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COMMENTARY

INOSITOL 1,3,4,5-TETRAKISPHOSPHATE AND REGULATION OF INTRACELLULAR CALCIUM

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Calcium is a divalent cation that serves as a primary transducer of information both outside and inside the cell. Regulation of intracellular Ca^{2+} function is achieved through the evanescent behaviour of Ca^{2+} in the cytoplasm after agonist stimulation. Thus, in the cell, exquisite regulatory mechanisms exist that both modulate and control the intracellular concentration of Ca^{2+}. Unstimulated cells have a low intracellular Ca^{2+} concentration in relation to the cell exterior and certain intracellular compartments, creating a steep electrochemical gradient with respect to the cytoplasm. The interaction of many types of agonists with specific cell surface receptors triggers a rapid increase in the production of intracellular second messengers and a rise in intracellular Ca^{2+}. Reversal of both the production and concentration of second messengers and Ca^{2+} occurs following agonist stimulation. Thus, normal physiological functions appear to be generated in response to both a rise and a corresponding fall of second messengers. Evidence for this concept of the evanescent nature of the second messenger for a complete physiological response are abundant in physiology. For example, Ca^{2+} released from the sarcoplasmic reticulum initiates muscle contraction. However, if the Ca^{2+} is not returned to basal levels, the muscle fails to undergo relaxation, producing the condition of tetany. Cyclic AMP is a second messenger and mediates many physiological responses, transiently accumulating during the successful transition of cycling cells through the G1 portion of the cell cycle. Cyclic AMP normally rises a few hours before the initiation of DNA synthesis and returns to basal levels prior to the initiation of DNA synthesis. However, if the cyclic AMP concentration remains elevated and does not return to basal levels, the proliferative response is aborted [1]. Protein function is also regulated through transient association with regulatory factors. For example, Ca^{2+} alters calmodulin function by transiently changing the protein conformation; phosphorylation and/or dephosphorylation of proteins transiently alters protein conformation and thus activity. These and other examples stress the importance of the transient nature of the second messenger for successful generation of physiological responses. This is the principle on which we have designed a model for the regulation of intracellular Ca^{2+} that involves two recently described and important second messengers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4].

There are several mechanisms involved in the regulation of intracellular Ca^{2+}, some which function to control Ca^{2+} entry into the cell through the plasma membrane voltage-dependent and receptor-operated Ca^{2+} channels and others involved in regulation of Ca^{2+} across intracellular membranes or organelles such as mitochondria, endoplasmic reticulum and perhaps even the nuclear membrane. It is the molecular nature of these mechanisms that will briefly concern us here. Considerable excitement resulted from the finding that Ca^{2+} can be released from an intracellular pool by the second messenger Ins(1,4,5)P_3 [2]. Ins(1,4,5)P_3 is generated along with diacylglycerol from a relatively minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PtdInsP_2), by the action of phospholipase C. Diacylglycerol remains in the membrane domain and activates protein kinase C, which in turn phosphorylates specific protein substrates. Recently, a novel inositol polyphosphate was identified and characterized as Ins(1,3,4,5)P_4 [3]. The mechanism of generation of Ins(1,3,4,5)P_4 is through phosphorylation of Ins(1,4,5)P_3 by an inositol-trisphosphate-3 kinase whose activity is regulated by a Ca^{2+}/calmodulin-dependent kinase [4]. Because of the rapid and transient production of Ins(1,3,4,5)P_4, the possibility of it being an additional second messenger emanating from receptor agonist coupling was considered.

Intracellular Ca^{2+} *rise*

It is now well documented that Ins(1,4,5)P_3 releases Ca^{2+} from intracellular stores, presumably the endoplasmic reticulum [2]. Snyder *et al.* [7] have demonstrated that the Ins(1,4,5)P_3 receptor is localized to the rough endoplasmic reticulum, often in close association with the nuclear membrane. In addition, Ins(1,4,5)P_3 receptors are also found on smooth endoplasmic reticulum and cisternae close to the plasma membrane presynaptic terminals [7]. Volpe *et al.* [8] have demonstrated that Ins(1,4,5)P_3 can release Ca^{2+} from subcellular fractions that do not contain endoplasmic reticulum, raising the possibility that a structure distinct from the endoplasmic reticulum may be the Ins(1,4,5)P_3-sensitive Ca^{2+} pool. The term calciosome has been coined for this unique Ca^{2+} storage site. In addition, Worley *et al.* [9] have isolated and characterized a membrane-associated protein that has high-affinity binding for

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$Ins(1,4,5)P_3$ compared to other inositol polyphosphates and is thought to be the receptor coupled to Ca^{2+} release. Although the exact molecular nature of the cascade of events that are responsible for $Ins(1,4,5)P_3$-induced Ca^{2+} release is not known, recent evidence illustrates several means of modifying this mechanism.

Heparin was originally shown by Worley *et al.* [9] to inhibit specific binding of $Ins(1,4,5)P_3$ to purified $Ins(1,4,5)P_3$ receptors, and Hill *et al.* [10] used heparin to block the $Ins(1,4,5)P_3$-induced release of Ca^{2+} by thrombin in intact cells and by $Ins(1,4,5)P_3$ in permeable rat liver epithelial cells. The release of Ca^{2+} by $Ins(1,4,5)P_3$ is also influenced by Ca^{2+}, by calmodulin inhibitors, and by phosphorylation. Danoff *et al.* [11] demonstrated in crude rat cerebellar membranes that the addition of Ca^{2+} inhibited the binding of $Ins(1,4,5)P_3$ to its receptor and that after purification of the receptor, Ca^{2+} was unable to affect $Ins(1,4,5)P_3$ binding. The protein mediating the effect of Ca^{2+} is of M_r 300,000, has been termed calmedin, and when added back to purified $Ins(1,4,5)P_3$ receptor conveys the ability of Ca^{2+} to inhibit $Ins(1,4,5)P_3$ binding to its receptor. Another suggestion that Ca^{2+}, working through calmodulin, affects the $Ins(1,4,5)P_3$-induced release of Ca^{2+} stems from our work with various calmodulin inhibitors [12]. Several naphthalenesulfonamides (i.e. W-7 and W-13) inhibit calmodulin function but are also known to block several protein kinases including protein kinase C and cyclic AMP-dependent protein kinase (protein kinase A). There is a new calmodulin inhibitor that appears to be more specific, although its solubility is still somewhat of a problem. This compound, CGS-9343B, potently blocks calmodulin-dependent protein kinase activity without affecting protein kinase A or protein kinase C. Each of these calmodulin antagonists blocks the ability of thrombin to release Ca^{2+} from intact T51B rat liver epithelial cells [12]. The focus of calmodulin action could be at the receptor, at hydrolysis of $PtdInsP_2$ by phospholipase C, or at the level of $Ins(1,4,5)P_3$ action. Results support the contention that calmodulin is involved at the $Ins(1,4,5)P_3$ site of action level because in electropermeable T51B cells the release of Ca^{2+} by addition of $Ins(1,4,5)P_3$ is blocked by each of these calmodulin antagonists without apparently affecting $Ins(1,4,5)P_3$ binding to its receptor [12, *]. It should be noted that the dose of antagonist used to inhibit Ca^{2+} release (i.e. W-7 = 50 μM; W-13 = 100 μM; and CGS-9343B = 110 μM) does not affect either protein kinase A or C activity [12]. The presence of calmodulin in the biochemical apparatus linking $Ins(1,4,5)P_3$ receptors to Ca^{2+} release channels implies that a membrane-bound protein regulated by calmodulin is an integral component of the Ca^{2+} mobilization mechanism as well. The function of calmodulin may be to alter protein conformation or to stimulate enzyme activity. Finally, it was reported that phosphorylation of the $Ins(1,4,5)P_3$ receptor by protein kinase A does not affect the binding of $Ins(1,4,5)P_3$ to its receptor but does influence the amount of Ca^{2+} released by $Ins(1,4,5)P_3$, shifting the dose–response curve to the right by one order of magnitude [7]. Because phosphorylation/dephosphorylation of proteins regulates their function, it is not unreasonable to suggest that a protein phosphatase may also play a critical role in the process of Ca^{2+} release.

Intracellular Ca^{2+} *fall*

The release of intracellular Ca^{2+} by $Ins(1,4,5)P_3$ is only one component of the Ca^{2+} transient that may be important for a physiological response. The other component is the return of the intracellular Ca^{2+} to basal levels. The decline in Ca^{2+} levels during the second portion of the biphasic Ca^{2+} response could be the result of Ca^{2+} being pumped out of the cells, Ca^{2+} being sequestered into mitochondria, or Ca^{2+} being pumped back into the calciosome or endoplasmic reticulum. The molecular mechanism by which Ca^{2+} is handled after release by $Ins(1,4,5)P_3$ is unknown but could involve the action of one of the metabolites of $Ins(1,4,5)P_3$ [i.e. $Ins(1,4)P_2$ or $Ins(1,3,4,5)P_4$].

Several lines of evidence support the hypothesis that $Ins(1,3,4,5)P_4$ functions by inducing the sequestration of intracellular Ca^{2+}. Several model systems have been developed in our laboratory to illustrate this effect of $Ins(1,3,4,5)P_4$ using both 261B/T51B rat liver cells and sea urchin eggs.

When 261B/T51B rat liver cells are permeabilized by electroporation, $Ins(1,4,5)P_3$ addition to a cell suspension induces Ca^{2+} release, and the response is biphasic, consisting of a rise and a fall [6]. Because we could repetitively add $Ins(1,4,5)P_3$ to these permeable cells and with each addition release Ca^{2+}, we concluded that receptor desensitization did not play a role in the return of Ca^{2+} to basal levels, but rather metabolism of $Ins(1,4,5)P_3$ may be important. $Ins(1,4,5)P_3$ is metabolized to either $Ins(1,4)P_2$ by a 5-phosphomonoesterase or to $Ins(1,3,4,5)P_4$ by the action of a Ca^{2+}/calmodulin-dependent inositol-trisphosphate 3-kinase [4]. When $Ins(1,4,5)P_3$ is added to permeable T51B cells, it is metabolized very rapidly, almost equally, to both of these metabolites, and the rate of metabolism correlates well with the rise and fall in Ca^{2+} concentration. Two separate experiments demonstrate that $Ins(1,3,4,5)P_4$ is responsible for initiating the sequestration phase of the Ca^{2+} transient. The Ca^{2+} rise without the fall or sequestration phase can be produced by the simple addition of about 600 nM Ca^{2+}. There must exist a feedback loop between the Ca^{2+} storage pool, the endoplasmic reticulum, and the receptor operated sequestration mechanism located within the membrane of this pool because another Ca^{2+} addition of 300 nM produced a Ca^{2+} rise but it was not sequestered (i.e. we find that usually two to three additions of 300 nM Ca^{2+} results in the condition where an additional 300 nM Ca^{2+} induces the rise in the buffer Ca^{2+} but not the fall; Fig. 1A). The effect of $Ins(1,3,4,5)P_4$ is illustrated by its addition (i.e. 2 μM) to the permeable cell suspension either before or after an additional 300 nM Ca^{2+}. This results in both the rise (due to the exogenous Ca^{2+} addition) and the fall [due to $Ins(1,3,4,5)P_4$] of buffer Ca^{2+} (Fig. 1B; [6]). The specificity of the $Ins(1,3,4,5)P_4$ effect

* Nakamoto SS, Zwiller J and Boynton AL, unpublished data.

is illustrated by the fact that no other inositol polyphosphate, including Ins(1,4)P_2, can mimic the response. These results suggest that metabolism of Ins(1,4,5)P_3 by action of the Ca^{2+}/calmodulin-dependent inositol-trisphosphate 3-kinase is important for inducing the sequestration of Ca^{2+}. This hypothesis is further illustrated by the fact that Ins(2,4,5)P_3, the non-metabolizable analogue of Ins(1,4,5)P_3 which still releases Ca^{2+}, induces a monophasic Ca^{2+} release, and the buffer Ca^{2+} remains elevated for an extended period of time (i.e. at least 20–30 min; Fig. 1C). However, the addition of 2 μM Ins(1,3,4,5)P_4 before the addition of Ins(2,4,5)P_3 enables the elevated Ca^{2+} to return to basal levels, and the normal biphasic Ca^{2+} response is completed in a time frame similar to the Ins(1,4,5)P_3-induced Ca^{2+} transient (Fig. 1D).

An additional model system utilized sea urchin eggs, *Trypneustes gratilla*, first microinjected with fura 2. Eggs were then microinjected with either Ins(1,4,5)P_3, Ins(2,4,5)P_3 or Ins(1,3,4,5)P_4, and the intracellular Ca^{2+} concentration was determined using a single cell digital imaging system. Microinjection of Ins(1,4,5)P_3, as in permeable T51B cells treated with Ins(1,4,5)P_3 and in intact T51B cells treated with various agonists such as thrombin and angiotensin, results in the rise and fall in intracellular Ca^{2+}, the transient lasting 3–4 min. On the other hand, the microinjection of Ins(2,4,5)P_3, like in permeable T51B cells, results in a rise of intracellular Ca^{2+} that remains elevated for a prolonged period of time (i.e. 20–30 min) that is presumably due to the inability of the cell to metabolize Ins(2,4,5)P_3. The critical experiment proving that Ins(1,3,4,5)P_4 induced sequestration of Ins(1,4,5)P_3 and Ins(2,4,5)P_3 released Ca^{2+} is illustrated by microinjecting into fura 2 loaded sea urchin eggs both Ins(2,4,5)P_3 and Ins(1,3,4,5)P_4. The intracellular Ca^{2+} rises, and this released Ca^{2+} is sequestered in a time course very similar to the pattern when Ins(1,4,5)P_3 alone is microinjected (i.e. within 5 min).* This result has an important implication in the interpretation of data generated using non-metabolizable analogues of Ins(1,4,5)P_3 and of Ins(1,3,4,5)P_4 and supports the concept that both a rise and fall in intracellular Ca^{2+} may be required in order to elicit a physiological response. The inability of sea urchin eggs microinjected with Ins(2,4,5)P_3 to reduce the elevated intracellular Ca^{2+} in the absence of Ins(1,3,4,5)P_4 brings several questions to mind concerning egg handling of cytoplasmic Ca^{2+}. First, why is the high intracellular Ca^{2+} not removed from the cell by plasma membrane Ca^{2+}-ATPases? Several explanations could account for this phenomenon. Ins(1,3,4,5)P_4 action may be required for plasma membrane Ca^{2+}-ATPase pumps in which case the observed effect of Ins(1,3,4,5)P_4 described above must induce the removal of Ca^{2+} from the cytoplasm through the plasma membrane and/or into an intracellular Ca^{2+} storage pool. It would appear, at first glance, that Ins(1,3,4,5)P_4 is filling an intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} pool (see below); however, if the Ca^{2+} were pumped out of the cell, the intracellular storage pool could again be filled according to the capacitative model of Ca^{2+} entry as proposed by Putney [13] and later by Irvine [14] see below). If this is the case, we would have to assume that Ins(1,3,4,5)P_4 is affecting plasma membrane Ca^{2+} ATPases. That this is not the case is supported by experiments with fura 2 loaded T51B cells maintained in a buffer solution containing only 100 nM extracellular Ca^{2+}, a concentration approximately equivalent to the intracellular resting concentration of Ca^{2+}. The addition of a maximum dose of thrombin release Ins(1,4,5)P_3, which in turn releases 500 nM Ca^{2+} and this cytoplasmic Ca^{2+} is reduced rapidly to basal levels. Within 30 sec, the addition of a maximum dose of ATP releases another 500 nM Ca^{2+} and again the cytoplasmic Ca^{2+} is returned rapidly to basal levels. It should be noted that the total amount of ionomycin releasable Ca^{2+} was not altered after the release and sequestration of intracellular Ca^{2+} induced by either thrombin or ATP. This is indicative of the released Ca^{2+} being sequestered into an intracellular storage pool as opposed to being pumped out of the cell by plasma membrane Ca^{2+}-ATPases. If the Ins(1,4,5)P_3-released Ca^{2+} were extruded from the cells by plasma membrane ATPases, the intracellular Ca^{2+} storage pools must have been refilled very rapidly and ultimately from the extracellular medium. This possibility seems remote because the Ca^{2+} pumped out of the cell would be diluted very rapidly and to such an extent that to recover essentially all of the Ca^{2+} back into the intracellular Ins(1,4,5)P_3-sensitive pool is unlikely. Experiments using Ca^{2+} microelectrodes placed in the extracellular medium are necessary to answer this fundamental question of the fate of Ins(1,4,5)P_3-released Ca^{2+}. The other entertaining possibility is that the Ins(1,4,5)P_3-released Ca^{2+} never sees plasma membrane Ca^{2+} pumps because of either compartmentation and/or extremely efficient Ca^{2+}-ATPases located in the intracellular membranes. Several pieces of evidence actually support this contention including the explanation just described above. An assumption we must make if this model is to be viable is that Ins(2,4,5)P_3 inhibits Ca^{2+}-ATPase activity because its presence in both permeable T51B cells and sea urchin eggs blocks the ability of cells to sequester Ca^{2+}, presumably via Ca^{2+}-ATPase activity. That Ins(2,4,5)P_3 can affect Ca^{2+}-ATPase activity is illustrated by the fact that it slows the ability of the pumps to remove Ca^{2+} from the cytoplasm of cells first chilled to 4° and subsequently warmed to 37°.* Alternatively, Ca^{2+}-ATPases might be functioning normally in response to high Ca^{2+} levels, but cells fail to accumulate Ca^{2+} due to the constant stimulation of the release mechanism by Ins(2,4,5)P_3. Therefore, Ca^{2+} returned to storage pools by Ca^{2+}-ATPases rapidly leaks back into the cytoplasm producing a net result of a prolonged high Ca^{2+} concentration. We must also at this point entertain the possibility that Ins(1,3,4,5)P_4 induces the sequestration of intracellular Ca^{2+} via a unique receptor operated channel located in the membrane of intracellular storage pools separate from the Ca^{2+} ATPases. Even though the evidence to date is sparse, the testable hypothesis that Ins(1,4,5)P_3-released Ca^{2+} never sees plasma membrane Ca^{2+}-ATPases remains attractive.

* Hill TD and Boynton AL, unpublished data.

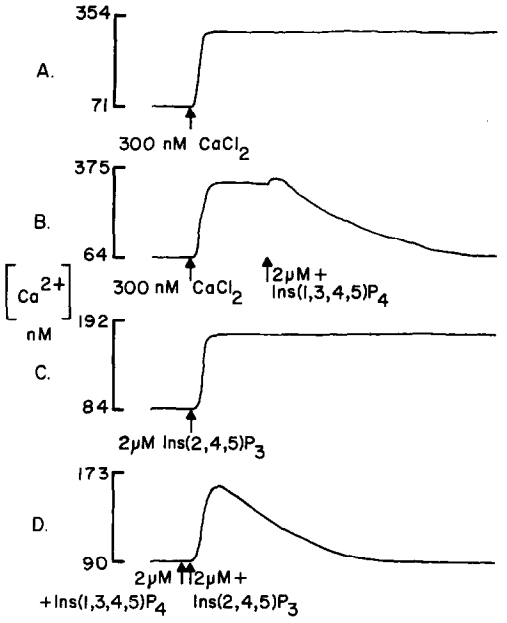

Fig. 1. Effect of $Ins(1,3,4,5)P_4$ on Ca^{2+} sequestration in permeable rat liver cells. 261B/T51B rat liver cells were electropermeabilized, and the buffer Ca^{2+} concentration was monitored with the Ca^{2+}-sensitive fluorescent probe fura 2. The sequestration mechanism responsible for removing elevated buffer Ca^{2+} could be down-regulated by the addition of about 600 nM Ca^{2+} to the buffer solution so that an additional addition of 300 nM Ca^{2+} would increase buffer Ca^2 without its sequestration (A). The high buffer Ca^{2+} concentration would remain elevated for extended periods of time (i.e. at least 30 min) unless $Ins(1,3,4,5)P_4$ was added which rapidly caused the buffer Ca^{2+} to fall (B). This Ca^{2+} was removed to an $Ins(1,4,5)P_3$ sensitive Ca^{2+} pool by the action of $Ins(1,3,4,5)P_4$ [5]. Addition of $Ins(2,4,5)P_3$ to these electropermeabilized cells also raised the buffer Ca^{2+} concentration without its subsequent sequestration (C). The addition of 2 μM $Ins(1,3,4,5)P_4$ before the addition of $Ins(2,4,5)P_3$ enabled the released Ca^{2+} to be sequestered (D). Reproduced, in part, with permission from *Science* **242**: 1176–1178, 1988. Copyright (1988) by the AAAS. [Ref. 6].

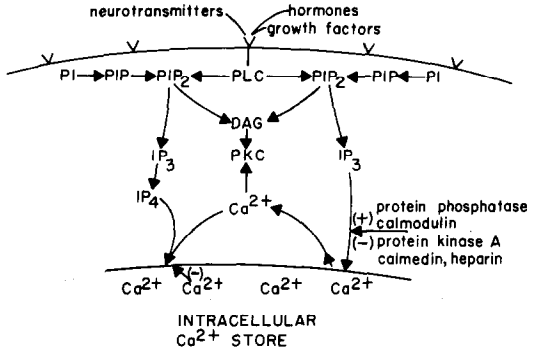

Fig. 2. A simplistic view of the cascade of events leading to the intracellular Ca^{2+} transient and the positive and negative factors that are known to influence this transient. Highly specific extracellular receptors for a variety of agonists, such as growth factors, neurotransmitters or hormones, are located on the exterior surface of the plasma membrane and are linked to the phosphatidylinositol 4,5-bisphosphate hydrolyzing phospholipase C. The result of agonist receptor binding is the production of diacylglycerol which activates protein kinase C and $Ins(1,4,5)P_3$ which releases Ca^{2+} from the intracellular stores. The release of Ca^{2+} by $Ins(1,4,5)P_3$ is positively affected, probably indirectly, by calmodulin and by a putative protein phosphatase and is negatively affected by protein kinase A and by Ca^{2+} itself through calmedin, a Ca^{2+}-binding protein. Metabolism of $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ by the action of a Ca^{2+}/calmodulin-dependent $Ins(1,4,5)P_3$-3 kinase results in the activation of the Ca^{2+} sequestration mechanism which removes the Ca^{2+} to an $Ins(1,4,5)P_3$-sensitive Ca^{2+} storage site, thus completing the transient. Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; IP_3, inositol 1,4,5-trisphosphate; and IP_4, inositol 1,3,4,5-tetrakisphosphate.

Storage pool of Ins(1,3,4,5)P_4-sequestered Ca^{2+}

The Ca^{2+} removed from the cytosol by the action of $Ins(1,3,4,5)P_4$ is directed in part into a cytosolic storage pool that is sensitive to $Ins(1,4,5)P_3$. Several lines of evidence support this contention. First, $Ins(1,4,5)P_3$ at a maximum dose released about 400 nM Ca^{2+} in permeable 261B/T51B cells. A submaximal dose of $Ins(2,4,5)P_3$ released 200 nM Ca^{2+} and a subsequent addition of a maximal dose of $Ins(1,4,5)P_3$ could release only 200 nM Ca^{2+}, suggesting that both isomers act on the same intracellular Ca^{2+} pool. The $Ins(2,4,5)P_3$-released Ca^{2+} is sequestered by the action of $Ins(1,3,4,5)P_4$, and this Ca^{2+} fills an intracellular Ca^{2+} pool sensitive to both $Ins(1,4,5)P_3$ and $Ins(2,4,5)P_3$. Thus, the same amount of Ca^{2+} was released by $Ins(1,4,5)P_3$ when added either before or after the Ca^{2+} release and reuptake in response to $Ins(2,4,5)P_3$ and $Ins(1,3,4,5)P_4$ [5]. Further support for this effect of $Ins(1,3,4,5)P_4$ comes from experiments using permeable rat liver cells in which the sequestration of added buffer Ca^{2+} could be stimulated by the action of $Ins(1,3,4,5)P_4$ as described above. The hypothesis is that the uptake of Ca^{2+} induced by $Ins(1,3,4,5)P_4$ is filling the same pool regulated by $Ins(1,4,5)P_3$. Results illustrate that Ca^{2+} released by $Ins(1,4,5)P_3$ following treatment with a combination of $Ins(1,3,4,5)P_4$ and 500 nM Ca^{2+} was more than twice the amount released prior to incubation with $Ins(1,3,4,5)P_4$ and Ca^{2+} [5]. $Ins(1,3,4,5)P_4$-induced Ca^{2+} uptake loaded the inositol-trisphosphate-sensitive pool to a greater extent than what occurs when cells are exposed to Ca^{2+} or $Ins(1,3,4,5)P_4$ alone. Thus, $Ins(1,4,5)P_3$, $Ins(2,4,5)P_3$ and $Ins(1,3,4,5)P_4$ all exert their influence on the same intracellular Ca^{2+} storage pool. It should be noted that $Ins(1,3,4,5)P_4$ induced the reuptake of all the added Ca^{2+} (i.e. 300 nM; Fig. 1B) to the buffer, but only an additional 150–175 nM Ca^{2+} was released by the subsequent addition of a maximal concentration of $Ins(1,4,5)P_3$. This suggests that an additional pool of intracellular Ca^{2+} exists to which $Ins(1,3,4,5)P_4$ also directs its action and which is not sensitive to $Ins(1,4,5)P_3$.

The intracellular Ca^{2+} storage pool and its filling

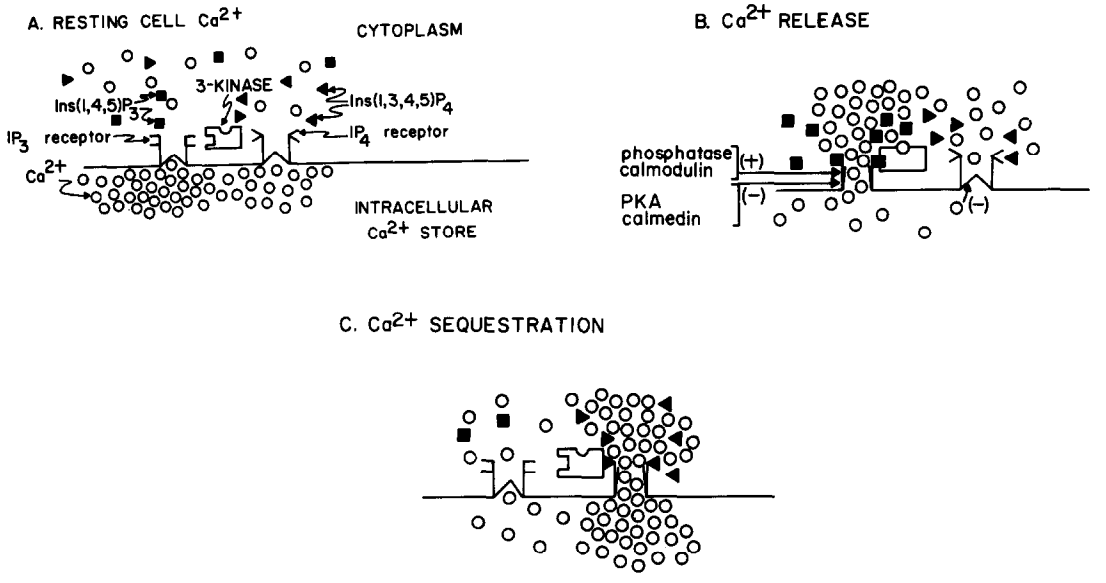

Fig. 3. A working diagram of the $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ receptors, how the Ca^{2+} transient occurs, and factors which affect the transient. (A) Depicted is the resting cell with the $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ receptors juxtaposed with the Ca^{2+}/calmodulin-dependent $Ins(1,4,5)P_3$-3 kinase in close association with the receptors. Ca^{2+} in the filled storage pools feeds back in a negative manner to the filling mechanism which presumably is associated with the $Ins(1,3,4,5)P_4$ receptor, and this mechanism is maintained in a down-regulated state. (B) In the normal course of events, agonist binding to cell surface receptors, via a phospholipase C induced hydrolysis of phosphatidylinositol 4,5-bisphosphate, produces the second messengers diacylglycerol and $Ins(1,4,5)P_3$. As depicted in this panel, Ins $(1,4,5)P_3$ binds to its receptor and Ca^{2+} is released from intracellular storage pools. It should be noted that the $Ins(1,4,5)P_3$ receptor itself is the Ca^{2+} channel as demonstrated by Snyder and colleagues in a reconstituted system [7]. The released Ca^{2+} performs many functions, turning on various physiological processes. In addition, the increased intracellular Ca^{2+} interacts with calmedin which, in turn, has a negative effect on the Ca^{2+} release mechanism. Ca^{2+} also interacts with the Ca^{2+}/calmodulin-dependent $Ins(1,4,5)P_3$-3 kinase turning on the enzyme and converting $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$. Another factor affecting the release of Ca^{2+} from internal stores is the phosphorylation state of the receptor. In the resting cell the receptor may be phosphorylated, thus reducing the efficiency by which $Ins(1,4,5)P_3$ can release Ca^{2+}. In the stimulated cell the receptor may be in the dephosphorylated state due to the action of a protein phosphatase, thus increasing the amount of Ca^{2+} released by $Ins(1,4,5)P_3$. Finally, calmodulin may positively affect the release of Ca^{2+} by $Ins(1,4,5)P_3$ because agents which antagonize calmodulin function block the ability of $Ins(1,4,5)P_3$ to release Ca^{2+}. (C) The Ca^{2+} released by the action of $Ins(1,4,5)P_3$ is rapidly returned to the $Ins(1,4,5)P_3$-sensitive Ca^{2+} storage pool by the action of $Ins(1,3,4,5)P_4$. The action of the Ca^{2+}/calmodulin-dependent $Ins(1,4,5)P_3$ kinase converts $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ and the Ca^{2+} release channel is rapidly closed; $Ins(1,3,4,5)P_4$ binds to its receptor which causes the channel responsible for refilling the $Ins(1,4,5)P_3$-sensitive storage pool to open, and the cytosolic Ca^{2+} is returned to basal levels. Ca^{2+} in the storage pool itself may modulate the filling mechanism because filling the pool with Ca^{2+} down-regulates the mechanism responsible for removing Ca^{2+} from the cytosol and into the $Ins(1,4,5)P_3$-sensitive storage pool.

have been the topic recently of vigorous discussion centered around what has been termed the capacitative model of Ca^{2+} entry as originally proposed by Putney [13]. Simply stated, the model originally described the concept that the filling of the $Ins(1,4,5)P_3$-sensitive pool after agonist depletion was through the plasma membrane and occurred without the Ca^{2+} first passing through the cytoplasm (i.e. there is no measurable increase in free intracellular levels of Ca^{2+}). This model has now evolved into a complex system of various intracellular Ca^{2+} pools regulated by Ca^{2+}, $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ [14]. The model now entertains that the primary $Ins(1,4,5)P_3$-sensitive Ca^{2+} store is the calciosome, and it is linked to a second Ca^{2+} pool, suggested to be the endoplasmic reticulum, that serves as the Ca^{2+} reserve for the calciosome and which is somehow linked to the cellular exterior. According to the model, it is between these two intracellular Ca^{2+} pools where $Ins(1,3,4,5)P_4$ functions by regulating and maintaining the Ca^{2+} concentration in the calciosome. Although our data with $Ins(1,3,4,5)P_4$ inducing the sequestration of Ca^{2+} does not directly support this model in its entirety, it does strongly support the idea of $Ins(1,3,4,5)P_4$ being responsible for filling an $Ins(1,4,5)P_3$-sensitive Ca^{2+} pool.

Conclusions

Many physiological responses are the result of

transient increases in critical primary transducing factors, such as hormones and growth factors, and second messengers, such as cyclic AMP, calmodulin and Ca^{2+}. Data are reviewed and presented here in which a model is built with respect to regulation of intracellular Ca^{2+} transients (Figs. 2 and 3). The foundation of the model is built on two recently discovered second messengers, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Put in its simplest form (Fig. 2), the model predicts that many agonists including growth factors, neurotransmitters and hormones interact with specific cell surface receptors which are linked in the plasma membrane to specific phospholipase C enzymes that, in turn, induce the hydrolysis of phosphatidylinositol 4,5-bisphosphate yielding two products, diacylglycerol and Ins(1,4,5)P_3. Diacylglycerol activates protein kinase C, while Ins(1,4,5)P_3, a soluble molecule with specific high-affinity binding sites located on the endoplasmic reticulum or perhaps the calciosome, releases Ca^{2+}, thus increasing the concentration of this ion in the intracellular environment. Interruption of calmodulin function has been demonstrated to block the ability of Ins(1,4,5)P_3 to release Ca^{2+} without affecting the binding of Ins(1,4,5)P_3 to its receptor. Thus, it is not unreasonable to conclude that calmodulin plays a positive role in the release mechanism involving Ins(1,4,5)P_3. Putative phosphorylation of the Ins(1,4,5)P_3 receptor by protein kinase A does not block the binding of Ins(1,4,5)P_3 to its receptor but does reduce by an order of magnitude the quantity of Ca^{2+} released. We would also like to propose at this point that the dephosphorylation of the protein kinase A phosphorylation site by a protein phosphatase may be a critical event in modulating the quantity of Ca^{2+} released by Ins(1,4,5)P_3. An additional negative influence on Ins(1,4,5)P_3-induced Ca^{2+} release is Ca^{2+} itself functioning through a rather large (M_r 300,000) Ca^{2+} binding protein termed calmedin. The model predicts that the increased intracellular level of Ca^{2+} sets into motion a variety of biochemical events that eventually lead to physiological responses. However, the completion of the train of events leading to the physiological response is dependent on the transient nature of the Ca^{2+} increase. Thus, the increased intracellular concentration of Ca^{2+} must be reduced, and evidence is presented, using both permeable rat liver cells and intact sea urchin eggs, that Ins(1,3,4,5)P_4 performs this function. Thus, a Ca^{2+} releasing agonist such as Ins(2,4,5)P_3, released stored Ca^{2+}, but the released Ca^{2+} is not sequestered because this inositol polyphosphate is not metabolized. However, exogenously added Ins(1,3,4,5)P_4 induces the sequestration of the released Ca^{2+}. The only factor found to affect the sequestration mechanism was Ca^{2+} itself. Thus, loading the intracellular Ca^{2+} pools in permeable cells by the simple addition of Ca^{2+} must somehow turn off the sequestration mechanism without affecting the Ins(1,3,4,5)P_4 receptor because a later addition of Ins(1,3,4,5)P_4 rapidly induces the sequestration of the cytosolic Ca^{2+} and moves it into an Ins(1,4,5)P_3-sensitive pool. Finally, evidence is presented that predicts that the Ins(1,4,5)P_3-released Ca^{2+} is sequestered, at least partially, by the action of Ins(1,3,4,5)P_4 into an Ins(1,4,5)P_3-sensitive Ca^{2+} pool and is not pumped out of the cell via plasma membrane Ca^{2+}-ATPases.

A more detailed model depicting the Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 receptor in relation to Ca^{2+} release and sequestration is illustratd in Fig. 3. The Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 receptors are juxtaposed on the endoplasmic reticulum with the Ca^{2+}/calmodulin-dependent Ins(1,4,5)P_3-3 kinase in close association. The model describes the release of Ca^{2+} from intracellular stores by the binding of Ins(1,4,5)P_3 to its specific and high-affinity receptor, and this releases Ca^{2+} which in turn signals the beginning of many cellular signals as well as activates the Ins(1,4,5)P_3-3-kinase. Activation of the kinase rapidly metabolizes Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 which binds to its specific and high-affinity receptor which, in turn, causes the high intracellular level of Ca^{2+} to be restored to prestimulation levels by refilling the Ins(1,4,5)P_3-sensitive Ca^{2+} store. Ins(1,3,4,5)P_4, in turn, is metabolized to Ins(1,3,4)P_3 by a 5-phosphomonoesterase, thus turning the sequestration phase of the Ca^{2+} transient off.

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