Depletion of Intracellular Calcium Stores Activates Smooth Muscle Cell Calcium-independent Phospholipase A$_2$

A NOVEL MECHANISM UNDERLYING ARACHIDONIC ACID MOBILIZATION*

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Herein we present multiple lines of evidence which demonstrate that depletion of internal calcium stores is both necessary and sufficient for the activation of calcium-independent phospholipase A$_2$ during arginine vasopressin (AVP)-mediated mobilization of arachidonic acid in A-10 smooth muscle cells. First, AVP-induced $[^{3}H]$arachidonic acid release was independent of increases in cytosolic calcium yet was decreased by pharmacological inhibition of the release of calcium ion from internal stores. Second, thapsigargin induced the dramatic release of $[^{3}H]$arachidonic acid from A-10 cells at a similar rate as the AVP-induced release of arachidonic acid, and the release of arachidonic acid by either AVP or thapsigargin was entirely inhibited by (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL). Third, the magnitude of thapsigargin-induced $[^{3}H]$arachidonic acid release was entirely independent of alterations in cytosolic calcium concentration. Fourth, A23187 resulted in the BEL-inhibitable release of $[^{3}H]$arachidonic acid from A-10 cells even when ionophore-induced increases in cytosolic calcium were completely prevented by calcium chelators. Fifth, pretreatment of A-10 cells with a calmodulin antagonist (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl) resulted in the time-dependent decrease of subsequent thapsigargin-induced $[^{3}H]$arachidonic acid release. Collectively, these results identify a novel paradigm which links alterations in calcium homeostasis to the calmodulin-mediated regulation of calcium-independent phospholipase A$_2$ through the depletion of internal calcium stores.

Alterations in cellular calcium homeostasis are a widely employed and potent regulatory mechanism that modulates many functionally distinct physiologic processes (1–6). Historically, attention has focused on alterations in cytosolic free calcium as the primary mechanism through which calcium mediates its downstream effects on cellular biochemical events (7–9). However, recent studies have identified intracellular calcium store depletion as an important mechanism mediating calcium signaling in activated cells (10–12). For example, intracellular calcium store depletion regulates transplasmalemmal calcium flux (13, 14), hormone secretion (15, 16), and cellular proliferation (17–19). Although the importance of intracellular calcium store depletion is now well established, the precise biochemical mechanisms which link the depletion of intracellular calcium stores to cellular signaling processes remains enigmatic.

Eicosanoids are critical lipid second messengers in stimulated cells whose production has traditionally been envisaged to result from increases in cytosolic calcium leading to the activation of one or more calcium-dependent phospholipases A$_2$ (e.g. cPLA$_2$, sPLA$_2$) (20, 21). Recently, we have demonstrated that the predominant phospholipase A$_2$ activity in A-10 muscle cells is not activated by calcium ion (i.e. it is a member of the iPLA$_2$ family) (22, 23) and that this iPLA$_2$ is physically associated with, and functionally coupled to, the intracellular calcium signal transducer, calmodulin (24). In this paradigm, iPLA$_2$ exists in a ternary complex with calcium and calmodulin in a catalytically inactive state. Pharmacologic removal of calmodulin from the phospholipase A$_2$ ternary complex by W-7 in intact A-10 smooth muscle cells results in the activation of iPLA$_2$ and the release of arachidonic acid (24). Accordingly, one mechanism potentially responsible for the release of arachidonic acid in activated cells is the depletion of calcium ion from specific subcellular loci and the resultant release of calmodulin-mediated inhibition of iPLA$_2$.

A-10 smooth muscle cells contain arginine vasopressin (AVP) receptors whose occupancy results in the activation of PLC (25), the generation of IP$_3$ (25, 26), and the subsequent release of calcium ion from internal stores (25–27). In previous studies, we utilized specific mechanism-based inhibition to demonstrate that AVP-induced release of arachidonic acid from A-10 smooth muscle cells occurs through the activation of iPLA$_2$ (22). We now report that AVP-induced release of arachidonic acid catalyzed by iPLA$_2$ is mediated by the depletion of internal calcium stores and that depletion of internal calcium stores, even in the absence of receptor occupancy or elevations of cytosolic free calcium, is sufficient for the activation of iPLA$_2$.

**EXPERIMENTAL PROCEDURES**

**Materials—**A-10 cells derived from rat aortic smooth muscle (ATCC no. CRL 1476) were obtained from ATCC. (E)-6-(Bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL), thapsigargin, cyclo-

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which are coupled to PLC and result in the IP₃-mediated mobilization of intracellular calcium stores (26, 27), the effects of pharmacologic inhibition of intracellular calcium pool depletion on AVP-mediated activation of smooth muscle cell iPLA₂ were investigated. Neomycin-mediated inhibition of PLC (29) decreased AVP-induced [³H]arachidonic acid release by 70% (Fig. 1a). Moreover, replacement of calcium in the incubation media with EGTA (1 mM) and BAPTA-AM (100 µM) did not attenuate AVP-induced [³H]arachidonic acid release, although alterations in [Ca²⁺]ᵢ, were completely prevented (Figs. 1, b and c).

To determine whether intracellular calcium store depletion in the absence of receptor occupancy and IP₃ production is a sufficient stimulus for iPLA₂ activation, two independent approaches were taken. In the first approach, three structurally disparate inhibitors of sarco/endoplasmic reticular calcium-ATPases (SERCA) were employed to deplete intracellular calcium pools (30, 31). Thapsigargin-induced the robust release of [³H]arachidonic acid from A-10 cells which was inhibited by BEL. The magnitude of both the release of [³H]arachidonic acid by thapsigargin and its inhibition by BEL were identical when cells were stimulated in media containing 2.5 mM calcium, EGTA alone, or EGTA and BAPTA-AM (Fig. 2a). Treatment of smooth muscle cells with thapsigargin in the presence of medium containing EGTA resulted in a small increase in cytosolic calcium ion concentration which could be completely chelated by BAPTA-AM (Fig. 2b). Although BAPTA-AM completely prevented the modest thapsigargin-induced increments in cytosolic calcium concentration, no alterations in thapsigargin-induced release of [³H]arachidonic acid were manifest (Fig. 2a).

Thus, thapsigargin-induced release of [³H]arachidonic acid is independent of alterations of cytosolic calcium ion concentration and is directly correlated with thapsigargin-induced depletion of intracellular calcium pools. Furthermore, the time course of thapsigargin-induced release of [³H]arachidonic acid was similar to that manifest by treatment of A-10 smooth muscle cells with AVP (Fig. 2c). Treatment of A-10 smooth muscle cells with two other structurally distinct SERCA inhibitors, cyclopaic acid (10 µM) (32) and BHQ (50 µM) (33), also resulted in similar increases in [³H]arachidonic acid release.

These increases were entirely inhibited by BEL and the mag-
The cyclopiazonic acid and BHQ-mediated [3H]arachidonic acid release was independent of alterations in [Ca\textsuperscript{2+}]\textsubscript{i} which were ablated by BAPTA-AM treatment utilizing medium containing EGTA (data not shown). Collectively, these findings demonstrate that the depletion of intracellular calcium stores in A-10 smooth muscle cells by SERCA inhibitors results in the activation of smooth muscle cell iPLA\textsubscript{2} independent of alterations in cytosolic calcium ion concentration.

In the second approach, the effects of intracellular calcium pool depletion on the activation of smooth muscle cell iPLA\textsubscript{2} were investigated employing the calcium ionophore, A23187 (34). This compound mediates the passive transport of calcium ion across both plasma and intracellular membranes down existing [Ca\textsuperscript{2+}] gradients, and thus can induce the depletion of calcium ion from intracellular sequestration sites. Treatment of A-10 smooth muscle cells with A23187 (10 \textmu M) for 5 min in the presence of 2.5 mM extracellular calcium resulted in the robust release of [3H]arachidonic acid which was entirely inhibited by BEL (Fig. 3a). Treatment of A-10 smooth muscle cells with A23187 (10 \textmu M) in the presence of extracellular EGTA (5 mM) resulted in similar increases in [3H]arachidonic acid release (which was BEL-inhibitable) as those manifest in the presence of extracellular calcium ion (Fig. 3a). Moreover, A23187-induced release of [3H]arachidonic acid in the presence of external EGTA was not altered by BAPTA-AM, although BAPTA-AM completely prevented A23187-induced increases in

![Arachidonic Acid Mobilization](image)
Cytoplasmic calcium ion (Fig. 3b). Collectively, these findings demonstrate that: 1) A23187 stimulation of A-10 smooth muscle cells mediates the release of [3H]arachidonic acid through iPLA2; 2) the A23187-induced release of [3H]arachidonic acid does not depend on the entry of extracellular calcium ion or ionophore-induced increases in cytosolic calcium ion concentration; and 3) iPLA2 is exquisitely sensitive to alterations in intracellular calcium ion compartmentation.

Recently, calmodulin has been demonstrated to be physically associated with, and functionally coupled to, iPLA2 (24). To determine if the physical association of calmodulin with iPLA2 is causally related to the activation of iPLA2 by intracellular calcium pool depletion, we exploited the principle of pharmacologically directed radiolabeled phospholipid pool depletion. If Ca2+-store depletion activated latent iPLA2 activity through dissociation of the calmodulin-iPLA2 complex, then prior exposure of cells to W-7 would deplete the [3H]arachidonic acid content of phospholipid pools which serve as substrates for iPLA2, and would result in decreased [3H]arachidonic acid release after subsequent exposure to thapsigargin. The results demonstrated that as the duration of pretreatment with W-7 increased, A-10 cells released progressively smaller amounts of [3H]arachidonic acid in response to subsequent thapsigargin treatment (Fig. 4). This suggests that activation of iPLA2, induced either by Ca2+-store depletion or by a calmodulin antagonist, results from the hydrolysis of a common pool of phosphoinositides.

Collectively, the results of the present study demonstrate that what has been nominally termed a “calcium-independent” phospholipase A2, based on in vitro activity assays is actually profoundly modulated by alterations in calcium homeostasis in its natural context in vivo. Moreover, these studies provide a rationale and experimental proof which integrates the receptor-mediated activation of phospholipase C with the subsequent phospholipase A2-mediated release of arachidonic acid through the IP3-mediated depletion of internal calcium stores.

A substantial body of literature has utilized the A23187-induced release of arachidonic acid as a bona fide indicator of the participation of calcium-dependent phospholipases A2 in arachidonic acid release (35–37). These results demonstrate that the interpretation of those experiments is more complex than previously anticipated.

The importance of alterations in calcium homeostasis and iPLA2 activation in the receptor-mediated release of arachidonic acid in many tissues is now well established (38–41). However, the mechanism through which calcium can modulate calcium-independent phospholipase A2 during signal transduction has previously represented a fundamental paradox. The present results provide a novel paradigm which links alterations in calcium homeostasis to the activation of iPLA2 activity through the calcium-calmodulin-mediated regulation of iPLA2 and the depletion of internal calcium stores. Thus, iPLA2 can function as a sensor of the filling state of internal calcium stores, and this sensor function is mediated by the calcium-dependent association of iPLA2 with calmodulin.

Finally, we point out that depletion of internal calcium pools has been causally linked to the activation of a plasma membrane calcium channel through an as yet unidentified second messenger (42–45). The demonstration of the coupling of iPLA2 activation with calcium pool depletion suggests that this second messenger is likely either a direct product of the iPLA2 reaction (e.g., arachidonic acid or lysolipids) or a downstream product whose synthesis is initiated by iPLA2 activation. It is our hope that the identification of the coupling of calcium pool depletion with the activation of iPLA2 will serve as a biochemical foundation to identify the second messenger(s), which facilitates the activation of store-operated plasma membrane calcium channels.

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