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COMMENTARY

Agonist-Releasable Intracellular Calcium Stores and the Phenomenon of Store-Dependent Calcium Entry

A NOVEL HYPOTHESIS BASED ON CALCIUM STORES IN ORGANELLES OF THE ENDO- AND EXOCYTOTIC APPARATUS

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ABSTRACT. Store-dependent calcium entry represents a little characterized calcium permeation pathway that is present in a variety of cell types. It is activated in an unknown way by depletion of intracellular calcium stores, for example in the course of phospholipase C stimulation. Current hypotheses propose that depleted calcium stores signal their filling state to this permeation pathway either by direct, protein-mediated interaction or by release of a small, diffusible messenger. The further characterization of store-dependent calcium entry will benefit from progress in the identification of the intracellular calcium storing compartments. Recent findings reviewed here suggest that these compartments include parts of the organelle system that is involved in endo- and exocytosis. This commentary describes a novel model of store-dependent calcium entry based on calcium stores belonging to the endo- and exocytotic organelle system. Such calcium stores could establish a tubule-like connection with the extracellular space, in analogy to the cellular compartments that contain the insulin-sensitive glucose transporter or the gastric proton pump. This connection will provide a pathway for store-dependent calcium entry. Under store depletion, extracellular calcium will permeate through the tubule-like connection into the store lumen and from there into the cytosol. The consequences of this model for the development of drugs modulating store-dependent calcium entry are discussed. BIOCHEM PHARMACOL 51;8:993–1001, 1996.

KEY WORDS. capacitative calcium entry; store-dependent calcium entry; calcium stores; vacuolar apparatus; inositol-1,4,5-triphosphate; calcium

Many central cellular processes, such as secretion of hormones and neurotransmitters, contraction and cell movement, and proliferation and cell division, are mediated by an elevation of the cytosolic free calcium ion concentration ($[Ca^{2+}]_{cyt}$). Cytosolic calcium can be elevated by increased calcium influx across the plasma membrane, by calcium release from intracellular calcium stores, or by a combination of both. The well characterized calcium antagonist nifedipine and related dihydropyridines act by inhibiting calcium influx via voltage-dependent calcium channels (calcium channels that are opened by depolarization). The medical use of these agents, for example antihypertensive inhibition of smooth muscle cell contraction in arterial vessels, demonstrates that selective pharmacological modulation of calcium-dependent cellular processes *in vivo* is possible.

Can more drugs be developed that interact with cellular calcium transport, selectively targeting other calcium-dependent processes, in other cells? To approach this issue, knowledge of cell type-specific calcium transport features is desirable. Considerable information is already available about the mechanisms of calcium influx in excitable cells, such as neurons, muscle, and endocrine cells. Calcium influx in these cells occurs mainly via VDCCs.† Several types of VDCCs are known. The VDCC types are distinguished by their different electrophysiological properties. The currently used calcium channel blockers, such as nifedipine and related dihydropyridines, verapamil, and diltiazem, act on so-called L-type VDCCs [1], the best characterized type of VDCC. Electrophysiological methods, particularly measurement of single ion channel activity with the patch-clamp technique, were applied very successfully for the characterization of calcium influx via L-type VDCCs [1]. In addition, L-type VDCCs could be purified [2], enabling biochemical characterization.

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† *Abbreviations:* CIF, calcium influx factor; CRAC, calcium release-activated current; ER, endoplasmic reticulum; IP_3, inositol, 1,4,5-triphosphate; IP_3-R, inositol 1,4,5-triphosphate receptor; LMWG, low molecular weight GTP-binding protein; PLC, phospholipase C; and VDCC, voltage-dependent calcium channel.

Much less is known about calcium influx in nonexcitable cells (cells that cannot generate action potentials), for example leukocytes, thrombocytes, hepatocytes, renal epithelial cells, and acinar cells of exocrine glands. Nonexcitable cells are devoid of VDCCs. In nonexcitable cells, calcium influx is induced typically by agents that activate phospholipase C, and thereby primarily release calcium from intracellular stores. It is unclear how these agents trigger calcium influx across the plasma membrane. There appears to be a mechanism by which depletion of intracellular calcium stores directly regulates calcium entry at the plasma membrane [3, 4]. This phenomenon is termed store dependent (since it is induced by calcium release from intracellular calcium stores) or capacitative calcium entry [3]. The term capacitative entry is now widely used. This term can be viewed as an analogy to the behaviour of an electrical capacitor, which can conduct a current only when it is uncharged. Charging of a capacitor by a continuous current is accompanied by increasing electrical resistance that eventually terminates the current. Like regulation of capacitor conductivity by its charging state, capacitative calcium entry in cells is thought to be determined by the filling state of calcium stores.

Capacitative calcium entry is not only present in nonexcitable cells, but also in at least some types of excitable cells, in addition to depolarization-induced calcium entry via VDCCs [5–8]. In nonexcitable cells, store-dependent calcium entry may be the dominant calcium influx pathway, in spite of the possible existence of other pathways such as receptor-operated and second messenger-operated calcium channels (ROCCs and SMOCCs; addressed in a recent commentary [9] in this journal). Understanding the mechanism of capacitative calcium entry could be a key to the development of new drugs that act by modifying calcium influx in nonexcitable cells.

Store-dependent, capacitative calcium entry is the subject of an intense and rather controversial debate. Besides giving basic facts of store-dependent calcium entry and outlining the major points that are discussed, the present commentary summarizes recent developments in the characterization of intracellular calcium stores with important implications for store-dependent calcium entry. We finally propose an alternative hypothesis for the mechanism of store-dependent calcium entry, resting on the existence of calcium stores in the organelles that participate in endo- and exocytosis.

CONCEPT OF STORE-DEPENDENT CALCIUM ENTRY

Early studies that contributed to the concept of store-dependent calcium entry examined $[Ca^{2+}]_{cyt}$ during treatment of nonexcitable cells with agents that stimulate PLC. Such agents usually bind to receptors that couple to and activate PLC via G proteins. This causes generation of the intracellular messenger IP_3 that opens calcium channels in the membrane of certain intracellular calcium-storing compartments, leading to calcium release from these stores. Release of intracellular calcium under these conditions was found to be typically accompanied by calcium influx across the plasma membrane into the cytosol. This calcium influx appeared to correspond closely to the filling state of the calcium stores, but less so to the presence of the agonist (reviewed in Ref. 3). This was concluded mainly from a persisting tendency to increased calcium influx after termination of agonist action by an antagonist, until the stores were filled again. Based on this rather indirect evidence, it was proposed for the first time that depletion of calcium stores could directly induce calcium influx, and that calcium influx caused by activators of PLC is mediated by this but not any other mechanism, such as opening of calcium channels by second messengers or by direct action of a G-protein. However, using agents that are agonists of receptors in the plasma membrane, involvement of such mechanisms cannot be excluded. Investigation of capacitative calcium entry was thus hindered by the lack of a substance depleting intracellular calcium stores without directly activating other intracellular processes.

The discovery of thapsigargin, a potent and specific inhibitor of intracellular calcium pumps [10, 11], made direct calcium store depletion possible, without prior activation of signal transduction pathways. Thapsigargin permeates easily into cells and mediates a complete inhibition of calcium pumps in intracellular calcium stores in nearly all cell types examined thus far. Calcium stores, in general, appear to have a permanent leak for calcium so that continuous calcium pumping is necessary to keep the stores filled. Upon pump inhibition by thapsigargin, the calcium stores are depleted rapidly via this little characterized leak.

Thapsigargin became a tool in the investigation of capacitative calcium entry. The availability of thapsigargin allowed extensive testing of earlier conclusions about store-dependent calcium entry. In fact, thapsigargin-mediated store depletion was found to induce calcium influx in a variety of cell types [11] to a degree similar to that of activators of phospholipase C. With thapsigargin as a tool, the wide occurrence of capacitative calcium entry in various cell types could thus be confirmed. In a few cases, for example in experiments with a neuroblastoma cell line [12], calcium stores were depleted by thapsigargin without any detectable concomitant calcium influx. It has been inferred from this that store-dependent calcium entry might be totally absent in some cells. However, in RINm5F insulinoma cells, which also did not show calcium influx after addition of thapsigargin, such influx could be observed after activation of protein kinase C by a phorbol ester [8]. This raises the question of whether store-dependent calcium entry exists in all cell types, but is in a latent state in some.

Later, application of electrophysiological measurements of calcium influx began to contribute to the investigation of store-dependent calcium entry. An inward calcium current that was triggered by depletion of intracellular calcium stores could be observed in rat mast cells [13]. This current, named I_{CRAC}, could be induced by diverse measures that

deplete intracellular calcium stores. It could also be observed in the absence of any cytosolic calcium elevation, confirming the notion that store-dependent calcium entry is not triggered by a rise of $[Ca^{2+}]_{cyt}$ but rather by the filling state of the stores. Such CRAC currents have now been recorded in a number of cell types [4]. These results provide some information on the characteristics of the store-dependent calcium influx pathway. It appears to have an extraordinarily high selectivity for calcium. Furthermore, it is remarkable that store depletion-induced currents could not be resolved into single-channel openings. Thus, the typical electrophysiological evidence for mediation of ion permeation by an ion channel is, in fact, still lacking for store-dependent calcium entry. Either the involved channels have a very low conductance, so that the amplitude of single-channel currents remains below the currently detectable levels [4], or store-dependent calcium entry may even not be mediated in the conventional way by an ion channel.

While I_{CRAC} can be found in many cell types, it may not be the only type of store depletion-activated current. Another type, which could be resolved into single channel openings, has been described in vascular endothelial cells [14]. Further investigations will have to show whether this current with a higher single channel conductance is of general importance.

PROPOSED MECHANISMS OF STORE-DEPENDENT CALCIUM ENTRY

The existence of store-dependent calcium entry is now widely accepted. Its mechanism, however, has remained unclear and is a matter of controversial and speculative discussion. The opinions on this issue follow two major lines. To give an overview, some investigators suggest that depleted calcium stores release CIF, a small, diffusible messenger of unknown structure (a molecule having, for example, the size of an amino acid) that activates remote, also yet to be identified calcium channels in the plasma membrane [15, 16]. An opposed group of hypotheses proposes transduction of a store depletion signal to a calcium influx pathway by a direct contact between calcium stores and the plasma membrane. In some of the latter hypotheses, calcium influx is viewed to occur through plasma membrane-attached calcium stores into the cytosol [17, 18]. Figure 1 shows the main possibilities that are discussed.

The diffusible messenger hypothesis mainly rests on the recent observation that cytosolic extracts obtained from cells after calcium store depletion could induce calcium influx in other cells [15]. The putative CIF in these extracts was partially purified and characterized. CIF is thought to possess a phosphate group and to have a molecular mass below 500 [15]. However, testing of similar cytosolic extracts by others led to the conclusion that such extracts affect cytosolic calcium in a less specific way than any mediator of store-dependent calcium entry should do [19]. Clearly, the CIF hypothesis remains vague until the complete purification of CIF and confirmation of its action.

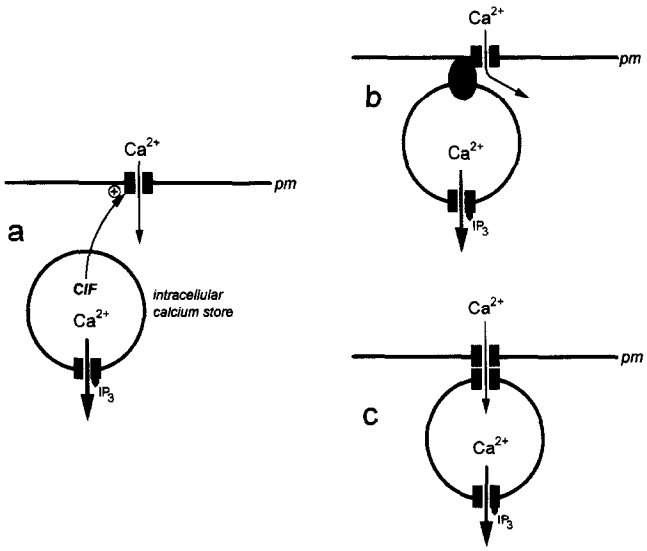

FIG. 1. Schematic reproduction of models that have been proposed for the mechanism of calcium store-dependent, capacitative calcium entry. (a) Diffusible messenger hypothesis [15]. Upon calcium store depletion by binding of IP_3 to the IP_3-R and subsequent opening of this channel, the stores release a small molecule called CIF. CIF diffuses to the plasma membrane (*pm*) and induces opening of specific channels. (b) Conformational cross-coupling hypothesis [17]. A transmembrane protein in calcium stores couples to a calcium channel in the plasma membrane. Store depletion changes the conformation of this protein. This conformational change induces opening of the calcium channel in the plasma membrane. (c) Gap junction-like coupling between calcium store and extracellular space [18]. Extracellular calcium permeates directly into the stores, via two coupled channels of unknown nature.

The alternative group of hypotheses, proposals of a direct interaction between calcium stores and the plasma membrane, is based on an analogy with skeletal muscle. There, plasma membrane calcium channels in the T-tubules are closely apposed to calcium release channels of the sarcoplasmic reticulum, resulting in functional coupling between these structures. In nonmuscle cells, special parts of the endoplasmic reticulum with potential function as calcium stores can also have close contact with the plasma membrane [20]. Store depletion could be signalled to the plasma membrane by a conformational change of certain transmembrane calcium store proteins that are in contact with plasma membrane calcium channels [17]. It has also been proposed that plasma membrane and calcium stores are connected by gap junction-like channels, which would allow direct permeation of extracellular calcium into the store lumen [18]. Like mediation of store-dependent calcium entry by CIF, all these interpretations are hypothetical. Evidence for the existence of any of the postulated store depletion signalling proteins, or for known proteins additionally having such a function, has yet to be presented.

Progress may come from the recent finding that store-dependent calcium entry appears to be activated by LMWGs [21, 22]. LMWGs comprise members of the subgroups

Ras, Rab, Rho, Arf and others. Many of these proteins function in the dynamic determination of subcellular structure. Rab and Arf are involved in intracellular vesicular trafficking, that is in the transport of contents from certain organelles to others via vesicles that bud from one compartment and finally fuse with the membrane of a different compartment, after transport across the cytosol [23]. Rho influences cytoskeletal architecture [23]. A role of an LMWG in store-dependent calcium entry could mean that a distinct change in subcellular structure is involved in activation of this calcium influx pathway. Knowledge about function and selective experimental manipulation of LMWGs is expanding rapidly. Therefore, it may soon be possible to identify and characterize an LMWG-dependent step in the induction of store-dependent calcium entry. This step may even be the central mechanism of store-dependent calcium entry itself.

Of interest is also the apparent regulation of store-dependent calcium entry by kinases. The influence of kinases on store-dependent calcium influx is, however, so diverse and cell type dependent that its investigation is less likely suited to reveal basic features of this process. Protein kinase C inhibits store-dependent calcium entry in neutrophil granulocytes [24] and in rat basophilic leukemia cells [25], whereas a stimulation is found in insulin-secreting cells [8, 26]. Cyclic AMP, presumably acting via protein kinase A, inhibits store-dependent entry in platelets [27], but it seems to amplify this pathway in hepatocytes [28]. Cyclic GMP, also known to act via a kinase, was suggested to induce store-dependent calcium influx in pancreatic acinar cells [29], but was also found to inhibit it in platelets [27]. Tyrosine kinases may be involved in maintaining store-dependent calcium entry in fibroblasts [30].

Hence, it appears that it will be difficult to identify the mechanisms of calcium store depletion-induced calcium entry solely by direct investigation of this phenomenon. In contrast, it is evident that research in this field will benefit from insights into structure and function of intracellular calcium stores.

WHAT IS KNOWN ABOUT THE NATURE OF AGONIST-RELEASABLE INTRACELLULAR CALCIUM STORES?

Store-dependent calcium entry is induced by depletion of such intracellular calcium stores that are released by the second messenger IP_3, generated by activation of PLC by agonists of receptors that can couple to PLC via G-proteins. Such stores are also designated as agonist-releasable stores. IP_3 opens a calcium-permeable channel in the membrane of these stores by binding to the channel protein (reviewed in Ref. 31). Thus, this channel is referred to as IP_3-R. At least four subtypes of IP_3-Rs exist [32]. The subcellular distribution of IP_3-Rs, revealed by immunocytochemical detection of these proteins with electron microscopy, should tell which organelles function as agonist-releasable calcium stores. This straightforward approach, however, is hindered by the very low expression of IP_3-Rs in most cell types and by other technical problems. Therefore, the characterization of agonist-releasable calcium stores requires an integration of results obtained with all methods that can provide information about these structures, including also subcellular fractionation and transport measurements with permeabilized cells. Summarizing knowledge derived from diverse approaches, it is now possible to tentatively outline a general scheme of intracellular IP_3-sensitive calcium storage. Namely, IP_3-Rs apparently can be present at two basically different intracellular locations.

One of the two locations of IP_3-Rs is the ER, including specialized subcompartments of this organelle. This has been demonstrated most convincingly by immunocytochemistry in cerebellar Purkinje neurons, which express a much higher quantity of IP_3-Rs than any other cell type [33]. IP_3-Rs were found in different parts of the ER, for example in rough ER, in smooth ER tubules, in smooth-surfaced cisternae located subplasmalemmal or perinuclear, and especially concentrated in certain stacked cisternae. The IP_3-R subtype expressed in Purkinje cells in such high amounts is IP_3-R [34]. This subtype, but as slightly different variants generated by alternative splicing, is also found in most cells of peripheral, nonneuronal tissues [34, 35]. Presumably, it is also localized in the ER in these cells, although low receptor density prevents confirmation of this by high resolution immunocytochemistry. However, results obtained with subcellular fractionation [36] or immunofluorescence [37] are at least compatible with such a localization.

The other location of IP_3-Rs is different from endoplasmic reticulum. Multiple evidence for such a non-ER localization is found, for example, in lymphocytes (Jurkat T-cell line). IP_3-Rs in lymphocytes were shown to contain sialic acid [38], which is atypical for ER proteins but suggests that these IP_3-Rs have passed the *trans*-Golgi network, where sialylation usually occurs [39]. Immunofluorescent labeling of IP_3-Rs in lymphocytes showed reactivity at or near the plasma membrane [40]. Localization of the majority of lymphocyte IP_3-Rs to the plasma membrane, however, is unlikely. Iodination of lymphocyte plasma membrane proteins was accompanied by only faint labeling of IP_3-Rs [40]. Patch-clamp measurements demonstrated an IP_3-activated calcium channel in excised patches from lymphocyte surfaces [41], whereas no IP_3-dependent single channel activity could be found in whole lymphocytes [42]. These results would be compatible with localization of lymphocyte IP_3-Rs in compartments beneath the plasma membrane that have a limited exchange or contact with the cellular surface.

A precedent for such a situation has been described in endothelial cells, where, using immunocytochemistry in combination with electron microscopy, IP_3-Rs were detected in caveolae [43]. Caveolae are small invaginations of the plasma membrane that occur in several cell types [44]. Caveolae have dynamic aspects, which are currently being investigated. It is thought that the connection of the ca-

veolar lumen with the extracellular space can close temporarily [45]. In other cells, caveolae may completely detach from the plasma membrane, forming separate vesicles that could function in endo- or transcytosis [46]. This would relate caveolae to other organelles involved in endo- and exocytosis.

There is also evidence for IP_3-Rs in other such organelles. IP_3-Rs have been detected in secretory granules of endocrine pancreatic cells [47], and isolated chromaffin granules show IP_3-dependent calcium release [48]. Figure 2 summarizes the different subcellular locations of IP_3-receptors that have been revealed by immunocytochemistry. Based on transport measurements with permeabilized cells and subcellular fractions, it has been suggested that the IP_3-sensitive calcium store of exocrine acinar cells is a compartment with vacuolar-type proton pumps, and thus also part of the organelles that perform endo- and exocytosis [49, 50]. A study using ion microscopy provided evidence for an agonist-releasable calcium store in the Golgi apparatus of a kidney cell line [51]. The acrosome, a specialized exocytotic organelle of sperm cells, was shown to represent a calcium store with IP_3-Rs [52].

In most cells that have been examined in this respect thus far, two or three of the IP_3-R subtypes are expressed in parallel [53, 54]. Like subtype I, the other subtypes will also have a specific subcellular localization. For subtype III, this has already been demonstrated [47, 55, 56]. The parallel presence of diverse IP_3-R subtypes in cells suggests that

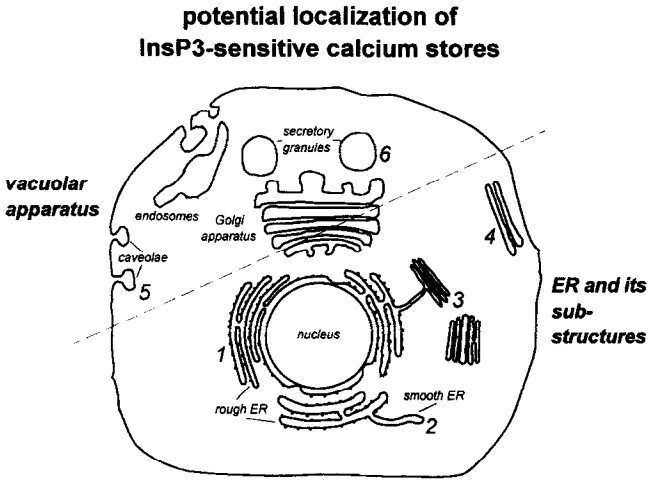

FIG. 2. Different intracellular locations of IP_3-sensitive calcium release channels that have been suggested thus far by immunocytochemical studies with various cell types. Locations 1–4 belong to the endoplasmic reticulum or its specialized substructures. (1) Rough ER; (2) smooth ER tubules in connection with rough ER; (3) membrane stacks in connection with rough ER; and (4) subplasmalemmal stacks derived from but not connected to ER. Locations 1–4 have been found in cerebellar Purkinje neurons and smooth muscle cells [33]. (5) Caveolae, small invaginations of the plasma membrane found in many cells. IP_3-Rs in caveolae have been found in smooth muscle and endothelial cells and in keratinocytes [43]. (6) Secretory granules of B- and D-cells in endocrine pancreas [47].

endoplasmic reticulum-localized and non-ER-associated IP_3-Rs may generally occur together in a cell. IP_3-R subtype III probably is the most likely candidate for the putative IP_3-receptor in a non-ER localization, since it is the IP_3-R that was detected on secretory granules. In some other epithelial cells, it is concentrated in the apical pole [56], close to sites of exocytosis.

ORGANELLES OF THE VACUOLAR APPARATUS AS IP_3-SENSITIVE CALCIUM STORES

Summarized, the findings concerning a non-ER localization of IP_3-Rs point to the general presence of IP_3-Rs in the so-called vacuolar apparatus. This term comprises all the compartments that participate in membrane traffic to and from the plasma membrane, namely endosomes, the *trans*-Golgi network and related structures, and secretory vesicles. The dynamic nature of these compartments and the behaviour of other transport proteins located therein provide clues for properties of IP_3-sensitive calcium stores in the vacuolar apparatus. Of particular interest is a number of well characterized transport proteins, including ion channels, that reside in specialized compartments, which, however, are also thought to belong to the vacuolar apparatus. These transport proteins include the water channel of the kidney collecting duct [57], the proton pump of gastric parietal cells [58], apical chloride channels in some epithelia [59], and the GLUT4 glucose transporter of adipocytes [60]. Induced by specific stimuli, the compartments containing these proteins all can display rapid establishment of continuous connections with the extracellular space or even exocytotic insertion into the plasma membrane.

It is attractive to speculate that IP_3-Rs may be concentrated in a similar compartment, especially regarding the resemblance with the IP_3-R bearing caveolae. A continuous connection between lumen of such a compartment and extracellular space, analogous to the neck of caveolae, would constitute a pathway for store-dependent calcium entry. In contrast to caveolae, however, such a compartment would not be a mere substructure of the plasma membrane but rather a separate intracellular organelle, being larger and possibly more remote from the cell surface.

The function of such a compartment as a calcium store would naturally also require the presence of a calcium pump. The intracellular calcium pumps that are characterized the best, thus far, are the members of the sarcoplasmic or endoplasmic reticulum calcium ATPase family (SERCA pumps). As indicated by the designation, they are thought to reside in the ER or its specialized subcompartments [1]. There is, however, also evidence, albeit less comprehensive, for calcium pumps in organelles of the vacuolar apparatus. High calcium concentrations are present in secretory granules [61] and probably also in the *trans*-Golgi network [62] from which the granules originate. With cytochemical methods, calcium-dependent ATPase activity has been detected on the membrane of these compartments [63, 64].

A POTENTIAL MECHANISM OF STORE-DEPENDENT CALCIUM ENTRY, MEDIATED BY CALCIUM STORES IN THE VACUOLAR APPARATUS

Based on the probable occurrence of calcium stores in organelles of the vacuolar apparatus, we propose the following novel model for the mechanism of store-dependent, capacitative calcium entry. Store-dependent calcium entry mediated by specialized subcompartments of the vacuolar apparatus serving as calcium stores could function as shown in Fig. 3. The lumen of such compartments could be connected with the extracellular space by a tubule-like structure, similar to the connections of gastric proton pump-carrying vesicles [58] or caveolae [44] with the cell surface. This tubule-like connection would represent a continuity of the store-delimiting membrane with the plasma membrane. It could be permanent or of temporary character, generated, for example, by store depletion. The connection could be stabilized by interaction with the cytoskeleton.

Upon depletion of the stores by IP_3, extracellular calcium could enter the store lumen via such a continuity with the extracellular space. Extracellular calcium would enter the cytosol by passing through the stores first. During experiments that induce store depletion by calcium pump inhibition, without a rise of IP_3, calcium would mainly reach the cytosol via the leak conductance of the stores. At least in some cell types, some opening of IP_3-sensitive channels by basal IP_3 concentrations contributes to this leak conductance [65, 66]. However, it is likely that besides IP_3-Rs other, yet uncharacterized, channels generate the leak conductance.

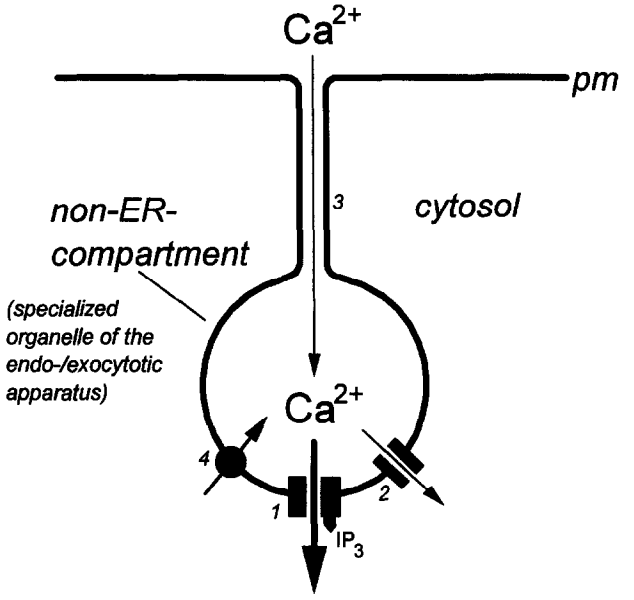

FIG. 3. A novel model for capacitative calcium entry, based on calcium stores in specialized substructures of the vacuolar apparatus, here tentatively designated as the non-ER-compartment. Extracellular calcium enters the calcium store via a tubule-like continuity (3) of the store-delimiting membrane with the plasma membrane (*pm*). Upon store depletion by opening of IP_3-sensitive calcium release channels (1), extracellular calcium can permeate through the tubule-like connection and then through these channels into the cytosol. When the calcium uptake pumps of the stores (4) are inhibited, the stores are depleted via yet uncharacterized calcium leak channels (2) in the store membrane, and possibly also by some opening of IP_3-sensitive channels by basal IP_3 concentrations. Upon store depletion by calcium pump inhibition, capacitative calcium entry would thus mainly occur via the leak channels (2).

Interestingly, store-dependent calcium entry via a continuity of the calcium store lumen with the extracellular space would be in accord with the postulated involvement of an LMWG, since such proteins are involved in the creation of similar structures. For example, rab 2 and rab 11 in gastric parietal cells are thought to function in the connection of proton pump-carrying intracellular vesicles with the extracellular space via tubules [67].

Any model for capacitative calcium entry should also be in accord with the reported electrophysiological properties of capacitative calcium entry, that is it should be in accord with the characteristics of I_{CRAC}, the inward calcium current that is associated with calcium store depletion. The characteristics of I_{CRAC} are a high selectivity for calcium over monovalent cations, and, in addition, a very low amplitude. In contrast, IP_3-opened calcium release channels appear to have opposite characteristics, namely a low selectivity for calcium and a high conductance [31]. I_{CRAC} could not be resolved into single channel openings [4]. Thus, the channels that are responsible for I_{CRAC} may have an extraordinarily low conductance. On the other hand, it is also possible that I_{CRAC} is generated in a more complex manner than other calcium influx currents. For example, the absence of detectable single channel openings might be caused by restricted diffusion to the channel pore. In this way, our model can provide an explanation for the low apparent conductance of any channels mediating I_{CRAC}. Namely, the tubule-like connection between extracellular space and calcium stores envisaged in our model could allow only limited diffusion of extracellular calcium into the stores. The lumen of the connection might be too small, might contain diffusion-limiting proteins, or might collapse intermittently. A restricted calcium supply to the intralumenal openings of IP_3-sensitive calcium channels in the stores thus could be limiting also for permeation of calcium through these channels, into the cytosol. This would result in a low amplitude of I_{CRAC} and could prevent the stepwise current increments that typically indicate single channel openings, conforming to the described features of I_{CRAC}.

For the other particularity of I_{CRAC}, high selectivity of calcium, our model (Fig. 3) offers no straightforward explanation. However, the high calcium selectivity might originate from characteristics of the yet unidentified calcium leak channels in the store membrane, provided that they contribute to a relevant extent to calcium release from the stores. Or, less likely, it might represent a special property of the IP_3-R subtype that is involved.

Finally, it is possible that the pathway we are proposing

and that of I_{CRAC} are different entities that occur together in cells.

SPECIALIZED SUBSTRUCTURES OF THE ENDOPLASMIC RETICULUM AS MEDIATORS OF STORE-DEPENDENT CALCIUM ENTRY?

Also of interest with respect to store-dependent calcium entry are subcompartments of the ER that are localized in close proximity to the plasma membrane. As stated above, in cerebellar Purkinje cells such structures contain IP_3-Rs [33]. In general, parts of the ER can be found so closely apposed to the plasmalemma [68] that this raises the question of direct interactions between these structures. Such interactions could be mediated by coupling of the cytoplasmic faces of transmembrane proteins. This is reminiscent of the hypotheses that explain store-dependent calcium entry by coupling of calcium store proteins to putative calcium channels in the plasma membrane (Fig. 1, b and c).

Complexity is added by the fact that subplasmalemmal ER has been reported to be coupled to caveolae [69]. It was concluded long before the discovery and characterization of IP_3-Rs that such structures could function in cellular calcium homeostasis. Taking all observations together, it is now even conceivable that IP_3-Rs in caveolae couple to calcium channels in the membrane of subplasmalemmal ER. Calcium then could enter the lumen of subplasmalemmal ER by sequential permeation through IP_3-sensitive channels in caveolae and calcium channels in the ER (similar to the model in Fig. 1c), without passing the cytosol. This may especially be the case for smooth muscle cells, with the sarcoplasmic reticulum as calcium store instead of the ER, and in the cardiac myocytes, regarding the data on IP_3-R localization in these cells [70]. For other cell types, however, such a configuration is more hypothetical, so that we favour our simpler interpretation of store-dependent calcium entry involving only IP_3-sensitive calcium stores outside the ER.

CONSEQUENCES FOR THE SEARCH FOR PHARMACOLOGICAL MODULATORS OF STORE-DEPENDENT CALCIUM ENTRY

Our model of capacitative calcium entry (Fig. 3) proposes a novel permeation pathway, namely a tubule-like connection between store lumen and extracellular space, a membrane continuity between the stores and the plasma membrane. Generation and maintenance of such a connection will most likely depend on cellular processes that generally determine the shape and interconnection of intracellular organelles. Thus, the cytoskeleton as well as the many proteins that regulate organelle structure and the so-called vesicular trafficking may be involved. This includes not only LMWGs but also the set of proteins that regulate exocytosis [71].

Our model, therefore, has two consequences for the discovery and evaluation of agents that modify capacitative calcium entry. The first consequence is that any agents that influence capacitative entry should be tested for actions on the cytoskeleton, on exocytosis, and on other aspects of vesicular trafficking. Such agents might be found to interfere nonspecifically with any of these points. On the other hand, substances with known actions in these fields can be used to test our model.

The second consequence of our model relates to its lack of a specific plasma membrane channel for capacitative calcium entry. Instead, capacitative calcium influx into the cytosol occurs by permeation through IP_3-sensitive calcium channels in the calcium store membrane. In our model, calcium release from intracellular stores and capacitative influx of extracellular calcium thus occur via the same channels. Therefore, the only agents that affect capacitative calcium entry by acting on channels might be substances that interact with IP_3-sensitive intracellular calcium channels. Such agents might be of interest, since as a drug they will probably have fewer side-effects than substances that can affect the tubule-like connection envisaged in our model. Concerning development of drugs that act on capacitative calcium entry, our model thus directs the attention to agents that interfere with IP_3-sensitive intracellular calcium release channels.

References

1. Pietrobon D, Di Virgilio F and Pozzan T, Structural and functional aspects of calcium homeostasis in eukaryotic cells. *Eur J Biochem* **193:** 599–622, 1990.
2. Flockerzi V, Oeken HJ and Hofmann F, Purification of a functional receptor for calcium-channel blockers from rabbit skeletal-muscle microsomes. *Eur J Biochem* **161:** 217–224, 1986.
3. Putney JW Jr, The capacitative model for receptor-activated calcium entry. *Adv Pharmacol* **22:** 251–269, 1991.
4. Penner R, Fasolato C and Hoth M, Calcium influx and its control by calcium release. *Curr Opin Neurobiol* **3:** 368–374, 1993.
5. Gamberucci A, Innocenti B, Fulceri R, Bànhegyi G, Giunti R, Pozzan T and Benedetti A, Modulation of Ca^{2+} influx dependent on store depletion by intracellular adenine-guanine nucleotide levels. *J Biol Chem* **269:** 23597–23602, 1994.
6. Robinson IM, Cheek TR and Burgoyne RD, Ca^{2+} influx induced by the Ca^{2+}-ATPase inhibitors 2,5-di-(*t*-butyl)-1,4-benzohydroquinone and thapsigargin in bovine adrenal chromaffin cells. *Biochem J* **288:** 457–463, 1992.
7. Ely JA, Ambroz C, Baukal AJ, Christensen SB, Balla T and Catt KJ, Relationship between agonist- and thapsigargin-sensitive pools in adrenal glomerulosa cells. Thapsigargin-induced Ca^{2+} mobilization and entry. *J Biol Chem* **266:** 18635–18641, 1991.
8. Bode HP and Göke B, Protein kinase C activates calcium entry in the insulin-secreting cell line RINm5F. *FEBS Lett* **339:** 307–311, 1994.
9. Felder CC, Singer-Lahat D and Mathes C, Voltage-independent calcium channels. Regulation by receptors and intracellular calcium stores. *Biochem Pharmacol* **48:** 1997–2004, 1994.
10. Thastrup O, Cullen PJ, Drobak BK, Hanley MR and Dawson AP, Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc Natl Acad Sci USA* **87:** 2466–2470, 1990.

11. Thomas D and Hanley MR, Pharmacological tools for perturbing intracellular calcium storage. *Methods Cell Biol* **40:** 65–89, 1994.
12. Lo TM and Thayer SA, Refilling the inositol 1,4,5-triphosphate-sensitive Ca^{2+} store in neuroblastoma X glioma hybrid NG108-15 cells. *Am J Physiol* **264:** C641–C653, 1993.
13. Hoth M and Penner R, Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* **355:** 353–356, 1992.
14. Vaca L and Kunze DL, Depletion of intracellular Ca^{2+} stores activates a Ca^{2+}-selective channel in vascular endothelium. *Am J Physiol* **267:** C920–C925, 1994.
15. Randriamampita C and Tsien RY, Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature* **364:** 809–814, 1993.
16. Randriamampita C and Tsien RY, Degradation of a calcium influx factor (CIF) can be blocked by phosphatase inhibitors or chelation of Ca^{2+}. *J Biol Chem* **270:** 29–32, 1995.
17. Irvine RF, Inositol phosphates and Ca^{2+} entry: Toward a proliferation or a simplification? *FASEB J* **6:** 3085–3091, 1992.
18. Putney JW Jr, A model for receptor-regulated calcium entry. *Cell Calcium* **7:** 1–12, 1986.
19. Bird GSJ, Bian X and Putney JW Jr, Calcium entry signal? *Nature* **373:** 481–482, 1995.
20. Henkart M, Landis DMD and Reese TS, Similarity of junctions between plasma membranes and endoplasmic reticulum in muscle and neurons. *J Cell Biol* **70:** 338–347, 1976.
21. Fasolato C, Hoth M and Penner R, A GTP-dependent step in the activation mechanism of capacitative calcium influx. *J Biol Chem* **268:** 20737–20740, 1993.
22. Bird GSJ and Putney JW Jr, Inhibition of thapsigargin-induced calcium entry by microinjected guanine nucleotide analogs. *J Biol Chem* **268:** 21486–21488, 1993.
23. Bokoch GM and Der CJ, Emerging concepts in the *Ras* superfamily of GTP-binding proteins. *FASEB J* **7:** 750–759, 1993.
24. Montero M, Garcia-Sancho J and Alvarez J, Transient inhibition by chemotactic peptide of a store-operated Ca^{2+} entry pathway in human neutrophils. *J Biol Chem* **268:** 13055–13061, 1993.
25. Parekh AB and Penner R, Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells. *Proc Natl Acad Sci USA* **92:** 7907–7911, 1995.
26. Bode HP and Göke B, Inhibition of capacitative calcium entry in clonal endocrine pancreatic β-cells by staurosporine. *Naunyn Schmiedebergs Arch Pharmacol* **351:** R98, 1995.
27. Brüne B and Ullrich V, Cyclic nucleotides and intracellular-calcium homeostasis in human platelets. *Eur J Biochem* **207:** 607–613, 1992.
28. Kass GEN, Llopis J, Chow SC, Duddy SK and Orrenius S, Receptor-operated calcium influx in rat hepatocytes. Identification and characterization using manganese. *J Biol Chem* **265:** 17486–17492, 1990.
29. Bahnson TD, Pandol SJ and Dinne VE, Cyclic GMP modulates depletion-activated Ca^{2+} entry in pancreatic acinar cells. *J Biol Chem* **268:** 10808–10812, 1993.
30. Lee KM, Toscas K and Villereal M, Inhibition of bradykinin- and thapsigargin-induced Ca^{2+} entry by tyrosine kinase inhibitors. *J Biol Chem* **268:** 9945–9948, 1993.
31. Ferris CD and Snyder SH, Inositol 1,4,5-triphosphate-activated calcium channels. *Annu Rev Physiol* **54:** 469–488, 1992.
32. Ross CA, Danoff SK, Schell MJ, Snyder SH and Ullrich A, Three additional inositol 1,4,5-triphosphate receptors: Molecular cloning and differential localization in brain and peripheral tissues. *Proc Natl Acad Sci USA* **89:** 4265–4269, 1992.
33. Satoh T, Ross CA, Villa A, Supattapone S, Pozzan T, Snyder SH and Meldolesi J, The inositol 1,4,5-triphosphate receptor in cerebellar Purkinje cells: Quantitative immunogold labeling reveals concentration in an ER subcompartment. *J Cell Biol* **111:** 615–624, 1990.
34. Newton CL, Mignery GA and Südhof TC, Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-triphosphate ($InsP_3$) receptors with distinct affinities for $InsP_3$. *J Biol Chem* **269:** 28613–28619, 1994.
35. Nakagawa T, Okano H, Furuichi T, Aruga J and Mikoshiba K, The subtypes of the mouse inositol 1,4,5-triphosphate receptor are expressed in a tissue-specific and developmentally specific manner. *Proc Natl Acad Sci USA* **88:** 6244–6248, 1991.
36. Sharp AH, Snyder SH and Nigam SK, Inositol 1,4,5-triphosphate receptors. Localization in epithelial tissue. *J Biol Chem* **267:** 7444–7449, 1992.
37. Lièvremont J-P, Hill A-M, Hilly M and Mauger J-P, The inositol 1,4,5-triphosphate receptor is localized on specialized sub-regions of the endoplasmic reticulum in rat liver. *Biochem J* **300:** 419–427, 1994.
38. Khan AA, Steiner JP and Snyder SH, Plasma membrane inositol 1,4,5-triphosphate receptor of lymphocytes: Selective enrichment in sialic acid and unique binding specificity. *Proc Natl Acad Sci USA* **89:** 2849–2853, 1992.
39. Berger EG and Hesford FJ, Localization of galactosyl- and sialyltransferase by immunofluorescence: Evidence for different sites. *Proc Natl Acad Sci USA* **82:** 4736–4739, 1985.
40. Khan AA, Steiner JP, Klein MG, Schneider MF and Snyder SH, IP_3 receptor: Localization to plasma membrane of T cells and cocapping with the T cell receptor. *Science* **257:** 815–818, 1992.
41. Kuno M and Gardner P, Ion channels activated by inositol 1,4,5-triphosphate in plasma membrane of human T-lymphocytes. *Nature* **326:** 301–304, 1987.
42. McDonald TV, Premack BA and Gardner P, Flash photolysis of caged inositol 1,4,5-triphosphate activates plasma membrane calcium current in human T cells. *J Biol Chem* **268:** 3889–3896, 1993.
43. Fujimoto T, Nakade S, Miyawaki A, Mikoshiba K and Ogawa K, Localization of inositol 1,4,5-triphosphate receptor-like protein in plasmalemmal caveolae. *J Cell Biol* **119:** 1507–1514, 1992.
44. Severs NJ, Caveolae: Static impocketings of the plasma membrane, dynamic vesicles or plain artifact? *J Cell Sci* **90:** 341–348, 1988.
45. Anderson RGW, Potocytosis of small molecules by ions by caveolae. *Trends Cell Biol* **3:** 69–72, 1993.
46. Dupree P, Parton RG, Raposo G, Kurzchalia TV and Simons K, Caveolae and sorting in the *trans*-Golgi network of epithelial cells. *EMBO J* **12:** 1597–1605, 1993.
47. Blondel O, Moody MM, Depaoli AM, Sharp AH, Ross CA, Swift H and Bell GI, Localization of inositol triphosphate receptor subtype 3 to insulin and somatostatin secretory granules and regulation of expression in islets and insulinoma cells. *Proc Natl Acad Sci USA* **91:** 7777–7781, 1994.
48. Yoo SH and Albanesi JP, Inositol 1,4,5-triphosphate-triggered Ca^{2+} release from bovine adrenal medullary secretory vesicles. *J Biol Chem* **265:** 13446–13448, 1990.
49. Thévenod F and Schulz I, H^+-dependent calcium uptake into an IP_3-sensitive calcium pool from rat parotid gland. *Am J Physiol* **255:** G429–G440, 1988.
50. Thévenod F, Dehlinger-Kremer M, Kemmer TP, Christian AL, Potter BVL and Schulz I, Characterization of inositol 1,4,5-triphosphate-sensitive (IsCaP) and -insensitive (IisCaP) nonmitochondrial Ca^{2+} pools in rat pancreatic acinar cells. *J Membr Biol* **109:** 173–186, 1989.
51. Zha X, Chandra S, Ridsdale AS and Morrison GH, Golgi apparatus is involved in intracellular Ca^{2+} regulation in epithelial LLC-PK_1 cells. *Am J Physiol* **268:** C1133–C1140, 1995.

52. Walensky LD and Snyder SH, Inositol 1,4,5-triphosphate receptors selectively localized to the acrosome of mammalian sperm. *J Cell Biol* **130:** 857–869, 1995.
53. Bush KT, Stuart RO, Li SH, Moura LA, Sharp AH, Ross CA and Nigam SK, Epithelial inositol 1,4,5-triphosphate receptors. Multiplicity of localization, solubility, and isoforms. *J Biol Chem* **269:** 23694–23699, 1994.
54. Sugiyama T, Yamamoto-Hino M, Miyawaki A, Furuichi T, Mikoshiba K and Hasegawa M, Subtypes of inositol 1,4,5-triphosphate receptor in human hematopoietic cell lines: Dynamic aspects of their cell-type specific expression. *FEBS Lett* **349:** 191–196, 1994.
55. Maranto M, Primary structure, ligand binding, and localization of the human type 3 inositol 1,4,5-triphosphate receptor expressed in intestinal epithelium. *J Biol Chem* **269:** 1222–1230, 1994.
56. Nathanson MH, Fallon MB, Padfield PJ and Maranto AR, Localization of the type 3 inositol 1,4,5-triphosphate receptor in the Ca^{2+} wave trigger zone of pancreatic acinar cells. *J Biol Chem* **269:** 4693–4696, 1994.
57. Sabolic I, Katsura T, Verbavatz JM and Brown D, The AQP2 water channel: Effect of vasopressin treatment, microtubule disruption, and distribution in neonatal rats. *J Membr Biol* **143:** 165–175, 1995.
58. Forte JG, Black JA, Forte TM, Machen T and Wolosin JM, Ultrastructural changes related to functional activity in gastric oxyntic cells. *Am J Physiol* **241:** G349–G358, 1981.
59. Dubinsky WP, Preston CL, Calenzo MA, White GJ and Decker ER, Immunolocalization of chloride-transporting membrane vesicles in tracheal epithelial cells. *Am J Physiol* **263:** C888–C895, 1992.
60. Slot JW, Geuze HJ, Gigengack S, Lienhard GE and James DE, Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J Cell Biol* **113:** 123–135, 1991.
61. Ornberg RL, Kuijpers GAJ and Leapman RD, Electron probe microanalysis of the subcellular compartments of bovine adrenal chromaffin cells. *J Biol Chem* **263:** 1488–1493, 1988.
62. Chanat E and Huttner WB, Milieu-induced, selective aggregation of regulated secretory proteins in the *trans*-Golgi network. *J Cell Biol* **115:** 1505–1519, 1991.
63. Mughal S, Cuschier A and Al-Bader AA, Intracellular distribution of Ca^{2+}-Mg^{2+} adenosine triphosphatase (ATPase) in various tissues. *J Anat* **162:** 111–124, 1989.
64. Peute J, van Linder ATM, Zandbergen MA and de Bruijn WC, Ultrastructural localization of calcium and Ca^{2+}-ATPase activity in gonadotrops and stellate cells of the catfish pituitary. *Histochemistry* **94:** 601–607, 1990.
65. Favre CJ, Lew DP and Krause KH, Rapid heparin-sensitive Ca^{2+} release following Ca^{2+}-ATPase inhibition in intact HL-60 granulocytes. *Biochem J* **302:** 155–162, 1994.
66. Toescu EC and Petersen OH, The thapsigargin-evoked increase in $[Ca^{2+}]_i$ involves an $InsP_3$-dependent Ca^{2+} release process in pancretic acinar cells. *Pflügers Arch* **427:** 325–331, 1994.
67. Wagner ACC and Williams JA, Low molecular weight GTP-binding proteins: Molecular switches regulating diverse cellular functions. *Am J Physiol* **266:** G1–G14, 1994.
68. Bergeron M, Thiéry G, Lenoir F, Giocondi MC and Le Grimellec C, Organization of the endoplasmic reticulum in renal cell lines MDCK and LLC-PK1. *Cell Tissue Res* **277:** 297–307, 1994.
69. Bundgaard M, The three-dimensional organization of smooth endoplasmic reticulum in capillary endothelia: Its possible role in regulation of free cytosolic calcium. *J Struct Biol* **107:** 76–85, 1991.
70. Kijima Y, Saito A, Jetton TL, Magnuson MA and Fleischer S, Different intracellular localization of inositol 1,4,5-triphosphate and ryanodine receptors in cardiomyocytes. *J Biol Chem* **268:** 3499–3506, 1993.
71. O'Connor V, Augstine GJ and Betz H, Synaptic vesicle exocytosis: Molecules and models. *Cell* **76:** 785–787, 1994.