}	7 pS (100 Ba^{2+})	$Ca^{2+} = Ba^{2+}$	$InsP_3$	Jurkat T cells	99
I_{InsP_3}	4–13 pS (110 Ca^{2+})	$Ca^{2+} = Ba^{2+}$	$InsP_3$, EGF	A 431 cells	143
$I_{GTP\gamma S}$	50 pS (Ringer) 10–20 pS (95 Ba^{2+})	$Na^{+} > Ba^{2+} > Ca^{2+} > Mn^{2+}$	GTPγS, substance P	Mast cells	126, 164
$I_{GTP\gamma S}$	1–2 pS (110 Ca^{2+})	$Ca^{2+} = Ba^{2+}$	GTPγS	A 431 cells	142
$I_{GTP\gamma S}$	4–17 pS (10 Ca^{2+})	$Ca^{2+} = Ba^{2+}$	GTPγS, ATP	Macrophages	145
		Receptor-operated channels			
I_{ATP}	5 pS (110 Ca^{2+})	$Ca^{2+} = Ba^{2+} > Na^{+}$	ATP	Smooth muscle	7
$I_{P_{2x1}}$	11 pS (110 Ca^{2+})	$Na^{+} > Ba^{2}$	ADP	Platelets	116, 117

EGF, epidermal growth factor; GTPγS, guanosine 5′-O-(3-thiotriphosphate). For current definitions, see text.

A. Properties of I_{CRAC}

Calcium release-activated Ca^{2+} current can be activated by a variety of procedures that share the common property of emptying the intracellular $InsP_3$-sensitive stores. Store depletion can be accomplished by both active and passive protocols. Active ways involve *1*) inclusion of $InsP_3$ in the patch pipette solution (77), *2*) external application of Ca^{2+} ionophores like ionomycin (77), and *3*) exposure to receptor agonists that elevate $InsP_3$ levels (158). Passive methods rely on the endogenous leak of Ca^{2+} from the stores. They prevent refilling so that the stores gradually lose their Ca^{2+}. These include *1*) dialyzing the cytoplasm with high concentrations of the Ca^{2+} chelators EGTA or BAPTA, which chelate Ca^{2+} and hence prevent store refilling (77, 78, 252); *2*) application of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors like thapsigargin which also prevent refilling (179, 159); and *3*) sensitizing the $InsP_3$ receptor to resting levels of $InsP_3$ with agents like thimerosal (157). Regardless of how the stores are depleted, I_{CRAC} activates with virtually identical properties, and I_{CRAC} has several diagnostic features.

1. Current-voltage relationship

Calcium release-activated Ca^{2+} current is a voltage-independent Ca^{2+} current in the sense that it is not gated by membrane voltage changes (78, 252). However, once activated, I_{CRAC} has a characteristic current-voltage relationship that shows relatively large currents at negative voltages and a reversal potential positive to +50 mV. The standard experimental paradigm to monitor I_{CRAC} is to apply voltage ramps spanning a range of about −100 to +100 mV. Under these conditions, I_{CRAC} has a steep current-voltage relationship that gives rise to prominent inward rectification at negative voltages. Part of this is attributable to the fact that asymmetric Ca^{2+} concentrations are used to measure the current (usually 10 mM Ca^{2+} outside and a few nM Ca^{2+} inside), which would give rise to some rectification as predicted by the Goldman-Hodgkin-Katz theory. A further accentuation of the rectification observed with the ramp protocol arises from Ca^{2+}-

dependent inactivation of I_{CRAC} (78, 253), which leads to an apparent steepening of the current-voltage curve at negative potentials. For a more detailed discussion of the negative-feedback inhibition of I_{CRAC} by intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), see section III*C*.

2. *Selectivity for Ca^{2+}*

As expected for selective Ca^{2+} currents (as opposed to Ca^{2+}-permeable nonselective cation currents), I_{CRAC} has a very positive reversal potential (greater than +50 mV). Changing external Na^+ has no significant effect on I_{CRAC}, demonstrating that little, if any, Na^+ permeates the channel in the presence of external Ca^{2+} (78, 104). Calcium release-activated Ca^{2+} current is remarkably selective for Ca^{2+}, and acute replacement of external Ca^{2+} with Ba^{2+} or Sr^{2+} results in substantially less current (77, 252). This divalent cation selectivity profile of $Ca^{2+} > Ba^{2+} = Sr^{2+}$ is not exhibited by voltage-operated Ca^{2+} channels, which are equally, if not more, permeable to Ba^{2+} and Sr^{2+}. In the sustained presence of Ba^{2+}, kinetically complex currents develop through Ca^+ release-activated Ca^{2+} (CRAC) channels (75, 78). After the initial transient decrease in current amplitude, there is a large increase in current that decays over several tens of seconds to attain a steady state. Replacing Ca^{2+} with Ba^{2+} now results in a transient increase in current amplitude before settling at a final steady-state level. These complex kinetic changes are only seen at negative voltages. Interestingly, some differences in the permeability to Ba^{2+} and Sr^{2+} have been noted when comparing I_{CRAC} in RBL cells and Jurkat T cells, which might point toward heterogeneity of I_{CRAC} in different cell types (75).

A further difference between the CRAC and voltage-operated Ca^{2+} channels is that the latter conduct outward Ca^+ or K^+ currents at potentials positive to the Ca^{2+} reversal potential. Calcium release-activated Ca^{2+} current does not conduct any significant outward monovalent cation currents unless external Ca^{2+} is lowered from 10 to 2 mM (75). Under these conditions, small outward K^+ (but not Cs^+) currents can be recorded through CRAC channels.

One good way to estimate the Ca^{2+} permeability of a channel is to relate the amount of Ca^{2+} entering per unit time (integral of the Ca^{2+} current) to the change in the Ca^{2+}-dependent wavelength of fura 2, when this dye is the dominant Ca^{2+} buffer in the cell (148, 147). The assumption is that all incoming Ca^{2+} binds to fura 2. With the use of this method, it was concluded that I_{CRAC} in mast and RBL cells is more selective for Ca^{2+} than voltage-operated Ca^{2+} channels (78). This is a remarkable property of CRAC channels when one bears in mind that voltage-operated Ca^{2+} channels are 1,000 times more selective for Ca^{2+} than Na^+.

3. *Ca^{2+}-dependent potentiation*

In addition to its inhibitory actions (see sect. III*C*), Ca^{2+} has been reported to potentiate Ca^{2+} influx. In *Xenopus* oocytes, it was found that raising external Ca^{2+} or hyperpolarizing the membrane resulted in a highly nonlinear increase in the Ca^{2+}-activated Cl^- current in thapsigargin-treated oocytes (168). It was suggested that Ca^{2+} entry elevated cytosolic Ca^{2+}, which then increased activity of CRAC channels. This would constitute a positive-feedback cycle by Ca^{2+} on its own influx. However, the highly nonlinear increase in Cl^- current on raising external Ca^{2+} was not observed in another study on oocytes (160). In Jurkat T cells, two groups independently reported that activity of CRAC channels was gated by external Ca^{2+} (30, 255). Readmission of Ca^{2+} resulted in a biphasic return of the current; an initial fast step was followed by a slower increase over several seconds. Similar results were observed when cells were held at positive potentials and then stepped to negative ones. These effects were observed in the presence of high concentrations of Ca^{2+} chelators and were blocked by Ni^{2+} acting extracellularly. It was concluded that the actions of Ca^{2+} were exerted on an extracellular site rather than a cytosolic one, in contrast to the conclusion from the oocyte experiments. In RBL cells, we have failed to see this potentiation when applying rapid changes in the membrane potential (unpublished observations). Whether or not Ca^{2+}-dependent potentiation is a widespread mechanism remains to be determined. Like the slight differences in selectivity for Ba^{2+} and Sr^{2+}, it might serve as a diagnostic tool in identifying different members of the presumed CRAC channel family.

4. *Single-channel conductance*

Calcium release-activated Ca^{2+} current has a very low single-channel conductance. Over a variety of voltages, Hoth and Penner (77) did not detect any increase in the variance as the current developed in mast cells. They estimated that if I_{CRAC} flowed through a channel mechanism, then the single-channel conductance would be substantially lower than 1 pS. Using noise analysis in Jurkat T cells, Zweifach and Lewis (252) estimated a single-channel conductance of 24 fS. This is almost three orders of magnitude smaller than single-channel conductances typical of most ionic channels. It was estimated that T cells would express 10^6 CRAC channels in the plasma membrane. Because I_{CRAC} has such a small conductance, one obvious question is whether the permeation pathway for CRAC reflects ion channel or carrier mechanisms. Resolution of this will have to await the cloning of the CRAC protein, but three lines of evidence are compatible with the permeation pathway being a channel. First, like voltage-gated Ca^{2+} channels, CRAC becomes nonselective in the absence of external divalent cations and then supports large Na^+ current. Second, I_{CRAC} exhibits anomalous mole fraction in the presence of different ratios of Ba^{2+} and Ca^{2+}. This is characteristic of Ca^{2+} channels and reflects at least

two Ca^{2+} binding sites with the pore. Finally, step hyperpolarizations evoke an instantaneous increase in conductance that is characterstic of a channel. Further evidence for I_{CRAC} being an ion channel mechanism comes from single-channel recordings of Na^+ flowing through I_{CRAC}. When I_{CRAC} carries monovalent ions, its unitary conductance increases to ~2 pS, allowing the study of its properties at the single-channel level (104).

5. *Pharmacological profile*

Although several compounds have been reported to inhibit store-operated Ca^{2+} influx (3, 34, 35, 58, 61, 191), there is other evidence that suggests these drugs may not be selective for I_{CRAC} (4, 57, 238). Some of the proposed inhibitors (e.g., econazole, SK&F-96365) not only block I_{CRAC} but also Cl^- and nonselective cation channels at similar concentrations (57). Hence, block of Ca^{2+} influx by these inhibitors is not diagnostic for I_{CRAC}. Clearly, the lack of specific inhibitors is a major obstacle to the development of the field.

Like voltage-operated Ca^{2+} channels, I_{CRAC} is blocked by a series of divalent cations with the following selectivity (all at 1 mM, Ref. 78): $Zn^{2+} > Cd^{2+} > Be^{2+} = Co^{2+} = Mn^{2+} > Ni^{2+} > Sr^{2+} > Ba^{2+}$. Interestingly, a similar divalent cation blocking profile on store-operated Ca^{2+} influx is observed in *Xenopus* oocytes (156), which might indicate that this divalent selectivity profile is typical of store-operated Ca^{2+} entry in a number of different cell types.

B. Activation Mechanisms of I_{CRAC}

1. *Stores*

It seems clear that depletion of cellular Ca^{2+} stores activates Ca^{2+} entry, but it is by no means apparent what the nature of these stores is and what exact quantitative relationship exists between degree of filling and activation of influx.

In addition to $InsP_3$-sensitive stores, nonexcitable cells can also possess $InsP_3$-insensitive ones. These include ryanodine-sensitive stores as well as stores that respond to novel intracellular messengers like sphingosine-1-phosphate (127, 215). Activation of I_{CRAC} seems restricted to the $InsP_3$-sensitive stores. The amplitude of I_{CRAC} after maximal depletion of the $InsP_3$ stores is similar to the amplitudes when all stores are emptied with either ionomycin or by dialyzing the cells with high-EGTA solution (78, 159). The amplitude of I_{CRAC} evoked by $InsP_3$ cannot be further enhanced by depletion of the $InsP_3$-insensitive stores with ionomycin. It therefore appears that I_{CRAC} is restricted to the $InsP_3$-sensitive stores.

Where are the $InsP_3$ stores that activate I_{CRAC} located in the cytosol? Paradiso et al. (154) carried out a clever set of experiments on human nasal airway epithelial cell monolayers. These are polarized cells where receptors are expressed on specialized cellular domains, like the apical and serosal surfaces. They found that stimulation of apical P_2 purinoceptors increased $InsP_3$, mobilized $InsP_3$ stores, and triggered Ca^{2+} influx only at the ipsilateral surface. No release of $InsP_3$-sensitive stores or Ca^{2+} influx was observed at the basolateral surface. Similarly, stimulation of basolateral ATP receptors resulted in Ca^{2+} release and Ca^{2+} influx that was confined to this region. No Ca^{2+} release or influx was seen in the contralateral membrane. This result suggests that in the polarized epithelial cells, receptor-mediated responses are confined to a specific region of the cell where the activated receptors reside. Production, diffusion and catabolism if $InsP_3$, as well as Ca^{2+} influx, are confined to this domain. It should be pointed out that the Ca^{2+} influx pathway in the experiments of Paradiso et al. (154) might not necessarily be a store-operated Ca^{2+} influx pathway. Recall that epithelial cells possess a Ca^{2+}-activated cation channel permeable to Ca^{2+} (23). This channel could underlie the P_2 receptor-mediated Ca^{2+} influx in the human epithelial cells. In pancreatic acinar cells, the situation appears to be different, since agonist stimulation or $InsP_3$ injection at the basal pole evokes the most marked $[Ca^{2+}]_i$ rise at the apical secretory pole (90, 227), suggesting that in these cells the spatial distribution of $InsP_3$ receptors determines the site of the primary Ca^{2+} release with $InsP_3$ acting as a mobile messenger.

Parekh and Penner (157) examined this issue using whole cell patch-clamp recordings in RBL cells. They took advantage of the sulfhydryl-containing organic compound thimerosal, which increases the sensitivity of the $InsP_3$ receptor for $InsP_3$ by two- to fivefold. In the absence of $InsP_3$ in the patch pipette solution, they found that application of thimerosal activated I_{CRAC}. This was prevented by inhibition of the $InsP_3$ receptor, as expected if the effects of thimerosal involved this receptor. Strikingly, Parekh and Penner (157) found that thimerosal could activate I_{CRAC} to its maximal amplitude even after 900-s dialysis with solutions lacking any exogenous $InsP_3$. Under these conditions, global $InsP_3$ levels would be very low. Inositol 1,4,5-trisphosphate has a lifetime of ~1 s in the cytosol and would diffuse out of the cell with a time constant of ~30 s. In whole cell recordings, high concentrations of $InsP_3$ are required to activate I_{CRAC} (~3 μM). For thimerosal to still activate I_{CRAC} under these conditions indicates that the $InsP_3$ receptors, and therefore the Ca^{2+} stores, that are sensitized by thimerosal must sense $InsP_3$ levels of several hundred nanomolar. It is difficult to see how this could be accomplished after 900-s whole cell recording in the absence of stimulation unless the stores were close to the site of $InsP_3$ production. Because this is at the plasma membrane, it would appear that the stores activating I_{CRAC} are also near the plasma membrane.

A similar conclusion about the close proximity of

stores and influx channels was drawn from studies in oocytes, where localized agonist application induced local Ca^{2+} release, which was followed by localized Ca^{2+} influx (170).

2. Ca^{2+} sensor

What links the Ca^{2+} content of the $InsP_3$-sensitive intracellular store to CRAC channel activity in the plasma membrane? Two components are necessary: a sensor of the store Ca^{2+} content and the activating signal. The molecular nature of the sensor is not known. However, two candidates have been proposed: the $InsP_3$ receptor and the Ca^{2+} binding protein calreticulin.

The $InsP_3$ receptor has remained something of an enigma. Although it has been established that cytosolic Ca^{2+} exerts a biphasic action on the Ca^{2+} release process with low concentrations facilitating $InsP_3$-mediated Ca^{2+} release and higher concentrations inhibiting it, several other key aspects of the release process are still unclear. Does the $InsP_3$ receptor inactivate in the presence of a fixed $InsP_3$ concentration? Conflicting results have been observed, with some groups reporting inactivation and others not. How is "quantal Ca^{2+} release" generated, and is the $InsP_3$ receptor regulated by luminal Ca^{2+}?

The idea that the $InsP_3$ receptor is sensitive to luminal Ca^{2+} was first postulated by Irvine (82). In an influential review, he proposed that the $InsP_3$ receptor on the stores directly coupled to an $InsP_4$ receptor in the plasma membrane and that the $InsP_4$ receptor was possibly the Ca^{2+} influx pathway. He conjectured that the $InsP_3$ receptor was directly gated by luminal Ca^{2+}. Emptying of the stores reduced luminal Ca^{2+}, which induced a conformational change in the $InsP_3$ receptor. This was then transferred to the $InsP_4$ receptor/Ca^{2+} channel by a conformational change resulting in Ca^{2+} influx. In Irvine's model, the $InsP_3$ receptor itself was the sensor of the stores Ca^{2+} content.

Subsequent experiments reported that the ability of $InsP_3$ to release Ca^{2+} from permeabilized cells was dependent on the luminal Ca^{2+} content (134, 150, 152). An increase or a decrease in luminal Ca^{2+} content enhanced or reduced, respectively, the sensitivity of the $InsP_3$ receptor for $InsP_3$. However, other reports failed to observe an effect of luminal Ca^{2+} on $InsP_3$-dependent Ca^{2+} release. In an exocrine gland, Shuttleworth (208) found that the sensitivity of the $InsP_3$ receptor to $InsP_3$ did not change when stores were loaded with different amounts of Ca^{2+}. A similar conclusion was drawn from studies in hepatocytes by Combettes et al. (36). In permeabilized RBL cells, Horne and Meyer (74) found that luminal Ca^{2+} regulated the $InsP_3$ receptor, but this was through an action on the cytosolic side that was prevented by high concentrations of the fast Ca^{2+} chelator BAPTA. Bezprozvanny and Ehrlich (15) reported that submillimolar concentrations of luminal Ca^{2+} had no effect at all on $InsP_3$-mediated channel opening when reconstituted in planar lipid bilayers. Many of the reports that have failed to document an effect of luminal Ca^{2+} on the $InsP_3$ receptor have been conducted on cell types that exhibit store-operated Ca^{2+} influx. These results would tend to argue against a role for the $InsP_3$ receptor that releases Ca^{2+} as the direct Ca^{2+} sensor of the stores.

Recent experiments have hinted that different $InsP_3$ receptors might have different functions. A currently popular idea is that the type 1 $InsP_3$ receptor is the major isoform that controls Ca^{2+} release, whereas the type 3 subtype might be more involved in Ca^{2+} influx (see sect. III*B4*). In some cell types, like *Xenopus* oocytes which have store-operated Ca^{2+} influx, the type 3 $InsP_3$ receptor is not expressed when examined using Western blots (39), which would argue against a role for the type 3 $InsP_3$ receptors as a ubiquitous Ca^{2+} sensor.

It seems a reasonable presumption that the sensor of the Ca^{2+} stores binds luminal Ca^{2+} and is therefore some sort of Ca^{2+}-binding protein. The first possible candidate was suggested to be calmodulin by Alvarez et al. (3). They found that calmodulin inhibitors increased Mn^{2+} uptake into thymocytes in the absence of store depletion. Importantly, these same calmodulin inhibitors apparently had no effect on Mn^{2+} uptake in cells whose stores had already been depleted (3). They conjectured that, in full stores, the Ca^{2+}-calmodulin complex inhibited the activation mechanism of Ca^{2+} influx (in their hands, cytochrome *P*-450, see sect. III*B3*). When stores were depleted, the inhibition was removed.

A major Ca^{2+}-binding protein within the stores is calreticulin. Mery et al. (131) overexpressed the protein in L fibroblasts and found that thapsigargin-evoked Ca^{2+} influx was abolished, whereas Ca^{2+} release was apparently unaffected. They suggested that calreticulin was involved in the activation mechanism through an action independent of its Ca^{2+}-binding properties. How calreticulin interferes with Ca^{2+} influx is not known.

3. Signal transduction

Despite intense investigation for several years, the nature of the activating signal that emanates from depleted stores is still unknown. Current models fall into two main categories (see Table 3): indirect coupling and direct coupling. Indirect coupling proposes an intermediate signal transduction pathway that could either result in the formation of a small messenger molecule acting as ligand to open the channels or trigger biochemical events that could covalently gate the channels, or even insert channels into the surface membrane. On the other hand, a direct coupling mechanism assumes a physical interaction between proteins in the plasma and organelle membrane. In this section, we briefly describe the main mechanisms that have been proposed to be involved in the activation of store-operated Ca^{2+} entry.

TABLE 3. *Proposed mechanisms of store-operated influx*

Direct Coupling Mechanism	Indirect Coupling		
	Second messenger	Kinase/phosphatase	Other
$InsP_3$ and $InsP_4$ receptor (82)	cGMP (6, 63, 153, 245)	CaM kinase II (60)	Fusion (214)
$InsP_3$ receptor type 3 (10)	CIF (62, 187, 225)	Tyrosine kinase (88, 89, 132, 198, 241, 247)	G protein (17, 53, 56, 244)
	$InsP_4$ (141, 172)	Protein kinase C (21)	Cytochrome *P*-450 (67)
		Protein phosphatase (94)	

Reference numbers are given in parentheses. cGMP, guanosine 3′,5′-cyclic monophosphate; CIF, Ca^{2+} influx factor; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; CaM, calmodulin.

A) CALCIUM INFLUX FACTORS. Randriamampita and Tsien (187) reported the isolation of a low-molecular-weight compound from Jurkat T lymphocytes that was released from depleted stores and which then activated Ca^{2+} influx in several different nonexcitable cells. They coined the term *Ca^{2+} influx factor* (CIF) for this novel messenger (187). Calcium influx factor was a nonprotein factor possessing a phosphate group. Breakdown of CIF could be prevented by okadaic acid, a protein phosphatase inhibitor (188). Independent studies by Hanley's group also resulted in the isolation of CIF from stimulated lymphocytes. Injection of CIF into oocytes evoked Ca^{2+} influx, which was potentiated by okadaic acid (93, 225). Earlier studies by Parekh et al. (161) had demonstrated that okadaic acid enhanced store-operated Ca^{2+} influx in oocytes after receptor stimulation. Based on the observation that okadaic acid enhanced the effects of CIF, Randriamampita and Tsien (187) linked the data from oocytes with that of CIF. It should be stressed that there is no evidence suggesting a role for an endogenous low-molecular-weight CIF in the oocyte, nor was a low-molecular-weight molecule postulated in the study of Parekh et al. In fact, Parekh et al. (161) pointed out that . . . "The molecule . . . may be a novel kinase and gate Ca^{2+} influx through a phosphorylation/dephosphorylation cycle."

Evidence against a major role for CIF was provided by Putney's group (16, 62). They extracted CIF from T cells and showed that it evoked Ca^{2+} influx in astrocytoma cells, independent of $InsP_3$ release. However, in mouse lacrimal acinar cells and rat hepatocytes, the extract caused Ca^{2+} release and then Ca^{2+} influx. The muscarinic receptor antagonist atropine abolished the effects of CIF, indicating that the extract possessed some ability to activate cell-surface muscarinic receptors (which couple to $InsP_3$ production). They concluded that the actions of the extract were not compatible with its containing the messenger for store-operated Ca^{2+} influx.

So far, the crucial experiment demonstrating activation of I_{CRAC} by any CIF-like molecule has not been reported. As reported in abstract form (180), CIF-containing extracts fail to activate I_{CRAC} in whole cell recordings from Jurkat T cells. Instead, CIF induced nonselective currents that are not normally observed in these cells, although it also upregulated I_{CRAC} activated by thapsigargin, suggesting a modulatory rather than a causal role for CIF.

B) GUANOSINE 3′,5′-CYCLIC MONOPHOSPHATE. In rat pancreatic acinar cells, Bahnson et al. (6) proposed that guanosine 3′,5′-cyclic monophosphate (cGMP) activated a store-operated Ca^{2+} current. Both muscarinic receptor stimulation and inclusion of $InsP_3$ in the patch pipette activated the same current through a step requiring an elevation in cGMP levels. They proposed that cGMP was the signal coupling stores to Ca^{2+} channels in the plasma membrane.

A similar conclusion was reached by Xu et al. (245) in studies on both intact and permeabilized pancreatic acinar cells. They found that pharmacological tools directed against both nitric oxide (NO) synthase and guanylyl cyclase inhibited store-operated Ca^{2+} entry, but influx could be restored by a membrane-permeable analog of cGMP. Furthermore, agents that depleted stores all increased cGMP levels, and this occurred with a low concentration of Ca^{2+} in the external solution. Nitric oxide synthase was only weakly activated by a large rise in cytosolic Ca^{2+}, but depletion of stores was very effective in activating the enzyme. They suggested that depletion of stores activated NO synthase, which then stimulated cytosolic guanylyl cyclase thereby generating cGMP. Guanosine 3′,5′-cyclic monophosphate then activated cGMP-dependent protein kinase, which activated Ca^{2+} influx.

Studies in human colonic epithelial cells by Bischof et al. (19) revealed that both NO and cGMP were important regulators of store-operated Ca^{2+} influx. Although these agents had only weak effects on cytosolic Ca^{2+} when stores were full, they increased the amount of Ca^{2+} influx after store depletion. This would indicate that these agents are important regulatory factors rather than constituting the activation mechanism itself. Interestingly, the effects of an NO donor (sodium nitroprusside) were found to be dose dependent in that low concentrations enhanced Ca^{2+} influx, whereas higher concentrations were inhibitory. A similar observation was made by Xu et al. (245), who found that low concentrations of cGMP increased store-operated Ca^{2+} influx, whereas higher concentrations of the cyclic nucleotide subsequently inhibited influx.

A subsequent report by Gilon et al. (63) failed to

observe any effect of cGMP on thapsigargin-evoked Ca^{2+} influx in both rat pancreatic acinar cells and Jurkat T cells. Similarly, in *Xenopus* oocytes and RBL cells, cGMP was without effect on store-operated Ca^{2+} influx (53, 161).

C) CYTOCHROME *P*-450 MESSENGERS. Using Mn^{2+} to monitor Ca^{2+} entry, Garcia-Sancho and colleagues (3) reported that store-operated Ca^{2+} influx in suspensions of rat thymocytes, Ehrlich ascites tumor cells, human platelets, and neutrophils was abolished by imidazole antimyotics like econazole that inhibit cytochrome *P*-450. They also found that calmodulin antagonists increased Mn^{2+} influx in cells whose stores were full. This influx was prevented by econazole. The calmodulin antagonists had no effect on Mn^{2+} uptake if applied after the stores had been depleted. They proposed that depletion of stores removes inhibition by the Ca^{2+}-calmodulin complex on microsomal cytochrome *P*-450 activity. This then results in the generation of a messenger that travels from the stores to the plasma membrane, opening the store-operated Ca^{2+} channels. In agreement with this were their findings that store-operated Ca^{2+} influx had a Q_{10} of 4–5, characteristic of enzymatic reactions and that the time course of Ca^{2+} influx was determined by a short-lived messenger (137).

Building on this work, Graier et al. (67) found that agents that interfered with cytochrome *P*-450 functioning affected store-operated Ca^{2+} influx in fura 2-loaded endothelial cells. They observed that 5,6-epoxyeicosatrienoic acid, synthesized from arachidonic acid by cytochrome *P*-450 monooxygenase, activated Ca^{2+} influx in the absence of store depletion. This influx was indistinguishable from bradykinin receptor-stimulated Ca^{2+} entry, but unlike bradykinin-stimulated Ca^{2+} influx, it was insensitive to cytochrome *P*-450 inhibitors. They suggested that depletion of stores activated cytochrome *P*-450 monooxygenase, resulting in the generation of the diffusible messenger 5,6-epoxyeicosatrienoic acid. This latter molecule, or a metabolite derived from it, activated Ca^{2+} influx.

Subsequent experiments using econazole revealed that it possessed numerous pharmacological actions not related to effects on cytrochome *P*-450. It blocks voltage-gated Ca^{2+} channels (238), Ca^{2+}-dependent K^+ channels (4), and most ominously in mast cells, it blocks I_{CRAC} as well as nonselective cation channels and Cl^- channels all over a similar concentration range (57). The inhibitors can therefore directly change the membrane potential, and hence the driving force for Ca^{2+} influx. Because the experiments using cytochrome *P*-450 inhibitors were conducted on non-voltage-clamped cells, effects on membrane potential cannot be ruled out. Furthermore, arachidonic acid and its metabolites regulate several types of ionic channels including K^+ currents (242), again raising the concern that their effects on store-operated Ca^{2+} influx might be mediated indirectly through changes in electrical driving force for Ca^{2+} influx.

D) SMALL-MOLECULAR-WEIGHT G PROTEINS. Fasolato et al. (53) observed that dialysis of RBL cells with nonhydrolyzable analogs of GTP [guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and 5′-guanylylimidodiphosphate] before depletion of stores prevented the activation of I_{CRAC} by ionomycin. Alone, GTPγS did not activate I_{CRAC}, whereas AlF_4^-, an activator of heterotrimeric G proteins but not of small G proteins, did evoke the current. This led them to suggest that a small G protein was involved in the activation mechanism. A similar conclusion was reached by Bird and Putney (17), who injected GTPγS directly into single lacrimal gland cells. They found that thapsigargin-evoked Ca^{2+} influx was abolished by preinjection of GTPγS or guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS). In rat megakaryocytes, Somasundaram et al. (214) found that GTPγS prevented the activation of I_{CRAC} to ionomycin and also suggested that a small G protein was involved. Fasolato et al. (53) and Bird and Putney (17) both found that GTP could overcome the inhibitory effects of GTPγS. Both groups also reported that, once activated, Ca^{2+} influx was less sensitive to GTPγS. The small-molecular-weight G protein is not known. Parekh and Penner (159) tested several possible candidates (ras, rac, rho, arf, cd42) but none activated I_{CRAC} nor interfered with the ability of ionomycin to evoke the current (159).

Petersen and Berridge (169) reported that GTPγS inhibited store-operated Ca^{2+} influx in *Xenopus* oocytes, but this was prevented by staurosporine, a broad protein kinase inhibitor. They concluded that the inhibitory actions of GTPγS were due to activation of protein kinases, like protein kinase C, rather than due to a small G protein (169). However, direct comparison with the results of Fasolato et al. (53) and Bird and Putney (17) is not straightforward. In the oocyte experiments, GTPγS was added after Ca^{2+} influx had been irreversibly activated. Petersen and Berridge (169) did not examine the effects of GTPγS on the activation mechanism, which was the focus of the experiments of Fasolato et al. (53) and Bird and Putney (17).

E) VESICLE FUSION. Because small G proteins are main players in vesicular trafficking, it is not surprising that vesicular fusion has been implicated in the activation of store-operated Ca^{2+} influx. There is precedence for such a mechanism in the recruitment of glucose transporters to the plasma membrane after stimulation with insulin via vesicular transport and fusion (73, 216). In analogy, Somasundaram et al. (214) have suggested that CRAC channels are stored in vesicles and then inserted into the plasma membrane of megakaryocytes after depletion of stores. This was based on the effects of the drug primaquine, which is known to inhibit vesicular transport. Pretreament with primaquine substantially reduced the ability of ionomycin to activate I_{CRAC}, whereas application of primaquine after I_{CRAC} had activated resulted in less block of current. From the effects of GTPγS, they suggested a diffusible small G protein was involved, and this regulated the primaquine-sensitive vesicular transport.

F) HETEROTRIMERIC G PROTEINS. Barritt and colleagues (13, 56) observed that the receptor agonist vasopressin, GTPγS, or thapsigargin all failed to evoke Ca^{2+} inflow in hepatocytes isolated from rats treated with pertussis toxin. In cells from control rats, these agents activated store-operated Ca^{2+} influx. Injection of GDPβS into the cells also inhibited thapsigargin-stimulated Ca^{2+} entry (13). They suggested that a pertussis toxin-sensitive heterotrimeric G protein was required for store-operated Ca^{2+} influx in hepatocytes. Injection of antibodies raised against the carboxy-terminal of the α-subunit of $G_{i\text{-}2}$, as well as direct injection of the synthetic $G\alpha_{i\text{-}2}$ peptide, abolished agonist- and thapsigargin-induced Ca^{2+} influx (14). The antibody did not affect agonist-induced Ca^{2+} release but, surprisingly, did reduce the amount of Ca^{2+} released by thapsigargin. Immunofluorescence studies revealed that $G\alpha_{i\text{-}2}$ was distributed in the cytoplasm whereas $G\alpha_{q11}$, which links receptors to phospholipase C-β, was exclusively located in the plasma membrane. The cytoplasmic distribution of $G\alpha_{i\text{-}2}$ would suggest it diffuses from stores to Ca^{2+} channels in the plasma membrane.

From studies on granulocytes, Jaconi et al. (86) also favored a role for a heterotrimeric G protein. They found that GTPγS blocked Ca^{2+} influx induced both by the receptor agonist formyl-Met-Leu-Phe and thapsigargin, without compromising the Ca^{2+} release induced by these agents. The inhibitory effect of GTPγS was not mimicked by GDPβS. Another heterotrimeric G protein activator, NaF, could substitute for GTPγS in inhibiting Ca^{2+} influx, leading them to suggest that the GTP-sensitive step encompassed a heterotrimeric G protein.

The experiments on hepatocytes and granulocytes were carried out using fura 2, under conditions where the membrane potential was not clamped. Because many channels are gated by heterotrimeric G proteins, like the inwarly rectifying K channel (31), it is likely that membrane potential will not stay constant. Additionally, effects of protein kinases on the Ca^{2+} influx pathway need to be considered (although protein kinase C was ruled out in the granulocyte experiments of Jaconi et al., Ref. 86). Finally, it is not clear whether store-operated Ca^{2+} influx is the only Ca^{2+} entry pathway in these cells. Llopis et al. (108) reported two independent influx pathways activated by receptor agonist in hepatocytes, only one of which was store activated.

G) TYROSINE KINASE. Tyrosine kinases are thought to be involved in a variety of different signal transduction pathways. Vostal et al. (241) provided the first evidence that tyrosine phosphorylation might be an important step in the activation of store-operated Ca^{2+} influx. From studies in platelets, they found that procedures that activated Ca^{2+} influx all caused tyrosine phosphorylation of a 130-kDa protein and to lesser extents 80- and 60-kDa proteins. The tyrosine kinase was specifically activated by Ca^{2+} release from the stores because phosphorylation occurred in the absence of external Ca^{2+} and was prevented by preincubating the cells with BAPTA acetoxymethyl ester (AM). Chilling the platelets also induced phosphorylation of the 130-kDa protein, apparently because the Ca^{2+}-ATPases on the stores ceased to function at the low temperature. Rewarming the chilled platelets in the absence of external Ca^{2+} did not result in dephosphorylation of the protein. Dephosphorylation only occurred if the cells were rewarmed in Ca^{2+}-containing solution, and this dephosphorylation could be prevented if thapsigargin was present. They suggested that the increased cytosolic Ca^{2+} after depletion of stores activated a cytosolic diffusible tyrosine kinase that phosphorylated a 130-kDa protein, which then resulted in Ca^{2+} influx. Refilling of the stores activated a protein tyrosine phosphatase that then dephosphorylated the 130-kDa protein resulting in cessation of Ca^{2+} influx.

The role of tyrosine kinases in platelets was also examined by Sage's group (196, 197), who tested the effects of kinase inhibitors on store-operated Ca^{2+} influx. They found that two tyrosine kinase inhibitors reduced Ca^{2+} influx in response to either receptor stimulation or thapsigargin, without affecting the Ca^{2+} release phase (196, 197). They observed tyrosine phosphorylation of the 130-kDa protein after store depletion, and this was prevented by the tyrosine kinase inhibitors. They also observed that tyrosine dephoshphorylation correlated with the extent of store refilling, compatible with the notion that the refilling process somehow activated a tyrosine phosphatase (88).

Reports suggesting a role for tyrosine kinases in store-operated Ca^{2+} influx are not confined to platelets. Villereal and co-workers (102) found that depletion of stores in human foreskin fibroblasts resulted in increased protein tyrosine phosphorylation. Again, predominant labeling was observed on a band corresponding to a protein of 130 kDa, remarkably similar to the observations in platelets. Store-operated Ca^{2+} influx in response to the receptor bradykinin or to thapsigargin was suppressed by tyrosine kinase inhibitors, whereas Ca^{2+} release was unaffected.

One of the key tenets of the tyrosine kinase model is that the activation of the relevant kinase requires a rise in cytosolic Ca^{2+}. Recall that in platelets, preloading of the cells with BAPTA-AM abolished tyrosine phosphorylation (241). In whole cell patch-clamp experiments on the other hand, where cytosolic Ca^{2+} is strongly buffered, I_{CRAC} can activate in the absence of a global increase in cytosolic Ca^{2+}. This might argue against a role for a Ca^{2+}-dependent tyrosine kinase in these cells. In platelets, the tyrosine kinase model predicts that an increase in cytosolic Ca^{2+}, in the absence of store depletion, should activate store-operated Ca^{2+} influx. It would be important to test this, perhaps in the presence of a tyrosine phosphatase inhibitor.

A further concern is that tyrosine kinases can regu-

late other ionic channels, including K^+ currents (79). Hence, inhibition of the kinases could affect the membrane potential, thereby altering the electrical gradient for Ca^{2+} influx. This would give the erroneous impression that the store-operated Ca^{2+} influx pathway itself was affected. Finally, high concentrations of the tyrosine kinase inhibitors are required and, at these doses, they are unlikely to be specific. One commonly used tyrosine kinase inhibitor, genistein, has been shown to block pharmacologically. In *Xenopus* oocytes, Parekh (155) has found that genistein inhibits the activation of store-operated Ca^{2+} influx. However, this effect is not mimicked by two structurally unrelated tyrosine kinase inhibitors, suggesting that the inhibition by genistein might not be related to tyrosine kinase block (unpublished observations).

H) CALCIUM/CALMODULIN-DEPENDENT KINASE. Although it has now been firmly established that a rise in cytosolic free Ca^{2+} is not necessary for the activation of I_{CRAC}, Ca^{2+}-activated kinases might regulate certain aspects of Ca^{2+} influx. In Chinese hamster ovary (CHO) cells, Gailly et al. (60) observed that calmodulin inhibitors attenuated the amount of Ca^{2+} influx that followed after depletion of agonist-sensitive stores either with a receptor agonist or thapsigargin. They suggested that Ca^{2+}/calmodulin kinase might regulate store-operated Ca^{2+} influx in these cells. The presence of a store-operated Ca^{2+} current in CHO cells has not been described, and it is conceivable that these cells express Ca^{2+}-activated influx pathways like Ca^{2+}-activated Ca^{2+}-permeable channels. It is interesting to recall the work of Braun and Schulman (23), who found that a Ca^{2+} activation of a Ca^{2+}-permeable channel in human epithelial cells was mediated by Ca^{2+}/calmodulin kinase (23).

In bovine vascular endothelial cells, inclusion of calmodulin in the patch pipette slowed the rate of activation of a store-operated Ca^{2+} current evoked by thapsigargin, reduced its peak amplitude, and accelerated its rate of inactivation (231). The effects of calmodulin were prevented by increasing the EGTA concentration in the patch pipette solution, indicating that the calmodulin actions required a rise in Ca^{2+}. How calmodulin is acting and whether it is through a kinase is not known.

I) PROTEIN KINASE C. Activation of phospholipase C will result in the production of both $InsP_3$ and diacylglycerol. Diacylglycerol can then activate various isozymes of protein kinase C. Although protein kinase C can inactivate I_{CRAC} (see sect. III*C4*), it has been suggested that the enzyme activated store-operated Ca^{2+} influx. Bode and Goeke (21) found that thapsigargin evoked only transient Ca^{2+} influx in populations of RINm5F cells, an insulinoma cell-line derived from pancreatic β-cells, preloaded with fura 2. Pretreatment with phorbol ester increased the amplitude and slowed the decay of Ca^{2+} influx, without altering the amount of Ca^{2+} released by thapsigargin. On the other hand, phorbol ester alone had no effect in the absence of store depletion, which suggests that protein kinase may not activate I_{CRAC} directly.

Parekh and Penner (159) found that pretreatment of RBL cells with bisindolylmaleimide, a specific protein kinase C inhibitor, had no effect on the activation of I_{CRAC}, suggesting that protein kinase C was not involved in the activation mechanism in these cells.

J) STAUROSPORINE-SENSITIVE STEP. Although protein kinase C appears not to be involved in the activation of I_{CRAC}, Parekh and Penner (159) found that pretreatment of RBL cells with the broad protein kinase inhibitor staurosporine prevented the activation of I_{CRAC} by $InsP_3$. Identical results were obtained when ionomycin was used to deplete the stores, ruling out an inhibitory action of staurosporine on Ca^{2+} release. Application of staurosporine, once I_{CRAC} had been activated, did not have any inhibitory effect on the current. This would argue against a pharmacological action of staurosporine on CRAC channels themselves. This also would suggest that, once activated, I_{CRAC} becomes independent of its activation mechanism. The inhibitory effects of staurosporine were not mimicked by bisindolylmaleimide, a relatively specific inhibitor of protein kinase C. Unraveling the molecular target of staurosporine might provide clues into the activation mechanism of I_{CRAC} in RBL cells.

K) DIRECT COUPLING. Based on an analogy with excitation-contraction coupling in skeletal muscle, where the ryanodine release channels of the sarcoplasmic reticulum physically interact with a subset of voltage-dependent Ca^{2+} channels in the plasma membrane, Irvine (82) postulated a similar mechanism for store-operated Ca^{2+} influx. In his hypothesis, the $InsP_3$ receptor on the endoplasmic reticulum physically coupled to an $InsP_4$ receptor in the plasma membrane. Interaction between these two proteins then controlled the Ca^{2+} influx pathway. A modified version of this model was forwarded by Berridge (10), who also proposed the involvement of $InsP_3$ receptors on the stores physically linked to the store-operated Ca^{2+} channels in the plasma membrane, but without the latter necessarily being $InsP_4$ receptors. The crux of this conformational-coupling model is that depletion of the Ca^{2+} stores changes the conformation of the $InsP_3$ receptor, and this leads directly to opening of the Ca^{2+} channels. So far, there has been no direct evidence in support of either conformational-coupling model. However, two indirect arguments have been cited: the analogy with excitation-contraction coupling and the presumed coupling mechanism of the trp protein.

As discussed above, the conformational-coupling models are based on an analogy with the ryanodine receptor-L-type Ca^{2+} channel complex in skeletal muscle. It therefore has a precedence in nature. It is important to keep in mind that skeletal muscle is a highly specialized tissue that contracts on a millisecond time scale. It therefore requires rapid signal transmission that can only be

provided on this time scale by direct protein-protein coupling. In both cardiac and smooth muscles, which contain similar ryanodine-sensitive and L-type Ca^{2+} channels to those in skeletal muscle, direct coupling does not occur. Instead, Ca^{2+} influx and Ca^{2+} release communicate via a diffusible messenger, namely, Ca^{2+}. Smooth muscle in particular is well-known for its slow responses, with contractions developing over a time of seconds. Such slow Ca^{2+}-dependent responses are routinely found in nonexcitable cells too. The endoplasmic reticulum in some smooth or cardiac muscle can lie very close to the plasma membrane at discrete points, yet skeletal muscle-type excitation-contraction coupling does not occur (59). The fact that the two membranes can be close therefore does not necessarily indicate a direct protein-protein interaction.

The TRP channel cloned from *Drosophila* photoreceptors is considered to be the first recombinant channel that can be activated by store depletion (see sect. VI). Furthermore, an antibody to trp in native *Drosophila* photoreceptors has localized the channel to the base of the rhabdomeres (178). This is a region where the plasma membrane and the endoplasmic reticulum lie very close to each other. It has been argued that this result is consistent with conformational coupling. However, it is not clear whether trp is indeed a store-operated Ca^{2+} channel in the photoreceptor (see sect. VI). Furthermore, a study employing different antibodies raised against trp failed to observe a preferential localization to the base of the rhabdomeres. This was used as an argument against conformational coupling between trp and a protein on the stores in the *Drosophila* photoreceptors (149).

The above arguments are balanced by some experimental findings that are difficult to reconcile with conformational coupling, which would be expected to occur rather quickly if protein-protein interactions took place. One of these findings is the significant delay after depletion of stores and the rather slow time course of of activation of I_{CRAC} (78). In exocrine gland cells, Ca^{2+} release was over within 5 s of agonist exposure, but Mn^{2+} influx did not commence for a further 30 s (209). A similar delay was observed when stores were emptied with thapsigargin. This delay was not due to Ca^{2+} inhibition of the Ca^{2+} influx pathway.

4. *Assessment of models*

The idea of a retrograde signal coming from the stores was first proposed by Takemura et al. (219). They suggested that an unknown second messenger was released from the stores which then activated Ca^{2+} influx. Three lines of evidence, all independently, point to a role for a retrograde messenger system in the activation of store-operated Ca^{2+} influx.

1) Slow activation of Ca^{2+} influx. In whole cell patch-clamp experiments, I_{CRAC} activates slowly with a time constant of 20–30 s (78, 128, 252). Similarly, in fura 2-loaded cells, Ca^{2+} influx switches on several seconds after the Ca^{2+} release phase is over (125). This time course is characteristic of biochemical signaling pathways.

2) Patch excision experiments. In *Xenopus* oocytes, Parekh et al. (161) recorded store-operated Ca^{2+} influx in cell-attached patches. Excision of the patch from the cell membrane resulted in rapid loss of the current, and this was not prevented by inclusion of $InsP_3$ in the bath solution. However, because of the large size of the oocyte, the excised patch could be reinserted into the cell at a spatially different point from where it had been excised, and the current quickly returned. This would suggest that the activation mechanism encompasses a somewhat mobile molecule, although its size was unknown. In epidermal cells, Lückhoff and Clapham (114) made a similar observation and showed that neither $InsP_3$ nor $InsP_4$, alone or in combination, prevented the rapid rundown of the excised patch current. In endothelial cells, Vaca and Kunze (234) found that excision of a cell-attached patch containing a store-operated current also ran down quickly after patch excision. It would be important to see whether the currents in epidermal and endothelial cells also return on reinserting the excised patch back into the parent cell.

3) Whole cell dialysis experiments. Fasolato et al. (53) found that the activation mechanism of I_{CRAC} washed out of the cell during whole cell patch-clamp experiments. As the time of whole cell recording increased, depletion of stores activated less I_{CRAC}. A similar conclusion was reached by Somasundaram et al. (214) from studies on rat megakaryocytes. In both cases, the molecule was estimated to be a small, diffusible protein. On the other hand, no such washout was observed in another study (157), where the most notable deviation in experimental conditions from Fasolato et al. (53) was the use of EGTA as the main Ca^{2+} buffer and inclusion of higher intracellular ATP levels. It remains to be determined more systematically which experimental parameters are responsible for the apparent washout or lack thereof in whole cell recordings.

Some features of store-operated Ca^{2+} entry are not easily accommodated in a scheme involving a second messenger-gated mechanism. For example, after depletion of stores in Ca^{2+}-free solution, readmission of external Ca^{2+} results in Ca^{2+} influx even if Ca^{2+} is readmitted several minutes after the stores have been depleted. Such experiments were first carried out by Takemura and Putney (219) and were used as evidence against a direct role for inositol polyphosphates in Ca^{2+} influx, since they would have been metabolized during such long time intervals. One might expect the same to be true for a second messenger, in that it would be metabolized and therefore would not be able to activate Ca^{2+} influx for extended periods of time. Although the long time course of acti-

vated store-operated Ca^{2+} channels might argue against a such a messenger, several pertinent points need to be borne in mind. First, the long time course does not argue against a messenger itself, but against a rapidly metabolizable one. In fact, a rapidly metabolizable messenger could conceivably activate a more stable molecule like a protein kinase. This would introduce a longer time course in the actions of the initial signal and is analogous to the adenosine 3′,5′-cyclic monophosphate (cAMP)/protein kinase A system. Short-term effects arise from cAMP actions and longer terms effects through protein phosphorylation. Second, the breakdown of one putative influx factor, CIF, is Ca^{2+} dependent. High cytosolic Ca^{2+} degrades the molecule (188). In low Ca^{2+}, as would occur when cells are maintained in Ca^{2+}-free solution for several minutes after depletion of stores, metabolism might be reduced.

The only study to address the time course of store-operated Ca^{2+} influx was conducted by Montero et al. (137). They concluded that the time course of Ca^{2+} entry was determined by a cytosolic labile message and not by the time course of store refilling. A further point of relevance to this is that the time course of I_{CRAC} might become independent of the activation mechanism. Parekh and Penner (159) found that pretreatment with staurosporine prevented the activation of I_{CRAC}, but staurosporine had no effect when applied after the current had developed. This would mean that, once activated, I_{CRAC} becomes independent of its activation mechanism. Recall the data of Fasolato et al. (53) and Bird and Putney (17) who found that GTPγS prevented the activation of I_{CRAC} only if applied before activation of the current. It had no effect once I_{CRAC} had developed. Hence, the long time course of active store-operated Ca^{2+} channels does not necessarily argue against a second messenger.

An attractive alternative to a second messenger acting as a direct ligand, and which could more easily account for the long-lived activation of store-operated Ca^{2+} entry, would be a covalent modification of the channels by phopsphorylation/dephosphorylation events or regenerative G protein cycles. Clearly, the substantial delay in the activation of I_{CRAC} and its slow development would be more consistent with a biochemical step linking store depletion to Ca^{2+} influx rather than a direct coupling model. Unfortunately, we are faced with a plethora of suggested mechanisms in this area, but no clear candidate mechanism has been demonstrated.

The major contender to the above-mentioned signaling cascades is the conformational coupling model. This model predicts that the $InsP_3$ receptor on the stores is physically coupled to CRAC channels. If this were true, then knock-out of the $InsP_3$ receptor should abolish store-operated Ca^{2+} influx. This was directly tested by Jayaraman et al. (87) in T lymphocytes. They stably transfected Jurkat T cells with an antisense oligonucleotide to the type 1 $InsP_3$ receptor cDNA. Receptor-evoked Ca^{2+} release and influx were abolished, as expected if $InsP_3$ was unable to deplete stores. However, thapsigargin was still able to evoke Ca^{2+} influx. Hence, the type 1 $InsP_3$ receptor, which was essential for Ca^{2+} release, was not necessary for Ca^{2+} influx. This important result has led Berridge to propose that the type 1 $InsP_3$ receptor controls Ca^{2+} release, whereas the type 3 $InsP_3$ receptor specifically couples to CRAC channels in the plasma membrane (11).

Is this model feasible? This question has been addressed by recent studies in *Xenopus* oocytes (39) and T lymphocytes (92), where overexpression of the type 3 $InsP_3$ receptor was found to substantially increase Ca^{2+} influx into the cell. In the T cells, this was associated with apoptosis, suggesting a crucial role for abnormalities in the type 3 receptor in disease. At first sight, these results would be consistent with the conformational model that involves type 3 $InsP_3$ receptors. However, Khan et al. (92) concluded that the type 3 receptor was inserted into the plasma membrane itself. Hence, it would function as a ligand-gated channel, being directly activated by cytosolic $InsP_3$ and conducting Ca^{2+} into the cell. It would not therefore constitute a store-operated pathway nor would the $InsP_3$ receptor function as a Ca^{2+} sensor in the stores. In the oocytes, Delisle et al. (39) showed that the type 3 $InsP_3$ receptor was not expressed in control cells. If it played a crucial role in store-operated Ca^{2+} entry, one might have expected it to be present. In any case, these experiments indicate that $InsP_3$ receptor subtypes might fulfill different functions.

It is worth bearing in mind that individual CRAC channels support only around an approximately femtoampere current (252). This is a very small amount, three orders of magnitude less than their voltage-dependent counterparts. To account for the macroscopic current in Jurkat T lymphocytes, it has been estimated that there must be >10,000 functional CRAC channels in the plasma membrane (252). This is a very large number. Voltage-dependent Ca^{2+} channels are present in copies of several hundred to 1,000-fold (single-channel current of 1 pA, whole cell current of several hundred picoamperes). Even if a protein on the stores (ostensibly the type 3 $InsP_3$ receptor) directly coupled to more than one CRAC channel, one would predict several thousand physical links, since there does not appear to be cooperativity in the activation mechanism of individual CRAC channels. Detailed immunocytochemical studies combined with electron microscopy will be helpful in examining this.

To summarize, the activation mechanism has remained largely elusive, although a confusing number of mechanisms have been proposed. It is not clear how much of the confusion can be attributed to the different experimental techniques employed. It is evident from the multitude of proposed activation mechanisms, either that there are indeed multiple mechanisms of activation specific to the cell type or that the mechanism is a general one, but

is under control of multiple regulatory steps. Because there now appears to be a diverse family of store-operated Ca^{2+} currents, with presumably subtly different physiological roles, one might venture to suggest that different cell types are endowed with different mechanisms for activating store-operated Ca^{2+} influx. Although this is a viable possibility, one cannot help but suspect that there is a ubiquitous mechanism. Virtually every cell throughout the phylogenetic tree releases Ca^{2+} from intracellular stores, and this is achieved by a ubiquitous messenger, $InsP_3$. Distinct patterns of Ca^{2+} release are accomplished, not by using different messengers, but rather by an elegant series of mechanisms that fine-tune the ability of the ubiquitous messenger to release Ca^{2+}.

C. Inactivation Mechanisms of I_{CRAC}

Several mechanisms that serve to inactivate I_{CRAC} have been characterized. They involve rapid and slow mechanisms and may be effective at different sites of the apparently complex signaling pathway between intracellular stores and plasma membrane channels.

1. Inactivation by Ca^{2+}

Calcium can regulate I_{CRAC} through three spatially and temporally distinct mechanisms. Like their voltage-dependent counterparts, CRAC channels are subject to feedback inactivation by Ca^{2+} from the cytosolic side. Hoth and Penner (78) found that, once I_{CRAC} had reached its maximum activity in rat mast cells dialyzed with EGTA, hyperpolarizing pulses resulted in an instantaneous increase in current followed by partial inactivation with time constants of ~10–100 ms (78). This inactivation was more effectively suppressed by inclusion of the fast Ca^{2+} chelator BAPTA in the pipette solution instead of EGTA. They proposed that CRAC channel inactivation resulted from the local rise of intracellular free Ca^{2+} as Ca^{2+} flowed into the cell. This would constitute a local fast negative feedback mechanism. Fast Ca^{2+}-feedback inactivation may fulfill an important role in controlling the time course and extent of Ca^{2+} influx and is likely to play an important role in determining the spatiotemporal pattern of Ca^{2+} signaling.

Zweifach and Lewis (253) showed that fast inactivation could also be observed in lymphocytes and was independent of the magnitude of the whole cell Ca^{2+} current. This implies that fast inactivation is specifically associated with the opening of individual Ca^{2+} channels. Fast inactivation was specific to Ca^{2+} because it was much less pronounced when Ba^{2+} was the charge carrier. Fast inactivation and recovery from this inactivation were both biexponential processes with fast and slow time constants in the range of 10 and 100 ms, respectively. They calculated that the Ca^{2+}-binding site was only a few nanometers from the pore. Such a local site of action suggests that CRAC channels are insensitive to global increases in cytosolic Ca^{2+} provided these excursions are not too large and if there is no store refilling (see sect. III*C2*). If global cytosolic Ca^{2+} is raised too high, as seen by photolytic release of Ca^{2+} from caged precursors, CRAC channels can quickly inactivate.

With the use of the two-electrode voltage-clamp technique in *Xenopus* oocytes, where Ca^{2+} influx is measured indirectly through the activity of Ca^{2+}-dependent Cl^- currents, Ca^{2+} inactivation of Ca^{2+} entry has been observed both after receptor stimulation and exposure to thapsigargin (155, 168).

2. Store refilling

If depletion of stores turns on I_{CRAC}, it seems likely that refilling of stores will turn off the current. Surprisingly, few studies have directly addressed this critical aspect of store-operated Ca^{2+} influx. Jacob (85) examined the temporal relationship between store refilling and Ca^{2+} entry in human endothelial cells, using the size of the histamine-evoked Ca^{2+} transient as an indicator of Ca^{2+} release and Mn^{2+} quenching of fura 2 to follow Ca^{2+} entry. He (85) found a close correlation between the Ca^{2+} content of the histamine-sensitive store and the time course of Ca^{2+} entry. A similar conclusion was reached by Montero et al. (136), from studies on human leukemia cells (HL-60 and U-937 types). They evoked Ca^{2+} influx by readmitting Ca^{2+} to cells whose stores had been depleted by prolonged incubation in Ca^{2+}-free solution. The content of the stores was assessed using the Ca^{2+} ionophore ionomycin. Montero et al. (136) found a good link between the amount of Ca^{2+} influx and the refilling of the stores, which refilled with a half time between 24 and 90 s depending on the cell type.

To date, only in T cells has it been shown that refilling of stores turns off I_{CRAC} (254). With 1.2 mM EGTA in the pipette solution, I_{CRAC} inactivated within 100 s. This inactivation was partially reversed by the presence of thapsigargin, which would prevent refilling of the stores. Hence, store refilling contributes to the inactivation of I_{CRAC}. Although store refilling can contribute to the time course of I_{CRAC}, several issues are still unclear. How does refilling of the stores inactivate I_{CRAC}? Does refilling simply remove the activating signal, or is an additional inhibitory mechanism specifically mobilized? Is the store that inactivates I_{CRAC} the same as the $InsP_3$-sensitive store that activates the current? Intracellular Ca^{2+} stores are known to fuse with each other in a GTP-dependent manner (224). It is conceivable that a subtype of $InsP_3$-insensitive Ca^{2+} stores first refills and then fuses with the $InsP_3$ stores, thereby replenishing the latter of their Ca^{2+} content.

3. Calcium-dependent but store-independent inactivation

Experiments on T lymphocytes suggest a third pathway that inactivates CRAC channels. Zweifach and Lewis

(254) found that even in the presence of thapsigargin I_{CRAC} still inactivated by ~50%. This means that I_{CRAC} can inactivate substantially even when stores cannot refill. This inactivation was Ca^{2+} dependent but could be separated from the fast Ca^{2+}-dependent inactivation operating on a milliseconds time scale. Although a role for protein phosphatases was unlikely, it would be interesting to see whether store-independent inactivation involves a protein kinase. A similar slow Ca^{2+}-dependent but store-independent inactivation is observed in NIH-3T3 cells (110) and in *Xenopus* oocytes (Parekh, unpublished observations). In the NIH-3T3 cells, two differences were observed compared with the results in the lymphocytes. First, okadaic acid had no effect on the slow inactivation, and second, a cytoplasmic rise in Ca^{2+} was not involved. Louzao et al. (110) suggested that slow inactivation arose from either Ca^{2+} binding to an external site on CRAC channels or within the pore such that the Ca^{2+} was inaccessible to BAPTA. The molecular processes that underlie this store-independent mechanism are currently not known, and future experiments need to address this issue.

Hence, three independent processes, all activated by Ca^{2+}, function to control the time course of CRAC channels. Fast inactivation operates on a milliseconds time scale, whereas the slower store-dependent and -independent mechanisms operate over several tens of seconds. These are likely to play important roles in determining the temporal properties of Ca^{2+} entry and hence shaping the pattern of Ca^{2+} signaling after receptor stimulation. It is important to note that the physiological amplitude and time course of I_{CRAC} are likely to be much smaller and shorter than suggested by the majority of biophysical experiments that are conducted with high concentrations of free EGTA or BAPTA in the recording pipette.

4. Protein kinase-mediated inactivation

A very important control mechanism that is used in a variety of biological systems is exerted through protein kinase-mediated phosphorylation. Store-operated Ca^{2+} entry is subject to such control.

Protein kinase C has disparate effects on store-operated Ca^{2+} influx, and this seems to be cell type specific. Early experiments employing fura 2 to track Ca^{2+} influx in suspensions of thyroid cells (228) and human neutrophils (138, 140) found that stimulation of protein kinase C by phorbol esters could attenuate Ca^{2+} influx evoked by thapsigargin and receptor agonists. These studies, however, could not rule out possible effects of phorbol esters on membrane potential. For example, it has been shown that receptor agonists and phorbol ester depolarizes neutrophils (98), which would also lead to a decrease in Ca^{2+} entry due to reduced driving force, but not necessarily through inactivation of the influx channels.

In RBL cells, I_{CRAC} inactivates when ATP is included in the patch pipette solution, and this inactivation is more pronounced when ATP is replaced by adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), an ATP analog that is readily used by protein kinases (158). Protein phosphatases cannot easily remove the thiophosphate moiety, which therefore results in "irreversible" protein phosphorylation. The effects of ATPγS are not mimicked by 5′-adenylylimidodiphosphate, another nonhydrolyzable analog but which is not utilized by kinases. The inactivation is accelerated by stimulation of protein kinase C and reduced in the presence of protein kinase C inhibitors, indicating a role for this kinase in the inactivation process.

In *Xenopus* oocytes, protein kinase C exerts a biphasic action. Acute stimulation with phorbol ester initially enhances thapsigargin-evoked Ca^{2+} entry, when monitored through the endogenous Ca^{2+}-dependent Cl^- current (168). Along similar lines, the time course of receptor-stimulated Ca^{2+} influx is substantially prolonged in the presence of ATPγS but not 5′-adenylylimidodiphosphate (160). Sustained stimulation of protein kinase C subsequently inactivates Ca^{2+} entry (168). The initial potentiation of Ca^{2+} influx by protein kinase C has not been observed in the other cell types where the kinase exerts an action on Ca^{2+} entry.

Because protein kinase C will be activated by receptors that increase $InsP_3$ levels, it can provide a powerful feedback mechanism on Ca^{2+} influx. Parekh and Penner (158) have found in RBL cells that the ability of certain receptor agonists to evoke Ca^{2+} entry is determined, at least in part, by the extent of protein kinase C stimulation. Activation of adenosine A_3 receptors that couple to the phosphoinositide signaling pathway evoked large Ca^{2+} release transients but very small Ca^{2+} plateaus, and this was associated with weak activation of I_{CRAC}. If the receptors were stimulated under conditions where protein kinase C was blocked, then a large noninactivating I_{CRAC} was produced which gave rise to prominent Ca^{2+} influx.

In lacrimal gland cells on the other hand, phorbol ester was reported to have no effect at all on store-operated Ca^{2+} influx (18). Similarly, Jurkat T cells also do not seem to utilize protein kinase C to modulate Ca^{2+} influx through I_{CRAC} (1, 199). This is not unlike the situation with voltage-dependent Ca^{2+} channels, some of which are modulated by PKC, whereas others are not. This might indicate that store-operated Ca^{2+} channels, although phenomenologically similar, may in fact exhibit a similar diversity as voltage-operated channels, possibly composed of subunits and regulated in different ways to meet the requirements of a given cell.

Although protein kinase C has been the best studied kinase, other kinases have been reported to regulate store-operated Ca^{2+} influx. In *Xenopus* oocytes, a membrane-permeable analog of cAMP was found to reduce Ca^{2+} entry (169). It was not clear whether this was a direct effect of the cAMP or via cAMP-dependent protein kinase A. In

RBL cells, cAMP has no significant impact on the properties of I_{CRAC} (158).

Guanosine 3′,5′-cyclic monophosphate itself or phosphorylation through cGMP-dependent protein kinase has been implicated in the activation mechanism (see sect. III*B3*), but several reports have failed to document a regulatory role. In RBL cells, cGMP did not affect inactivation of I_{CRAC} (158). Similarly, in *Xenopus* oocytes, membrane-permeable analogs of cGMP did not alter store-operated Ca^{2+} entry (161, 169). In pancreatic acinar cells and T lymphocytes, cGMP was also found to have no role in store-operated Ca^{2+} influx (63).

5. *Adenosine and guanosine nucleotides*

Fasolato and co-workers (80) described an inhibitory action of cytosplasmic ADP on I_{CRAC} in RBL cells that provided a link between the metabolic status of the cell and CRAC channels in the plasma membrane. This mechanism was found to be temperature dependent and Ca^{2+} dependent and specific for ADP compared with other nucleotides. The inhibition by ADP was rapidly lost on obtaining the whole cell configuration, indicating that it required a small diffusible factor.

IV. OTHER CALCIUM INFLUX PATHWAYS

A. Store Operated

Although I_{CRAC} was the first store-operated Ca^{2+} current to be described, other currents have been observed. These currents have a different ionic selectivity compared with I_{CRAC} and are not as selective for Ca^{2+}. They have been observed in A 431 endothelial cells (114), *Xenopus* oocytes (161), and endothelial cells (234). These are summarized in Table 2. In pancreatic β-cells, an excitable cell that expresses voltage-gated Ca^{2+} channels, depletion of stores has been reported to activate a nonselective cation current that is permeable to Ca^{2+} (243).

With the use of perforated patch recordings at 37°C in RBL cells, antigenic stimulation and thapsigargin were both found to activate an inwardly rectifying Ca^{2+} current (248). This Ca^{2+} current had very different selectivity compared with I_{CRAC} in that the permeation preference was determined to be $Sr^{2+} > Ba^{2+} > Ca^{2+}$. Interestingly, pretreatment of the cells with BAPTA-AM reversed the conductivity profile so that now $Ca^{2+} > Ba^{2+}, Sr^{2+}$. The reason for this BAPTA-dependent switch is not clear but needs to be addressed because it is might yield important insight into mechanisms that determine selectivity of store-operated channels.

It has also been proposed that the selectivity of CRAC channels changes in a manner dependent on the external Ca^{2+} concentration. When Ca^{2+} was lowered from 10 to 2 mM, some outward K^+ currents, presumed to flow through CRAC channels, were observed (75). Selectivity of store-operated Ca^{2+} currents might be dependent on the ionic composition of the solutions used, and therefore, the different selectivities might merely reflect the different experimental conditions. However, this is unlikely to account for those preparations where single-channel currents have been measured. In A 431 epidermal cells, a single-channel conductance of 2 pS (in 200 mM Ca^{2+}) was observed (114). This Ca^{2+} current was only measured in cell-attached patches and was not observed in the whole cell configuration. The reason for this is not clear but might reflect the presence of only a few channels in the entire cell, that the signal activating the channels is rapidly lost on obtaining the whole cell configuration, or the Ca^{2+} channels are rapidly inactivated by Ca^{2+} influx. In endothelial cells, a single-channel conductance of 11 pS was observed (233).

B. Store Independent

It now is widely accepted that store-operated Ca^{2+} influx is the predominant voltage-independent Ca^{2+} entry pathway. However, other Ca^{2+} influx pathways have been described. Although these pathways are not as ubiquitously distributed, they can coexist with store-operated influx in the same cell. It is therefore essential to rule out possible contributions of these other influx pathways to Ca^{2+} influx to examine store-operated Ca^{2+} influx in relative isolation.

1. *$InsP_3$-gated Ca^{2+} channels*

Calcium channels directly gated by $InsP_3$ were first described by Kuno and Gardner (99) in excised patches taken from Jurkat T lymphocytes. These channels had a single-channel conductance of 7 pS and were freely permeable to Ca^{2+} and Ba^{2+}. The $InsP_3$-activated Ca^{2+} currents have also been reported in A 431 cells (143), endothelial cells (234), and olfactory neurons (48). It is noteworthy that, with the exception of olfactory neurons, all studies that report $InsP_3$-gated ion channels in the plasma membrane have only been observed in single-channel patches.

In mast and RBL cells, $InsP_3$-, thapsigargin-, and ionomycin-activated I_{CRAC} are not additive (78, 159). Hence, $InsP_3$ does not appear to evoke additional Ca^{2+} influx other than through depletion of the stores. Similarly, whole cell experiments in Jurkat T cells only revealed I_{CRAC} after either receptor activation or application of thapsigargin and failed to document the presence of $InsP_3$-gated channels (252).

2. *$InsP_4$ and Ca^{2+} influx*

Inositol 1,4,5-trisphosphate is metabolized to $InsP_4$ by a Ca^{2+}-activated 3-kinase. Inositol 1,3,4,5-tetrakisphos-

phate would therefore provide an attractive mechanism for stimulating Ca^{2+} influx after $InsP_3$-mediated Ca^{2+} release. Initial experiments on sea urchin eggs found that although $InsP_3$ and $InsP_4$ alone were ineffective in evoking Ca^{2+} influx, a combination of $InsP_3$ and $InsP_4$ induced entry (83). These results were supported by patch-clamp experiments on lacrimal gland cells (141). In *Xenopus* oocytes, it was also reported that $InsP_3$ and $InsP_4$ act synergistically to promote Ca^{2+} influx (41). However, these reports were challenged by groups using the same cells and techniques and which concluded that $InsP_4$ was not necessary for Ca^{2+} influx. In rat mast cells, $InsP_4$ did not enhance Ca^{2+} influx induced by $InsP_3$ (125). Other experiments in *Xenopus* oocytes employing nonmetabolizable analogs of $InsP_3$ showed that $InsP_3$ alone was sufficient to induce Ca^{2+} entry (37, 132, 213). In fact, overexpressing the 3-kinase, which converts $InsP_3$ to $InsP_4$, was found to reduce Ca^{2+} influx in the oocyte, apparently by reducing $InsP_3$ levels and hence the extent of store depletion (237).

In A 431 epidermal cells, Lückhoff and Clapham (113) reported the existence of an $InsP_4$-modulated Mn^{2+}-permeable channel in excised patches. This channel required high cytosolic Ca^{2+} for activation. Modulation by $InsP_4$ was not observed when Ca^{2+} was clamped at low levels. The $InsP_4$-modulated Ca^{2+} channels have also been reported in excised patches from olfactory neurons (49). In our own experiments in RBL cells and mast cells, $InsP_4$ alone does not activate any currents at low or high cytosolic Ca^{2+} (1 μM), nor does $InsP_4$ affect the amplitude or time course of I_{CRAC} when activated by $InsP_3$ or ionomycin. Although a role for $InsP_4$ in promoting Ca^{2+} influx after receptor stimulation is controversial, it appears that $InsP_4$ does not contribute in a major way to store-operated Ca^{2+} influx.

3. Ca^{2+}-activated cation channels

Von Tscharner et al. (240) described the presence of large-conductance Ca^{2+}-activated channels permeable to Ca^{2+} in human neutrophils and proposed that these channels would be activated by Ca^{2+} release from $InsP_3$-sensitive stores (240). They would therefore contribute to the plateau phase of Ca^{2+} entry. Calcium-activated channels have also been reported in human epithelial cells (23), endothelial cells (113), brown fat cells (95), collecting duct cells (96), lung epithelial cells (122), pancreatic acinar cells (174), and rat insulinoma cells (190). These channels do not discriminate well between Ca^{2+} and Na^+, so that under physiological conditions, the current will be carried mainly by Na^+, and large currents will be required to significantly elevate cytosolic Ca^{2+}. Activation of these currents will result in a substantial depolarization of the membrane potential, and this will significantly reduce the electrical driving force for Ca^{2+} entry through the more effective store-operated pathway.

4. Ligand-gated cation channels

Ligand-gated channels, epitomized by the nicotinic acetylcholine receptor at the neuromuscular junction, are nonselective cation channels with a limited permeability to Ca^{2+}. These channels are activated by the binding of specific extracellular agonists. In smooth muscle, Benham and Tsien (7) described an ATP-gated channel with a P_{Ca}/P_{Na} of 3. Sage and co-workers (116, 117) described an ADP-gated channel in platelets that was permeable to Na^+, Ca^{2+}, and Mn^{2+}. These channels discriminate poorly between Ca^{2+} and Na^+, and therefore, large whole cell currents are required to elevate Ca^{2+}.

5. Leukotriene-activated Ca^{2+} channels

Growth factors like epidermal growth factor induce Ca^{2+} influx in many cell types. In A 431 carcinoma cells, epidermal growth factor activated 10-pS Ca^{2+} channels in excised or cell-attached patches, and this was eliminated by lipoxygenase inhibition (167). Leukotriene C_4 mimicked the effects of epidermal growth factor in excised patches. Similar eicosanoid-activated Ca^{2+} channels in other tissues have not been well documented.

6. Kinase-activated Ca^{2+} channels

In human B lymphocytes, Akha et al. (2) described an anti-immunoglobulin-induced voltage-insensitive Ca^{2+} influx pathway (2). This pathway exhibited a pharmacological profile similar to that of the L-type Ca^{2+} channel but was activated through a cGMP-dependent protein kinase pathway. It was still activated despite the presence of thapsigargin, which was interpreted as evidence against a store-dependent mechanism.

7. G protein-activated Ca^{2+} channels

In response to receptor stimulation, e.g., with compound 48/80, nonselective cation channels can be activated in rat peritoneal mast cells (but not in the tumor mast cell line RBL-2H3). The channel has a unitary conductance of 50 pS and induces small whole cell currents (5–50 pA at a holding potential of −40 mV; Ref. 164). Channel activation is likely mediated by a G protein, since GTPγS mimicks and GDPβS inhibits receptor-mediated activation of these channels. Channel activity is subject to negative-feedback inhibition through protein kinase C and high $[Ca^{2+}]_i$. Although activation of 50-pS channels is often associated with Ca^{2+} mobilization from intracellular stores, the 50-pS channel is not directly activated by either Ca^{2+} or $InsP_3$. Because I_{CRAC} is also present in mast cells, it was necessary to assess the relative contributions of the two influx pathways. The conclusion from these studies was that 50-pS cation channels account for usually less than about one-third of Ca^{2+} influx activated by receptors (52).

V. PROBING STORE-OPERATED CALCIUM INFLUX

Several different methods are employed to study store-operated Ca^{2+} influx, and it may be appropriate here to briefly delineate the advantages and disadvantages of each technique.

A. Importance of SERCA Inhibitors

The discovery of the naturally occurring plant alkaloid thapsigargin in the late 1980s focused widespread attention on store-operated Ca^{2+} influx (218, 223). Thapsigargin is a sequiterpene lactone derived from the plant *Thapsia garganica*. It is a potent inhibitor of the SERCA and has become the major tool in the study of store-dependent Ca^{2+} influx (222).

Application of thapsigargin results in elevation of cytosolic free Ca^{2+} due to slow depletion of the stores. Hence, if the depletion of stores activates Ca^{2+} entry, one would expect that thapsigargin treatment should result in prominent Ca^{2+} influx. This was soon shown to be the case first in parotid cells (218) and then in a plethora of different cell types. Because Ca^{2+} entry evoked by thapsigargin was not associated with any elevation in inositol polyphosphates (but see below), it was the depletion of the stores per se that triggered Ca^{2+} influx.

Other SERCA inhibitors like cyclopiazonic acid (CPA) and di-*tert*-butylhydroquinone (TBHQ) were also able to mimic the effects of thapsigargin, although some differences in efficacy, reversibility, and selectivity have been reported (123). The usual protocol to induce store-operated Ca^{2+} influx is to apply the SERCA inhibitor in the absence of external Ca^{2+} and then, after a few minutes when the release phase is over, to readmit extracellular Ca^{2+}. This latter maneuver results in Ca^{2+} influx, the so-called off-response (219). Calcium entry evoked by thapsigargin or other SERCA inhibitors is now taken to be diagnostic for the presence of store-operated Ca^{2+} influx, and some of the references listed in Table 1 use this as the sole indicator for store-operated Ca^{2+} influx. This begs the question as to whether this is indeed true or if additional mechanisms might contribute. This issue has been discussed controversially in hepatocytes, where receptor stimulation and thapsigargin treatment have differential effects on Ca^{2+} influx as assessed by Mn^{2+} quenching of fura 2 fluorescence (65, 108). In addition, other pharmacological effects of thapsigargin unrelated to store depletion have been documented (194).

Another concern with the use of thapsigargin is that the depletion of stores in intact cells is usually associated with a rise in cytosolic Ca^{2+}. Only in whole cell patch-clamp experiments, where high Ca^{2+} buffer is included in the pipette solution, can the rise in Ca^{2+} be effectively suppressed. Calcium-activated Ca^{2+}-permeable cation channels have been described (113, 240) and therefore could conceivably contribute to Ca^{2+} influx. Although cytosolic Ca^{2+} can be quite low during continuous application of thapsigargin in Ca^{2+}-free solution, it should be kept in mind that Ca^{2+} currents can be enhanced by Ca^{2+}-activated enzymes like calmodulin-dependent kinase. This could result in a form of "memory" in that a transient Ca^{2+} release is converted into a long-lasting activation of the Ca^{2+} entry pathway by protein phosphorylation. Of particular relevance to this issue is the recent finding that Ca^{2+} activation of cation channels in epithelial cells is mediated by calmodulin kinase, and the activation can long outlast the elevation in cytosolic free Ca^{2+} (23).

A further difficulty is that not all studies employing thapsigargin have ruled out elevation of inositol polyphosphates. If the levels of the latter rise, then one cannot definitively conclude that it is the depletion of stores per se that activates Ca^{2+} influx. Of relevance to this issue is the fact that certain isozymes of phospholipase C, the enzyme that hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate $InsP_3$, are activated by a rise in cytosolic Ca^{2+} (47). In fact, this positive feedback by Ca^{2+} on phospholipase C (which would generate more $InsP_3$ and hence Ca^{2+} release) has been proposed as a mechanism for generating cytosolic Ca^{2+} oscillations in a variety of cell types (72, 133). Although several studies have documented the absence of an increase in $InsP_3$ levels after thapsigargin treatment (66, 84, 100, 218), this important experiment has not always been conducted.

B. Cytosolic Ca^{2+} Measurements With Indicator Dyes

This method is currently the popular choice largely because cells can easily be loaded with the dye. Experiments are conducted either on cell populations or individual cells. Cell populations have the major drawback that the total Ca^{2+} signal is the average from thousands of cells, and important kinetic information is lost. Calcium oscillations were not observed in population studies, but routinely seen at the level of the individual cell.

The method has the major advantage that it is noninvasive, and therefore, important cytosolic molecules are not lost from the cell during an expeiment, as occurs in whole cell patch-clamp recordings. However, there are several limitations.

1) The membrane potential is rarely controlled and is usually a free parameter in these cells. Calcium influx is very sensitive to the membrane potential, and fluctuations in electrical driving force can have considerable effects on Ca^{2+} influx (125, 164). Furthermore, many cells express several other ion channels like Ca^{2+}-activated K^+ and Cl^- channels, and these can be activated by receptor

stimulation. In rat mast cells, it was shown that activation of a cAMP-gated Cl^- current clamped the membrane potential at negative potentials, which maintained a favorable driving force for Ca^{2+} influx (126, 164). Regulation of this Cl^- current would indirectly regulate Ca^{2+} influx through changes in the membrane potential.

2) Fura 2 experiments do not distinguish between store-operated Ca^{2+} influx and contributions from other Ca^{2+} entry pathways, which would complicate the overall interpretation.

3) The Ca^{2+} signal is determined by the balance between Ca^{2+} influx and Ca^{2+} extrusion. Changes in pump activity, Na^+/Ca^{2+} exchange, and cytosolic buffering capacity, can all give the erroneous impression of changes in Ca^{2+} influx.

4) Dye compartmentalization can occur so that the dye accumulates in an internal store. Different results have been reported when fura 2 was directly injected into the cells compared with loading through the ester form (65, 108).

C. Mn^{2+} Influx and Fluorescence Quench

To circumvent some of the above problems, especially *point 3*, some researchers have used Mn^{2+} as an indicator of unidirectional Ca^{2+} influx (130). Manganese can permeate through CRAC channels and, on entering the cell, it binds to and quenches fura 2 (54). This is easily recorded as a steep decline in the fluorescent signal at 360-nm wavelength. Manganese quenching does not overcome the problem of changes in membrane potential. Furthermore, Mn^{2+} is also permeable to nonselective cation channels and can enter the $InsP_3$-sensitive store, since the $InsP_3$ receptor/channel complex is permeable to Mn^{2+} as well. Manganese can then be released into the cytosol after receptor activation (54). Hence, Mn^{2+} quenching of fura 2 cannot be unequivocably attributed to Ca^{2+} influx through store-operated pathways. In addition, the entered Mn^{2+} might affect regulatory elements of store-operated influx.

D. Ca^{2+}-Activated Currents as Reporters

This method has been used in cells which express Ca^{2+}-activated currents (e.g., pancreatic acinar cells, *Xenopus* oocytes, HeLa cells, exocrine gland cells). *Xenopus* oocytes, for example, have numerous Ca^{2+}-dependent Cl^- channels in the plasma membrane, and Ca^{2+} influx can be monitored through the currents these channels subsequently generate. Pancreatic acinar cells (173) and avian salt gland cells (121) have both Ca^{2+}-dependent K^+ and Cl^- channels. This method also enables the membrane potential to be controlled. The main limitation is that Ca^{2+} is measured only indirectly, and changes in either Ca^{2+} buffers or the properties of the Cl^- channels themselves need to be ruled out.

E. Patch-Clamp Measurements of I_{CRAC}

The most direct way to study store-operated Ca^{2+} influx is to measure the Ca^{2+} current itself. This can only be accomplished by using the patch-clamp technique. Direct monitoring of the Ca^{2+} current greatly simplifies interpretation. For example, agonist-induced reduction in the current cannot be attributed to changes in the membrane potential or Ca^{2+} pump activity. A further advantage with the patch-clamp technique is that, in whole cell dialysis experiments, the experimenter can control the ionic composition of the cytosol by judicious choice of pipette solution. Conditions can be used that eliminate other Ca^{2+} influx pathways so that store-operated Ca^{2+} influx can be studied in relative isolation. A main limitation with the whole cell patch-clamp technique is that potentially important molecules are washed out from the cell during dialysis. The perforated patch technique avoids this problem while still enabling excellent voltage clamp. However, a high Ca^{2+}-buffering capacity is required for measuring I_{CRAC} (several mM), and this is not possible in perforated patch recordings because only small ions equilibrate between the pipette solution and the cytosol. Although it is possible to preload the cytosol with chelators by incubation with acetoxymethyl derivatives (e.g., BAPTA-AM), the actual loading is low and rarely exceeds a few hundred micromoles per liter.

The different interpretations that might be drawn using different techniques can be seen from our work on Ca^{2+} influx in RBL cells (158). In fura 2-AM-loaded cells, the amount of Ca^{2+} influx is similar following activation of either antigen or adenosine A_3 receptors. However, I_{CRAC} was smaller after antigen stimulation (-1.6 pA/pF) compared with adenosine receptor stimulation (-4 pA/pF). This means that the size of Ca^{2+} influx in a fura 2-AM-loaded cell does not accurately indicate the amplitude of I_{CRAC}. The reason for this discrepancy likely arises from the fact that antigen activates inwardly rectifying K^+ channels in RBL cells. This would clamp the membrane potential at negative potentials, thereby creating a favorable driving force for Ca^{2+} influx. Few conducting CRAC channels at negative potentials will be as effective in raising Ca^{2+} as many conducting channels at more depolarized potentials.

VI. MOLECULAR BIOLOGY OF STORE-OPERATED CHANNELS

The CRAC channel has not yet been cloned, and nothing is known about the structural components of the channel that determine gating and selectivity. Recently, much

excitement has been aroused by the findings that the *Drosophila* photoreceptor *trp* gene might encode a store-operated channel.

In invertebrates, absorption of a photon by rhodopsin initiates a cascade of events culminating in $InsP_3$ production. This is associated with a depolarization of the photoreceptor cells due to activation of an inward cationic current that generates the so-called receptor potential. The receptor potential has two components: an initial rapid depolarization due to a Na^+ current and, if light is maintained, a smaller but sustained depolarization that arises from a current carried by both Na^+ and Ca^{2+}. In one type of mutant fly, called transient receptor potential or trp, light activates only the transient current, and the slow sustained component is missing (189, 251).

Molecular cloning of the *Drosophila* trp (135), and the related protein trpl (176), subsequently revealed some homology to voltage-gated Ca^{2+} channels. Local hydrophobicity plots suggested six transmembrane-spanning regions as observed for voltage-dependent Na^+ and Ca^{2+} channels. Strikingly, homology was strongest in the S5-S6 linker region that constitutes the channel pore. Interestingly, the high density of charged amino acids in the S4 segment, thought to constitute the voltage sensor, was missing in trp. These findings led Hardie and Minke (71) to speculate that trp might encode a Ca^{2+} channel related to those involved in store-operated Ca^{2+} influx. Like voltage-gated Ca^{2+} channels, trp may be composed of multiple subunits. One such protein is INAD, which was cloned from mutant flies (207). The INAD mutants lack the prolonged depolarizing after potential which follows stimulation by intense blue light. Antibodies against InAD coimmunoprecipitate with trp, demonstrating a tight physical association.

Expression of *Drosophila* trp in Sf9 insect cells showed that the protein indeed encoded a Ca^{2+}-permeable channel that could be activated by thapsigargin (235). Similarly, in *Xenopus* oocytes, *Drosophila* trp was found to increase the amplitude of Ca^{2+}-entry activated Cl^- currents (171). Unlike trp, trpl which is a nonselective cation current, was not activated by thapsigargin but instead required GTPγS or $InsP_3$ (45). Schilling and colleagues (212) observed that trp and trpl differed largely in their carboxy-terminal tail; trp contained a long, proline-rich tail that was completely absent in trpl. They speculated that this proline-rich segment might confer the sensitivity to thapsigargin. To test this directly, they constructed chimeras between trp and trpl. When the carboxy-tail of trp was replaced with that from trpl, the trp chimera was no longer sensitive to thapsigargin. However, thapsigargin sensitivity was conferred onto trpl when it contained the carboxy-terminal of trp. It was therefore proposed that sensitivity to thapsigargin-induced Ca^{2+} store depletion resided in the carboxy-terminal tail (212).

Recently, several human homologs of trp have been identified (175, 249, 250). Transient expression of two of these clones (*Htrp1* and *Htrp3*) in COS and L cells enhanced Ca^{2+} influx up to twofold after muscarinic receptor stimulation (249). Unexpectedly, Ca^{2+} influx after thapsigargin treatment increased to a lesser extent in the *Htrp3*-transfected cells than was the case with receptor stimulation. It seemed that *Htrp3* was more sensitive to agonist-induced store depletion than thapsigargin-induced depletion. This raises the possibility that receptor activation might activate additional signaling pathways in addition to depletion of stores that control store-operated Ca^{2+} influx. Experiments using antisense oligonucleotides to the *trp* genes revealed that endogenous store-operated Ca^{2+} influx was abolished in six of nine clones that had been transfected. Zitt et al. (250) have also identified a human homologue to trp, *TRPC1A*, from a human fetal brain cDNA library. It appears to be a splice variant of *Htrp1*. Expression of *TRPC1A* in CHO cells induced nonselective cation currents with similar permeabilities for Na^+, Ca^{2+}, and Cs^+ in response to intracellular infusion of *myo*-$InsP_3$ or thapsigargin (250). A further trp homolog, termed *bCCE*, was identified by Philipp et al. (175) by screening bovine cDNA libraries. When expressing the gene product in human embrionic kidney cells, large inward currents carried by Ca^{2+} were activated when challenging the cells with thapsigargin or $InsP_3$. Together, these results suggest that an endogenous trp-like protein is involved in Ca^{2+} influx after receptor stimulation. The human trp homologs have very variable carboxy-terminals yet are apparently activated by thapsigargin. Thus the thapsigargin sensitivity of mammalian trp homologs may not be only localized to the carboxy-terminal.

In *Drosophila* photoreceptors, Niemayer at al. (149) demonstrated that the light-activated conductance was actually a complex consisting of both trp and trpl (149). Although each channel could be activated independently, they served partially overlapping functions. Using antibodies and immunofluorescent staining, they clearly demonstrated that trp localized to rhabdomeres. They did not find any evidence for preferential localization of trp to regions adjacent to the plasma membrane.

Does trp encode for I_{CRAC}? Although evidence is accumulating that trp can be activated by thapsigargin, the results described below suggest that trp is not I_{CRAC}.

1) The trp channel is moderately selective for Ca^{2+} over Na^+ and exhibits a P_{Ca}/P_{Na} of ~40 (70). Calcium release-activated Ca^{2+} current has a P_{Ca}/P_{Na} of ~1,000.

2) Noise analysis estimates the single-channel conductance of trp to be 4.5 pS (70). This is almost three orders of magnitude larger than the estimates for I_{CRAC}.

3) It has still not been demonstrated in photoreceptors that trp is activated by store depletion. Other sensory neurons express $InsP_3$- and $InsP_4$-gated Ca^{2+} channels, and these might conceivably control trp in the native tissue (48, 49). A complicating factor is that the surface mem-

brane of the photoreceptors is highly invaginated, and therefore, free diffusion is hampered. It is difficult to adequately control the ionic solution experimentally, so Ca^{2+} chelators like BAPTA are not readily able to access these sites. The trp channel could be activated by a rapid local rise in cytosolic Ca^{2+} rather than store depletion. In light of this latter possibility, it is interesting to note that trp has calmodulin binding sequences in the primary amino acid sequence. In mammalian photoreceptors, cGMP activates a channel permeable to Na^+ and Ca^{2+}. It has been suggested that elevated Ca^{2+} (due to release from stores) stimulates guanylyl cyclase, resulting in cGMP production, which then gates the mammalian analog of *Drosophila* trp (97). The only studies to directly examine whether light could induce depletion of stores were negative so far, indicating that activation of trp in its native cell was not associated with depletion of stores (69, 189).

4) Recombinant trp in Sf9 insect cells shows some properties not expected of I_{CRAC}. The Ca^{2+} current does not inactivate during long hyperpolarizing pulses of 400-ms duration despite the presence of 50 mM Ca^{2+} in the extracellular solution and modest intracellular Ca^{2+} buffering (235).

5) Large Ca^{2+} currents of almost 1-nA amplitude can be measured in the Sf9 cells after thapsigargin treatment with only a few hundred micromolar of free EGTA in the recording pipette (235). Assuming 99% of incoming Ca^{2+} is buffered, and the Sf9 cell is a typical round cell of 10-μm diameter, this means that cytosolic Ca^{2+} should rise at a rate of 10 μM/s. This is a massive rate, and the buffers would not be able to adequately control free Ca^{2+}. A crucial experiment to establish that recombinant trp is a store-operated Ca^{2+} current requires its transfection in a mammalian cell line, and the demonstration that several independent methods of store depletion activate the same Ca^{2+} current in the presence of high intracellular Ca^{2+} chelators.

6) The report that trp is localized in the plasma membrane adjacent to the Ca^{2+} stores has been taken as evidence in support of the conformational-coupling model for store-operated Ca^{2+} influx (178). A recent finding using polyclonal antibodies to trp and trpl has demonstrated that the proteins are localized to the rhabdomeres, which are devoid of intracellular stores (149). If trp is indeed activated by store depletion in the photoreceptors, then this latter result is difficult to reconcile with conformational coupling. Clarification of this issue is required to carry the conformational-coupling hypothesis further.

VII. PHYSIOLOGICAL FUNCTIONS OF CALCIUM RELEASE-ACTIVATED CALCIUM CURRENT

Although we know little about the molecular biology of CRAC channels, recent experiments have clearly established that I_{CRAC} fulfills numerous important physiological functions.

A. Refilling of Stores

One obvious role of I_{CRAC} would be to replenish the intracellular Ca^{2+} stores. In fact, most of the crucial experiments that led to the discovery of store-operated Ca^{2+} entry focused on the refilling kinetics of depleted stores (218, 219). For example, in *Xenopus* oocytes, Parekh et al. (156) found that the store-operated Ca^{2+} entry pathway was essential for refilling of the agonist-sensitive internal stores. If Ca^{2+} influx was compromised, subsequent Ca^{2+} release by a receptor agonist was reduced. Similar conclusions have been drawn from studies in endothelial cells (85) and neutrophils (137). Although some cells are quite effective in recycling their released Ca^{2+}, many cell types require influx from the extracellular space to refill their stores, since a major part of the Ca^{2+} released from intracellular stores is exported from the cell by the plasma membrane Ca^{2+}-ATPase. In pancreatic acinar cells, the amount of Ca^{2+} extruded from a cell during a single spike corresponds to ~40% of the total mobilizable intracellular Ca^{2+} pool (221). The large extrusion highlights the need for compensatory Ca^{2+} entry and explains why only a few Ca^{2+} transients can be generated in the absence of store-operated Ca^{2+} entry.

B. Ca^{2+} Oscillations and Waves

In many diverse cell types, Ca^{2+} influx has been reported to alter the pattern of Ca^{2+} oscillations. In *Xenopus* oocytes, Ca^{2+} influx accelerated the speed at which the spiral Ca^{2+} wave propagated through the cytoplasm and modified the pattern of cytosolic Ca^{2+} oscillations (64, 246). In HeLa cells and avian exocrine nasal gland, Ca^{2+} influx was necessary to drive the intrinsic intracellular oscillatory mechanism (120, 226). In T lymphocytes, Ca^{2+} oscillations are critically dependent on Ca^{2+} influx, and it has been suggested that they arise from cyclical interaction between intracellular Ca^{2+} stores and CRAC channels in the plasma membrane (44).

These results clearly demonstrate an important role for Ca^{2+} influx in determining the pattern of Ca^{2+} oscillations and wave propagation, and it will be important to assess the relevant contribution of store-operated Ca^{2+} influx pathways during the various phases of oscillatory Ca^{2+} signaling in different cell types. A recent report argues that store-operated Ca^{2+} influx is not involved in generating cytosolic Ca^{2+} oscillations. Oscillations are driven instead by a store-independent receptor-activated pathway (210).

C. Secretion

In many nonexcitable cells, cytosolic Ca^{2+} is an important factor controlling regulated exocytosis. In mast cells, I_{CRAC} provides the major contribution to the elevated Ca^{2+} plateau following receptor stimulation (52). Although secretion in mast cells involves a GTP-dependent step (165), Ca^{2+} influx through I_{CRAC} accelerates the rate of GTP-dependent exocytosis (146, 166). In RBL cells, I_{CRAC} is the direct source of Ca^{2+} for secretion (158, 248).

D. Adenylate Cyclase

Several Ca^{2+}-dependent isozymes of adenylyl cyclase have been described. Activation of Ca^{2+} influx after thapsigargin treatment in C6–2B glioma cells has been found to inhibit the type VI adenylyl cyclase isozyme and activate the type I form (29, 37). This Ca^{2+}-dependent regulation was specific to Ca^{2+} influx because Ca^{2+} release was without effect on enzyme activity. Functional studies have led to the suggestion that the Ca^{2+}-sensitive adenylyl cyclases might colocalize with the Ca^{2+} entry channels (50). Changes in Ca^{2+} influx could then induce rapid changes in the levels of cAMP. Recently, it has been found that cAMP-dependent protein kinase can phosphorylate serine residues on phospholipase C-β_2, thereby inhibiting enzymatic activity (107). This also raises the possibility of a novel feedback mechanism between Ca^{2+} release and store-operated Ca^{2+} influx, mediated by cAMP levels. If the type I adenylyl cyclase colocalizes with the Ca^{2+} influx pathway, then Ca^{2+} entry will increase cAMP levels because this isozyme is Ca^{2+} activated. Elevation of cAMP and subsequent activation of cAMP-dependent protein kinase could then phosphorylate phospholipase C-β_2, resulting in cessation of $InsP_3$ production. Stores will now refill, and store-operated Ca^{2+} influx will turn off. However, if the type VI adenylyl cyclase isozyme colocalizes with the Ca^{2+} entry pathway, Ca^{2+} influx will reduce cAMP levels and presumably phospholipase C-β_2 activity will therefore increase. This could result in more sustained store depletion due to continuous $InsP_3$ production and hence prolonged store-operated Ca^{2+} influx. The time course of store-operated Ca^{2+} influx could be subtly controlled by the isozyme of adenylyl cyclase that colocalizes with the Ca^{2+} influx pathway.

Given that cAMP can regulate multiple spatially and temporally distinct processes, changes in Ca^{2+} entry could indirectly control many diverse cell functions.

E. Gene Transcription

In lymphocytes, activation of the T-cell receptor ultimately results in increased synthesis and secretion of both interleukin-2 (IL-2) and its receptor. The molecular mechanisms whereby this is accomplished are now reasonably well understood (38). T-cell receptor activation elevates $InsP_3$ levels that deplete stores. This results in activation of I_{CRAC} and thus sustained Ca^{2+} influx. Elevated cytosolic Ca^{2+} then activates the Ca^{2+}-dependent phosphatase calcineurin. Calcineurin enters the nucleus and dephosphorylates the transcriptor factor NF-AT, resulting in increased transcription of the IL-2 gene as well as its receptor. Studies on mutant lymphocytes that were defective in I_{CRAC} established a close correlation between the reduction in Ca^{2+} influx due to I_{CRAC} and subsequent Ca^{2+}-dependent gene transcription (51).

F. Cell Cycle and Proliferation

Most cells in a multicellular system are in a quiescent, nondividing G_0 state. Upon stimulation with appropriate mitogens/growth factors, the cells can resume the cell cycle that culminates in mitosis. Two critical junctures are at the G_1/S phase, which precedes the onset of DNA synthesis, and the G_2/M phase. It now appears that release of stored Ca^{2+} by $InsP_3$ plays a crucial role in determining the entry into these stages (9). The role for Ca^{2+} influx is more obscure. In both Swiss 3T3 and mouse NIH-3T3 cells, reducing Ca^{2+} entry during mitogen stimulation inhibits cell growth, although the nature of the Ca^{2+} entry pathway is not known (115, 220). Just how Ca^{2+} elevation controls DNA synthesis is not clear, although the available evidence points to a role for calmodulin in stimulating certain transcription factors resulting in protein synthesis.

At the G_2/M phase, a transient increase in free Ca^{2+} is important for the generation of maturation-promoting factor, which orchestrates the final events leading to mitosis. Interestingly, during the mitotic state, it appears that the coupling between depletion of stores and subsequent Ca^{2+} influx is severed (181). Both histamine- and thapsigargin-evoked Ca^{2+} entry is lost, whereas the Ca^{2+} release is largely unaffected. The mechanism that underlies this uncoupling is not known. In view of the dramatic changes that occur in cell morphology, remodeling of the cytoskeleton, distribution of stores, and presence of numerous new signaling molecules, it is likely that several processes can contribute to the dissociation between Ca^{2+} release and influx.

G. Apoptosis

Activation of sphingomyelinase generates an array of signaling molecules that have recently been shown to play a prominent role in cell growth and differentiation as well as programmed cell death, or apoptosis (215). The metabolites produced from sphingomyelin cleavage, e.g., sphingosine, sphingosine-1-phosphate, and ceramide, stimulate or inhibit numerous effector systems, including stimula-

tion of a ceramide-activated protein kinase, a ceramide-activated protein phosphatase, a mitogen-activated protein kinase, inhibition of protein kinase C, and release of $[Ca^{2+}]_i$. The latter two in particular may have important implications for the observations that link Ca^{2+} entry and protein kinase C to growth arrest and cell death (112), because the observed increase in $[Ca^{2+}]_i$ after stimulation of sphingolipid signaling pathways may be mediated in part by Ca^{2+} release and depletion of intracellular stores with subsequent activation of I_{CRAC}. At the same time, I_{CRAC} might be boosted by removal of negative-feedback inhibition due to inhibition of protein kinase C, leading to severely enhanced and prolonged elevations of $[Ca^{2+}]_i$ that ultimately may result in apoptotic cell death.

H. Pathophysiology

Given that I_{CRAC} can control a diverse array of physiological processes, it comes as no surprise that abnormalities in the current give rise to human diseases. The most vivid demonstration of this was the finding that I_{CRAC} was completely absent from the T cells of a patient suffering from a primary immunodeficiency (162). This was associated with defective T-cell proliferation after stimulation with TCR-CD3 complex. Calcium release was not compromised in the disorder. Proliferation was restored by addition of a Ca^{2+} ionophore to the medium. Hence, the immune disorder specifically lay in the absence of I_{CRAC}. The child was not able to mount an immune response and died.

Abnormalities in I_{CRAC} have also been implicated in hyperglycemia (192). Elevated glucose in the culture medium was found to inhibit thapsigargin-evoked Ca^{2+} influx compared with incubations in lower glucose medium. In the hyperglycemic cells, thapsigargin released Ca^{2+} at a faster rate in Ca^{2+}-free solution, indicating that the loss of influx was not simply due to less Ca^{2+} release from the stores.

VIII. CONCLUDING REMARKS

The past few years have witnessed a dramatic increase in our understanding of the processes that underlie Ca^{2+} influx in nonexcitable cells. It is now firmly established that the predominant Ca^{2+} entry pathway is the store-operated one. Electrophysiological studies point to the existence of a family of store-operated Ca^{2+} currents, rather than a homogeneous population of a ubiquitously distributed channel. In this respect, store-operated Ca^{2+} currents might resemble voltage-dependent Ca^{2+} currents in excitable cells. Calcium release-activated Ca^{2+} current appears to be the most widely distributed store-operated Ca^{2+} current, and certainly the most selective for Ca^{2+}. Although the CRAC channels have not yet been cloned, the *trp* gene appears to be the first candidate that encodes a store-operated channel.

Now that the basic aspects of store-operated Ca^{2+} influx have been established, perhaps the greatest challenge facing researchers in the field is to understand, at a molecular level, how the current is activated. Just how depletion of stores activates Ca^{2+} channels remains a mystery. At least two steps are required: a molecule that senses the Ca^{2+} content of the stores and a signal that links the store content to Ca^{2+} channels in the plasma membrane. Unfortunately, and despite intense research, little is known about the properties of either process. Although numerous candidates have been proposed, the activation mechanism still remains elusive.

We are beginning to appreciate the crucial role store-operated Ca^{2+} influx fulfills in physiological systems. This Ca^{2+} entry pathway does more than merely replenish the stores. Store-operated Ca^{2+} influx cannot only induce short-term effects through changes in the spatiotemporal pattern of Ca^{2+} signaling, but also long-lasting effects through regulation of gene transcription. As the burgeoning list of processes regulated by store-operated Ca^{2+} entry continues, it is likely that disturbances in the entry pathway will be linked to human disorders. The store-operated Ca^{2+} influx pathway may therefore represent an important, and hitherto unexplored, target for the design of therapeutic agents.

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