CALCIUM HOMEOSTASIS AND THE ACTIVATION OF CALCIUM CHANNELS IN CELLS OF THE IMMUNE SYSTEM*

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IT is now widely accepted that calcium plays a central role in the development of the immune response. Changes in the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) are thought to be essential for responses as varied as bacterial killing by neutrophils and the synthesis and secretion of antibodies by lymphoid cells. Three lines of evidence support this contention. First, an elevation in $[Ca^{2+}]_i$ is a nearly universal feature associated with activation of cells of the immune system.[1,2] Second, many responses normally elicited by physiological stimuli can be mimicked by simply elevating $[Ca^{2+}]_i$ by means of exogenous ionophores. These latter agents, which selectively increase the permeability of the membrane to divalent cations, have been reported to induce degranulation[3] and superoxide generation[4] in neutrophils, proliferation of lymphocytes,[5] and a variety of other components of the immune response. Finally, it has been demonstrated in several systems that precluding the stimulus-induced changes in $[Ca^{2+}]_i$ results in obliteration of the subsequent physiological response, suggesting a causal relationship. Thus, lymphoproliferation and neutrophil activation are greatly inhibited or even totally impaired when stimulation does not produce the normal elevation of $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ can be minimized in several systems by removal of extracellular calcium, particularly if this maneuver is combined with the introduction of calcium-buffering agents into the cells, to neutralize

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the mobilization of calcium from internal stores (see below).

EFFECTS OF CALCIUM CHANNEL BLOCKERS ON IMMUNE CELL FUNCTION

Cell type	*Response*	*Inhibitor/effect (comments)*	*Ref.*
Human neutrophils	OAG*-induced superoxide production	Inhibited by verapamil (100 μM-400 μM)	30
	Killing of Pseudomonas aeruginosa	Inhibited by verapamil (1 μM) (also inhibits A23187-induced effect)	31
	Zymosan-induced formation of PAF	Inhibited by nifedipine (5 μM) (also inhibits A23187-induced effect)	32
Human basophils	Ragweed antigen-induced histamine release	Inhibited by verapamil (100 μM)	32
	Con A-induced histamine release	No effect of verapamil or dilitiazem	
Human B lymphocytes	Anti-μ-induced progression through cell cycle	Inhibited by verapamil, nitrendipine (100 μM)	33
Human lymphocytes	PHA-induced or A23187-induced proliferation	Inhibited by verapamil, nifedipine (>20 μM) (calcium uptake inhibited by verapamil)	34
Human T lymphocytes	Tetanus toxoid or IL-2 binding-dependent proliferation	Inhibited by verapamil, nifedipine, diltiazem (>10 μM)	35
Bovine lymphocytes	Lectin ± phorbol ester-induced mitogenesis	Inhibited by verapamil, nicardipine (10 μM)	36
	Phorbol ester + A23187-induced mitogenesis	Inhibited by nicardipine but not by verapamil	
Cytolytic T cell line	Antigen-induced $[Ca^{2+}]_i$ Increase	Insensitive to verapamil	37
	Cell killing effect	Inhibited by nifedipine, verapamil (100 μM)	38
Human lymphoid-cell cultures	Interferon production	Inhibited by nitrendipine, nisoldipine, verapamil (10–100 μM)	39

*OAG: oleoyl-acetyl glycerol; PAF: platelet activating factor; PHA: phytohemagglutinin; IL-2: interleukin-2; Con A: concanavalin A

The accurate measurement of $[Ca^{2+}]_i$ changes in the comparatively small cells of the mammalian immune system became possible only recently, with the introduction of fluorescent probes capable of monitoring $[Ca^{2+}]_i$ in intact, viable cells. Early measurements using the "first generation" probe, quin-2 revealed a single monotonic stimulus-induced $[Ca^{2+}]_i$ increase. It

subsequently became apparent that the substantial calcium buffering power introduced by the probe itself was preventing the resolution of the individual components of the response. This deficiency was at least partly alleviated by the introduction of the "second generation" probes fura-2 and indo-1, which can be used at much lower concentrations, consequently contributing less to the overall buffering capacity. Using these fluorophores, two components of the $[Ca^{2+}]_i$ response have been clearly identified: an early, large and transient response followed by a smaller but more sustained phase. When measured in cell suspensions, the former is generally over within a couple of minutes, whereas the second phase can be sustained for tens of minutes. The nature and properties of these components of the $[Ca^{2+}]_i$ response are the subject of the remainder of this article.

The early phase of the $[Ca^{2+}]_i$ elevation has been generally attributed to the mobilization of calcium from intracellular stores to the cytoplasmic compartment. Consistent with this interpretation, the transient $[Ca^{2+}]_i$ peak elicited by stimulation persists in media devoid of calcium, indicating that entry of the cation across the plasma membrane is not necessary for this component of the response. The location of the calcium stores mobilized during activation has not been definitively identified. By analogy with the sarcoplasmic reticulum of skeletal muscle, it was initially believed that the endoplasmic reticulum, in general, was the compartment involved. However, more recent evidence indicates that a special submembranal vesicular component of the reticulum, tentatively named "calciosomes," may be the source of the calcium mobilized in the early phase. Though a direct interaction between this compartment and the plasma membrane has been suggested,[7] convincing experimental evidence is not yet available. Instead, it is clear that, following interaction of the surface receptors with their respective ligands, a soluble second messenger is delivered to the calciosomes, signalling the release of calcium. This second messenger has been identified as inositol-1,4,5,-trisphosphate, a product of the hydrolysis of phosphatidylinositol bis-phosphate by phospholipase C. In support of this notion, it has been repeatedly reported that, in cells where the plasma membrane has been rendered permeable by means of detergents, addition of exogenous inositol-1,4,5,-trisphosphate releases calcium accumulated intracellularly, in the absence of receptor-ligand interactions.[8,9]

A different process appears to underlie the slower, sustained phase of $[Ca^{2+}]_i$ rise that accompanies stimulation of cells of the immune system. This phase is stringently dependent on the availability of extracellular Ca^{2+}, indicating that entry of the cation across the plasma membrane is involved.

Despite extensive study, however, little is known regarding the precise mechanism whereby calcium enters stimulated cells. It is generally believed that calcium channels open when the cells are activated. Yet, to our knowledge, in cells of the immune system there is only one instance where increased calcium channel activity in response to addition of ligand was directly recorded. In 1986 Kuno et al.[10] reported activation of calcium-permeable (though not calcium-specific) channels in T lymphocytes stimulated with mitogenic lectins. Unfortunately, comparable results in other cell types are not yet available, and confirmation of the above results in T cells has not been forthcoming.

In view of its large, inside positive reversal potential, the entry of calcium into cells through a conductive channel would be expected to produce a significant depolarization of the membrane potential. Though a delayed depolarization has been reported to occur following stimulation in B lymphocytes[11] and in neutrophils,[12] the potential change does not seem to correlate with the elevation in $[Ca^{2+}]_i$. Moreover, in other cells,[13] the only discernible membrane potential change is in the hyperpolarizing direction. In the latter cells it is conceivable that the inward calcium current is masked by an outward potassium flow. During stimulation, potassium exits the cells through calcium-activated channels[14] as well as through voltage-sensitive channels which can also be responsive to the formation of receptor-ligand complexes.[15] If this were the case, the conductive calcium flux could, in principle, be unmasked by preventing or at least delaying the opening of potassium channels. In fact, a small yet reproducible depolarization was detected upon stimulation of B lymphocytes loaded with 1,2-bis-(aminophenoxy)-ethane-N,N,N′,N′-tetraacetate (BAPTA), a calcium buffering agent that delays the ligand-induced rise in $[Ca^{2+}]_i$.[16] In thymic T lymphocytes we have recently detected a depolarization when the cells were stimulated with concanavalin A, a polyclonal mitogen, but only when the cells were simultaneously exposed to charybdotoxin, a potent blocker of calcium activated potassium channels (J.D. Smith and S. Grinstein, unpublished observations). These observations suggest that conductive calcium channels may indeed open during activation of cells of the immune system.

Another indication that the fluxes triggered by activators may be conductive was obtained analysing the membrane potential dependence of the $[Ca^{2+}]_i$ increase. In lymphocytes, neutrophils, and basophilic leukemia cells the sustained phase of $[Ca^{2+}]_i$ increase is greatly reduced when the membrane is artificially depolarized during stimulation.[17–19] A comparable reduction was observed whether the cells were depolarized by elevating the

external potassium concentration or by means of sodium-conductive ionophores. These findings are consistent with the hypothesis that calcium enters the cells through conductive channels so that influx is reduced as the internally negative potential, and hence the inward driving force, are reduced. However, this is not the only possible interpretation and a potential-sensitive, yet nonconductive transport mechanism could conceivably be invoked.

Three different types of calcium channels have been proposed to exist,[20] based on their mechanism of activation: the classical voltage-operated channels (VOCs), which are activated by depolarization of the membrane and were first described in excitable tissues; receptor-operated channels (ROCs), in which the receptor and channel functions reside in the same molecule or in adjacent molecules; and second messenger-operated channels (SMOCs), which are activated following the formation of receptor-ligand complexes, but in which the channel is not directly linked functionally (and possibly also anatomically) to the receptor. Whereas direct information exists demonstrating the existence of VOCs and ROCs, the evidence supporting the presence of SMOCs in the plasma membrane is largely indirect. The properties of these channels are summarized briefly below in an attempt to assign the calcium "channels" of cells of the immune system to one of the groups.

VOCs, which have been extensively studied in nerve and muscle cells, have been subdivided into three different groups, primarily on the basis of their kinetic and pharmacologic properties.[21,22] The L-type channels are the most commonly found. They are characterized by their large conductance, long open times, and long-lasting increase in the open state probability. L-type channels are blocked by dihydropyridines and by a variety of other organic and inorganic calcium channel antagonists. In addition to being modulated by the transmembrane voltage, L-type channels are also regulated by the level of intracellular calcium and, in some cell types, by hormones and neurotransmitters. Because of their large conductance and long open times, these channels are thought to play an important role in the regulation of $[Ca^{2+}]_i$. In contrast to the L channels, the T-type channels open only transiently, due to a voltage-dependent inactivation process. Their conductance is also smaller than that of L-type channels. In combination, these properties limit the amount of calcium that enters the cell through T channels, which have probably little effect on $[Ca^{2+}]_i$. Instead, they probably function to modulate the membrane potential, e.g., to initiate action potentials. Finally, a third type of channel with intermediate conductance and kinetic properties has been described. These so called N channels open only under rather restrictive conditions: upon large depolarizations from very negative resting

(holding) potentials. The N-type channels can be modulated by neurotransmitters but, unlike L channels, are insensitive to dihydropyridines. The latter property is also shared by T channels.

With the exception of myeloma and B lymphoid hybridoma cells,[23] there is practically no evidence of calcium-selective VOCs in cells of the immune system. If present, these channels would seem to have little impact on $[Ca^{2+}]_i$ in unstimulated cells. In this regard, it has been demonstrated in several cell types (e.g., lymphocytes and neutrophils) that sustained depolarization has no detectable effect on $[Ca^{2+}]_i$. Similarily, opening of VOCs does not appear to underlie the stimulus-induced change in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ rise proceeds under conditions where voltage is unaltered or even hyperpolarized. In addition, concentrations of antagonists known to block VOCs (such as nanomolar levels of dihydropyridines) do not preclude the change in $[Ca^{2+}]_i$ elicited by ligands in immune cells.

It has been suggested that in lymphocytes calcium can enter the cells through voltage-activated potassium channels, thought to open in response to the addition of mitogens.[15] This suggestion is based mainly on the observation that cellular proliferation, a calcium-dependent process, can be inhibited by potassium channel blockers. However, a direct effect of these blockers on the entry of calcium has not been substantiated and it is unclear whether the antiproliferative effect of the channel antagonists is specific. Taken together, the available information does not support a role of calcium (or potassium) VOCs in the activation of cells of the immune system.

ROCs are activated as a result of the interaction of a receptor with its specific ligand. In this case, the channels are either part of or immediately adjacent to the receptor itself, i.e., there is no mediation by other molecules. Though admittedly arbitrary, this definition clearly sets ROCs apart from SMOCs, where intervening messengers are involved. The ATP-induced calcium current in smooth muscle described by Benham and Tsien[24] probably typifies the properties of ROCs. ATP opens these calcium-conducting channels even at very negative potentials. Unlike VOCs, these channels are not blocked by either nifedipine or cadmium. They can be also distinguished from SMOCs in that they are activated very rapidly (within 1 s) after addition of the ligand, without involvement of readily diffusible mediators. The latter was concluded because the responses are observed in outside-out excised membrane patches, but not in cell attached patches when ATP was added to the bach (i.e., to the rest of the cell).

The channels that mediate the $[Ca^{2+}]_i$ changes in cells of the immune system are unlikely to belong to the ROC category. The single exception may be the dichromoglycate-sensitive channel of mast cells,[25] which has been

reported to open upon ligand binding under conditions where second messenger participation is unlikely. However, these studies have not been confirmed by more recent patch clamp analysis and will therefore not be considered further. Other calcium transporting pathways, such as those in lymphocytes and neutrophils, depart from the simple ROC model in two respects. First, a significant lag time is often observed between the formation of the receptor-ligand complex and the increase in permeability to calcium. This has been clearly illustrated using Mn^{2+} as a calcium substitute in chemotactic peptide-treated neutrophils.[26] Though binding of the peptide to its receptors occurs within seconds (as evidenced by the rapid mobilization of internal calcium) entry of divalent cations is only detectable about 1 min later. Second, in neutrophils and lymphocytes the ligand-induced increases in $[Ca^{2+}]_i$ can be inhibited by treatment of the cells with pertussis or cholera toxin. These toxins are thought to act by catalyzing the ADP-ribosylation of GTP-binding (or G) proteins. The latter are known to function as transducing elements in the stimulus-response cascade, often inducing the formation of secondary messengers. Because interference with G protein operation impairs the elevation of $[Ca^{2+}]_i$, it seems unlikely that the receptors and channels are directly coupled.

In view of the slow onset of the external calcium-dependent $[Ca^{2+}]_i$ increase, and having excluded VOCs and ROCs as the possible mechanism, it is considered most likely that ligand-induced $[Ca^{2+}]_i$ changes in immune cells are mediated by SMOCs. Indeed, specific second messengers have been tentatively identified in the case of neutrophils and lymphocytes. Von Tscharner et al.[27] have provided evidence that calcium itself may be the signal that opens plasma membrane channels in activated neutrophils. According to their model, ligand-receptor complex formation would promote the generation of inositol 1,4,5-trisphosphate, with consequent release of calcium from the internal stores. The resulting transient increase in $[Ca^{2+}]_i$ would itself render plasmalemmal channels open, accounting for the secondary phase of $[Ca^{2+}]_i$ elevation. Despite its elegance, this model is not in keeping with more recent data obtained using fluorescent dyes.[26] In the latter studies it was shown that chemotactic peptides will stimulate entry of extracellular calcium even under conditions where $[Ca^{2+}]_i$ is minimally affected by release of intracellular calcium. Conversely, entry of divalent cations across the membrane could not be induced in the absence of physiological stimuli by simply elevating $[Ca^{2+}]_i$. Thus, it remains to be established whether calcium itself is the second messenger that activates SMOCs in neutrophils.

Using lymphocytes, Kuno and Gardner[28] presented evidence that inositol

1,4,5-trisphosphate, the signal that triggers release from the calciosomes, can also activate calcium-transporting channels in the plasma membrane. These authors hypothesized that stimulation of phospholipase C would therefore have a dual effect on $[Ca^{2+}]_i$ by mobilizing internal calcium as well as facilitating entry through plasma membrane channels. This economic model of second messenger mediated activation has not yet been confirmed in other cell types. In fact, in the neutrophil study discussed above[27] no channel gating was detected with inositol 1,4,5-trisphosphate under conditions similar to those used by Kuno and Gardner.[28] Thus, confirmation of a role of inositol 1,4,5-trisphosphate in plasma membrane channel opening must await further results.

Very recent data obtained in other cell types provides indirect support for inositol 1,3,4,5-tetrakisphosphate as a possible activator of calcium channels. Microinjection of this inositol polyphosphate caused raising of the fertilization envelope in sea urchin eggs.[29] Because this effect is dependent on external calcium, it was postulated that inositol, 1,3,4,5-tetrakisphosphate binds to and activates calcium channels at the plasma membrane. A similar conclusion was reached measuring membrane potential changes in salivary gland cells. In this system, microinjection of inositol 1,3,4,5-tetrakisphosphate resulted in hyperpolarization, which was attributed to calcium-activated potassium channels. The latter presumably opened in response to elevated $[Ca^{2+}]_i$ that was the result of opening plasma membrane calcium channels. To our knowledge, there is presently no evidence favoring the existence of inositol 1,3,4,5-tetrakisphosphate-activated calcium channels in the plasma membrane of cells of the immune system. It is clear, however, that these cells have the capability of generating inositol, 1,3,4,5-tetrakisphosphate by phosphorylation of inositol, 1,4,5-trisphosphate. The possible role of the tetraphosphate in calcium homeostasis in immune cells will, in all likelihood, be established in the near future.

Even though calcium channels in immune cells do not appear to conform to the classical properties of VOCs, a number of laboratories have reported blockade of calcium-dependent functions by verapamil, dihydropyridines, and other VOC inhibitors. An incomplete yet representative summary is presented in Table I. The responses found to be inhibited by the channel blockers range from superoxide production in neutrophils to histamine release from basophils and mitogenesis in lymphoid cells. However, the effects of the channel antagonists are not universal. Thus, concanavalin A-induced release of histamine by human basophils was unaffected by verapamil and diltiazem (see table). Moreover, even in those instances where the channel

blockers are effective, the concentrations required to observe the biological effect were generally several orders of magnitude higher than those required to block L type VOCs. Whereas excitable cell calcium channels are blocked at nM concentrations of dihydropyridines, the inhibitory effects in cells of the immune system require doses>10 μM and often 100–400 μM. This could simply imply that the pharmacologic properties of SMOCs and VOCs are somewhat different. However, it is also conceivable that the inhibition of the biological responses measured was nonspecific, i.e., unrelated to blockade of calcium entry into the cells. In this regard, it is noteworthy that calcium fluxes or $[Ca^{2+}]_i$ changes were measured in only a few instances. Perhaps more important, in several studies the blockers inhibited responses under conditions where $[Ca^{2+}]_i$ increases are not normally observed or required, and blockade of calcium ionophore-induced responses has also been observed. Because blocking calcium channels is not expected to inhibit these responses, the occurrence of nonspecific effects must be suspected. In conclusion, calcium channel antagonists at elevated concentrations can interfere with the development of several immunological responses. Whether this interference is related to inhibition of calcium channels and is due to the reduction or elimination of the stimulus-induced increase in $[Ca^{2+}]_i$ remains to be determined.

SUMMARY

In summary, calcium seems to play a central role in the activation of cells of the immune system. When the cells are stimulated, $[Ca^{2+}]_i$ generally increases as a result of entry from the external medium, as well as mobilization of calcium from intracellular membrane-bound compartments. It is generally acknowledged that inositol 1,4,5-trisphosphate mediates the release of calcium from internal stores. By comparison, relatively little is known about the mechanism underlying entry of calcium across the plasma membrane. Currently available evidence seems to rule out the participation of classic voltage-gated channels. Similarly, it appears unlikely that the interaction of ligands with their surface receptors directly activates the channels. Instead, it is more likely that a second messenger produced by the formation of the receptor-ligand complex is responsible for gating the channels. Several mediators, including calcium itself and inositol phosphates, have been proposed to effect gating of the channels, but conclusive evidence is not yet available. Ongoing work is likely to reveal the nature of the putative second messenger(s) in the near future.

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