Evidence for a Phorbol Ester-insensitive Phosphorylation Step in Capacitative Calcium Entry in Rat Thymic Lymphocytes*

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Experiments were undertaken to investigate the regulation of capacitative Ca$^{2+}$ entry by phorbol ester-sensitive protein kinase C and serine/threonine protein phosphatase activity. The thapsigargin-activated Ca$^{2+}$ entry pathway was probed in control cells and cells treated with phosphatase type 1/2A inhibitors, okadaic acid and calyculin A, or with the phorbol ester, phorbol 12-myristate 13-acetate. The permeability state of this pathway was monitored in the presence or absence of these agents using fluorometric measurements of intracellular Ca$^{2+}$ concentration, unidirectional Mn$^{2+}$ entry, and membrane potential and unidirectional measurements of Ca$^{2+}$ uptake using $^{45}$Ca$^{2+}$. The results of these studies demonstrate that modification of the phosphorylation state of target protein(s) on serine/threonine amino acid residues by inhibition of phosphatase type 1/2A inhibits the capacitative Ca$^{2+}$ entry pathway in rat thymic lymphocytes. Importantly, the capacitative Ca$^{2+}$ entry pathway in rat thymic lymphocytes is not modulated by activation of phorbol ester-sensitive protein kinase C.

While it is generally accepted that the Ca$^{2+}$ permeability of the plasma membrane in nonexcitable cells is regulated in large part by the degree of filling of the intracellular storing compartments (capacitative Ca$^{2+}$ entry), the mechanisms underlying this phenomenon remain obscure. A wide range of mechanisms have been proposed or implicated in the communication step and have been discussed in detail in recent reviews on this subject (1, 2).

Several of the proposed mechanisms describe events whereby protein phosphorylation plays an important modulatory role in the control of capacitative Ca$^{2+}$ entry, either at the level of the communication step or at the Ca$^{2+}$ entry pathway. However, many of the reports implicating phosphorylation/dephosphorylation events in the regulation of capacitative Ca$^{2+}$ entry appear to be contradictory. For example, a number of studies have demonstrated an enhancement of the Ca$^{2+}$ entry pathway using phosphatase inhibitors that promote serine/threonine phosphorylation (3–5). Consistent with this notion, it has been reported that modest activation of phorbol ester-sensitive PKC enhances capacitative Ca$^{2+}$ entry in oocytes and a pancreatic cell line (6, 7). In contrast, phorbol ester activation of PKC has been shown to inhibit capacitative Ca$^{2+}$ entry in the lymphocytic cell line Jurkat, human neutrophils, and peripheral T-lymphocytes, Drosophila photoreceptors, and rat basophilic leukemia cells (8–12). In accordance with an inhibition of capacitative Ca$^{2+}$ entry by hyperphosphorylation of serine/threonine residues, serine/threonine phosphatase inhibitors have been reported to inhibit this pathway (13). Clearly, the role of phosphorylation in the modulation of capacitative Ca$^{2+}$ entry is not consistent, and it may represent differences between cell types.

The existence of capacitative Ca$^{2+}$ entry in rat thymic lymphocytes has been well documented. Release of endosomal Ca$^{2+}$ via inhibition of endosomal Ca$^{2+}$-ATPase activity, elevations in inositol 1,4,5-trisphosphate, or brief incubation in Ca$^{2+}$-free medium activates an indistinguishable plasma membrane Ca$^{2+}$ influx pathway (14–18). Recent work from our laboratory is indicative of a role of high energy phosphate donors in the activation and sustained activation of the capacitative Ca$^{2+}$ entry pathway in this cell type (19). Such a finding may be indicative of an important modulatory role of protein phosphorylation in the control of capacitative Ca$^{2+}$ entry in rat thymic lymphocytes. The purpose of the present experiments was to investigate the regulation of capacitative Ca$^{2+}$ entry by phorbol ester-sensitive PKC and serine/threonine protein phosphatase activity. To address these questions we have utilized non-invasive fluorometric and isotopic techniques.

**EXPERIMENTAL PROCEDURES**

Reagents and Solutions—The AM derivatives of indo-1 and BCECF were purchased from Tefabs (Austin, TX). The AM derivatives of Bapta and EGTA, and bis-oxonol were purchased from Molecular Probes (Eugene, OR). Ionomycin, thapsigargin, and HEPES were obtained from Calbiochem. Gramicidin and EGTA were purchased from Sigma. Charybdotoxin was purchased from Peninsula Laboratories (Belmont, CA). NMG, NiCl$_2$, hexahydrate, and Mg$_2$SO$_4$ were purchased from Aldrich. NaCl, KCl, CaCl$_2$, MgCl$_2$, MnCl$_2$, D-glucose, NaOH, and KOH were purchased from Fisher. Nor-okadaone, calyculin A, and Na$^+$ salt of okadaic acid were purchased from LC Laboratories (Woburn, MA). PMA was purchased from multiple vendors. Stock solutions of indo-1-AM, Bapta-AM, thapsigargin, PMA, and gramicidin were made up in Me$_2$SO. Ionomycin, nor-okadaone and calyculin A were dissolved in ethanol.

The basic Na$^+$ medium employed in the fluorescence experiments contained 140 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM D-glucose, and 20 mM HEPES-free acid, titrated to pH 7.3 at 37 °C with NaOH. Ca$^{2+}$-free solutions were made by omitting Ca$^{2+}$ and adding 200 μM EGTA. NMG$^+$ medium was made by equimolar replacement of sodium. When Ca$^{2+}$ and Mn$^{2+}$ were added to cell suspensions, these divalents were added as chloride salts. All solutions and stocks were stored at −20 °C.

**Cell Isolation and Bapta Loading**—Thymic lymphocytes were iso-
lated from 120–200-g male Wistar rats (Charles River Breeding Laboratories) as described previously (20). The cells were counted using a model ZM Coulter Counter (Coulter Electronics, Hialeah, FL) and maintained at room temperature in basic Na+ medium.

Where required, the rate of change of [Ca2+]i, was slowed by loading cells with the Ca2+- chelator Bapta as previously reported (15, 16, 21). This procedure also facilitated the detection of \( E_{Ca} \), and prolonged the linear phase of Ca2+ uptake in \( Ca^{2+} \) experiments. Cells were loaded by incubation with 20 \( \mu M \) of the AM derivative of Bapta at 37°C in basic Na+ medium. To control for differences in the efficacy of Bapta loading, all comparisons between experimental conditions were made in cells taken from the same batch of Bapta-loaded cells.

**Fluorescence Determinations**—All experiments were performed at 37°C using a Photon Technology International fluorescence spectro-photometer (Delta Scan) equipped with a magnetic stirrer and temperature control. The cells were counted immediately after the last manipulation, prior to addition to the cuvette, to ensure that the appropriate number were added.

**Determination of Free Cytoplasmic Calcium Concentration**—[\( Ca^{2+} \)], was determined by measuring the fluorescence of indo-1 as previously reported by our laboratory (15, 16). The excitation and emission wavelengths were 351 nm (3-nm slit width) and 405 nm (10-nm slit width), respectively. Cells suspensions (25 \( \times \) 10^6 cells/ml) were added with indo-1 by incubation with a 4 \( \mu M \) concentration of the AM precursor for 30 min at 37°C in basic Na+ medium. When necessary, cells were simultaneously loaded with Bapta. The cells were then sedimented, resuspended in basic Na+ medium, and kept at room temperature until required. Cells were added to the cuvette at a final concentration of 2 \( \times \) 10^6 cells/ml. Fluorescence was calibrated using ionomycin and Mn2+ as described previously (15, 16, 22) using a dissociation constant of 250 nm (23).

**Determination of Mn2+-Influx**—Mn2+ uptake was monitored as the rate of quenching of indo-1 fluorescence at the isosbestic point for the Ca2+-indo-1 complex (excitation 331 nm (3-nm slit width), emission 443–447 nm (10-nm slit width)) determined daily under our experimental conditions. When measured at the isosbestic wavelength, the rate of fluorescence decrease is insensitive to changes in the [\( Ca^{2+} \)], and is proportional to the rate of Mn2+ accumulation in the cytosol (15, 16).

**Determination of Cytoplasmic pH**—Fluorimetrically measured free cytoplasmic pH was measured using BCECF as previously reported (19, 26). The excitation and emission wavelengths used were 495 nm (3-nm slit width) and 525 nm (10-nm slit width), respectively. Loading with BCECF was achieved by incubating 25 \( \times \) 10^6 cells/ml with 2 \( \mu M \) of the AM precursor for 30 min at 37°C. To monitor fluorescence, aliquots containing the required cell number were sedimented, resuspended in the appropriate medium devoid of Ca2+-, and added to the cuvette at a final concentration of 2 \( \times \) 10^6 cells/ml. Calibration curves of pH against fluorescence were generated by lysing the cells with Triton X-100 (0.05% (v/v) final) at the end of the experiment and titrating the medium by the addition of small amounts of NaOH or HEPES while measuring the solution pH with a semimicro combination pH electrode (Accu-pHast, Fisher Scientific). A correction factor was independently determined for each batch of BCECF-loaded cells to offset the red shift undergone by the dye inside the cells (20, 25).

**Results**

We have monitored the effects of phorbol ester stimulation of PKC on capactative Ca2+ entry activated by the addition of the endosomal Ca2+-ATPase inhibitor thapsigargin. The increase in [\( Ca^{2+} \)], measured fluorometrically with indo-1 following the addition of 33 nm thapsigargin to cells suspended in NMG+-medium containing 1 mm Ca2+-, is shown in Fig. 1A. Thapsigargin treatment results in a marked increase in [\( Ca^{2+} \)], that declined somewhat to a sustained plateau level. At this stable value, the phorbol ester, PMA (100 nm) was added. Fig. 1A shows that the addition of PMA results in a modest reduction in [\( Ca^{2+} \)], consistent with either a minor decrease in Ca2+ entry or a PMA-mediated increase in Ca2+ efflux in accordance with the documented stimulation of the plasma membrane Ca2+- pump by phorbol esters (27, 28).

To address whether the small changes in [\( Ca^{2+} \)], occur as a result of changes in Ca2+- influx or by altered efflux mechanisms, we have monitored unidirectional Mn2+ influx as measured by indo-1 quench. When measured at the isosbestic wavelength, the rate of fluorescence decrease is insensitive to changes in the [\( Ca^{2+} \)], and is proportional to the rate of Mn2+ accumulation in the cytosol (15, 16). Representative experi-
ments are presented in Fig. 1B. Indo-1-loaded cells were exposed to 33 nM thapsigargin in NMG medium containing 1 mM Ca$^{2+}$ and 400 μM Mn$^{2+}$. The addition of thapsigargin resulted in a stimulated rate of decrease of indo-1 fluorescence, consistent with stimulation of Mn$^{2+}$ influx (Fig. 1B). The subsequent addition of 1 mM ionomycin, which can transport Mn$^{2+}$, abolished the remaining fluorescence. The addition of either 100 nM PMA or vehicle alone had no effect on the rate or magnitude of the fall in indo-1 fluorescence (Fig. 1B). To ensure that cells were exposed to PMA for a sufficient time to stimulate PKC, similar experiments were conducted in which cells were treated with PMA for 100 s prior to the addition of Mn$^{2+}$. As shown in Fig. 1C, exposure to PMA for 100 s, under conditions where [Ca$^{2+}$], is elevated due to capacitative Ca$^{2+}$ entry, failed to alter the rate or magnitude of Mn$^{2+}$ influx. Such results are not consistent with inhibition of the capacitative Ca$^{2+}$ entry pathway by PMA.

Given the lack of effect of phorbol esters, it was necessary to test the efficacy of this dose of PMA to stimulate PKC. To address this issue, identical experiments to those described above were conducted to monitor the activity of the Na$^{+}$/H$^{+}$ antiporter known to be present in this cell type (20). Previous studies have demonstrated the ability of phorbol ester-stimulated PKC activity to enhance basal activity of the Na$^{+}$/H$^{+}$ antiporter, resulting in modest alkalinization of cells (29–31), which can be inhibited either by PKC depletion (30, 31) or by pharmacological inhibitors of PKC (29, 31). The pH changes associated with the addition of PMA were measured using the pH-sensitive fluorescent dye BCECF. As is evident in Fig. 1D, the addition of PMA following activation of capacitative Ca$^{2+}$ entry with thapsigargin results in an increase in intracellular pH. In the absence of external Na$^{+}$ (equimolar substitution of Na$^{+}$ by NMG$^-$), no change in intracellular pH was detected following PMA addition (data not shown), consistent with the alkalization observed in Na$^{+}$-medium occurring as a consequence of increased Na$^{+}$/H$^{+}$ antiporter activity as previously reported (29–31). These data confirm the efficacy of PMA to stimulate PKC activity under our experimental conditions.

To directly address the question of whether the modest fall in [Ca$^{2+}$], seen in Fig. 1A was due to altered influx via the capacitative Ca$^{2+}$ entry pathway, we have measured unidirectional 45Ca$^{2+}$ uptake in thapsigargin-treated Bapta-loaded cells in the absence and presence of 100 nM PMA. Cells were incubated for 5 min at 37 °C in Ca$^{2+}$-free NMG$^-$ solution in the presence of 100 nM thapsigargin to ensure maximal depletion of intracellular Ca$^{2+}$ stores. Control cells were incubated under identical conditions in the absence of thapsigargin. Uptake was initiated by the addition of 4 mM Ca$^{2+}$ containing 10 μCi of 45Ca$^{2+}$, in the absence or presence of PMA. Fig. 2A shows the time course of 45Ca$^{2+}$ uptake under these experimental conditions (n = 4 experiments). Unidirectional uptake rates were calculated from the linear time course data presented in Fig. 2A and are shown in Fig. 2B. Unidirectional uptake rates show no effect of PMA in either thapsigargin-stimulated or control cells (Fig. 2B). To address the concern that the Ca$^{2+}$ chelator, Bapta, itself alters PKC activity or interferes with the actions of PMA, we have performed similar experiments in which cells were loaded with EGTA in place of Bapta and have found no significant effect of PMA on unidirectional Ca$^{2+}$ entry (data not shown). Taken in concert, these experiments are consistent with the hypothesis that activation of phorbol ester-sensitive PKC does not modulate thapsigargin-mediated Ca$^{2+}$ influx.

Furthermore, phorbol ester treatment does not alter basal plasma membrane Ca$^{2+}$ permeability in rat thymic lymphocytes. However, these data do not preclude the involvement of phorbol ester-insensitive serine/threonine phosphorylation/dephosphorylation events in capacitative Ca$^{2+}$ entry.

To further investigate the role of serine/threonine protein phosphorylation in the regulation of the capacitative Ca$^{2+}$ entry pathway, we have investigated the effects of okadaic acid, a potent type 1/2A phosphatase inhibitor (32), on this pathway. Fluorescence experiments were conducted to monitor the changes in [Ca$^{2+}$], in the presence and absence of okadaic acid (Fig. 3A). Following activation of the capacitative Ca$^{2+}$ entry pathway, reintroduction of 4 mM Ca$^{2+}$ to Bapta-loaded control cells resulted in an increase in [Ca$^{2+}$], from 172 ± 6 mM to 1023 ± 71 mM (± S.E., n = 12 experiments). In contrast, Ca$^{2+}$ uptake via capacitative Ca$^{2+}$ entry in cells treated with 500 nM okadaic acid for 30 min was markedly inhibited, showing an increase in [Ca$^{2+}$], to only 548 ± 21 mM from a resting value of 165 ± 6 mM (n = 12, p < 0.05) (Fig. 3A). To ensure that this inhibitory effect was due to the ability of okadaic acid to inhibit protein phosphatases and not some ancillary effect, identical experiments were performed using the related analogue norokadaone, which exhibits little or no inhibitory effect upon 1/2A phosphatase activity (33). Fig. 3A shows the results of such an experiment. These data illustrate the lack of effect of norokadaone upon the Ca$^{2+}$ entry pathway. Experiments were undertaken to address the possibility that prior incubation with okadaic acid interferes with the ability of thapsigargin to
deplete intracellular Ca\(^{2+}\) stores and hence, the activation of capacitative Ca\(^{2+}\) entry. Indo-1-loaded cells were treated with okadaic acid for 30 min in normal Na\(^+\) medium prior to exposure to thapsigargin in Ca\(^{2+}\)-free medium. Treatment with okadaic acid failed to alter the magnitude or kinetics of the transient increase in [Ca\(^{2+}\)]\(_i\), attributable to the release of the intracellular Ca\(^{2+}\) stores by thapsigargin (data not shown). These findings are consistent with the conclusion that okadaic acid does not interfere with the ability of thapsigargin to deplete intracellular Ca\(^{2+}\) stores and, hence, its ability to activate capacitative Ca\(^{2+}\) entry.

To exclude the possibility that the inhibition by okadaic acid of the increase in [Ca\(^{2+}\)]\(_i\), presented in Fig. 3A is due to stimulation of Ca\(^{2+}\) extrusion mechanisms, measurements of unidirectional Ca\(^{2+}\) uptake were performed. Bapta-loaded cells were incubated in basic Na\(^+\) medium for 30 min at 37 °C in the presence or absence of 500 nM okadaic acid. Cells were further incubated in the presence or absence of okadaic acid for 5 min at 37 °C in Ca\(^{2+}\)-free Na\(^+\) medium in the presence of 100 nM thapsigargin to ensure maximal depletion of intracellular Ca\(^{2+}\) stores. To investigate the effect of okadaic acid on basal Ca\(^{2+}\) influx, cells that were not treated with thapsigargin were incubated under identical conditions in the presence and absence of okadaic acid. In all cases, uptake was initiated by the addition of 4 mM Ca\(^{2+}\) containing 10 μCi of \(^{45}\)Ca\(^{2+}\). Fig. 3B shows the time course of \(^{45}\)Ca\(^{2+}\) influx upon readdition of 4 mM Ca\(^{2+}\) under these experimental conditions (n = 4 experiments). Uptake rates were calculated from the time course data and are presented in Fig. 3C. The unidirectional uptake rate accompanying activation of capacitative Ca\(^{2+}\) entry by thapsigargin was markedly reduced from a value of 830 ± 80 pmol/min/15 × 10⁶ cells (±S.E., n = 4) to a value of 160 ± 10 pmol/min/15 × 10⁶ cells (±S.E., n = 4, p = 0.05) in cells pretreated with okadaic acid. Control cells that were not exposed to thapsigargin, and hence displayed no stimulated capacitative Ca\(^{2+}\) entry, showed no difference in basal Ca\(^{2+}\) uptake rates between okadaic acid-treated and -untreated cells (Fig. 3B). The inhibitory effect of okadaic acid on capacitative Ca\(^{2+}\) entry is consistent with a requirement for sustained protein phosphatase activity for the continued activation of the Ca\(^{2+}\) influx pathway.

To further address this hypothesis we have investigated the effect of calyculin A, a phosphatase inhibitor structurally distinct from okadaic acid (32), on capacitative Ca\(^{2+}\) entry. A 30-min prior exposure to 100 nM calyculin A resulted in almost total inhibition of the changes in [Ca\(^{2+}\)]\(_i\), measured fluorometrically following reintroduction of 4 mM Ca\(^{2+}\) to thapsigargin-treated Bapta-loaded cells (Fig. 4A). Reintroduction of Ca\(^{2+}\) to cells following incubation with calyculin A resulted in an increase in [Ca\(^{2+}\)]\(_i\), to only 183 ± 12 nM from a resting value of 153 ± 12 nM, compared with the increase in [Ca\(^{2+}\)]\(_i\), seen in control cells, from a value of 166 ± 9 to 1011 ± 182 nM (n = 3, p = 0.05) (Fig. 3A). In agreement with the observations using okadaic acid, pretreatment of indo-1-loaded cells with calyculin A for 30 min failed to alter the magnitude or kinetics of the transient increase in [Ca\(^{2+}\)]\(_i\), attributable to the release of the intracellular Ca\(^{2+}\) stores by thapsigargin in Ca\(^{2+}\)-free medium (data not shown). These data indicate that calyculin A does not interfere with the ability of thapsigargin to deplete intracellular Ca\(^{2+}\) stores and, hence, its ability to activate capacitative Ca\(^{2+}\) entry. In addition, 30-min preexposure to 200 nM calyculin A in normal Na\(^+\) medium totally abolished thapsigargin-mediated \(^{45}\)Ca\(^{2+}\) influx (n = 3 experiments) (Fig. 4B). Uptake rates were calculated from the time course data and are presented in Fig. 4C. Control cells that were not exposed to thapsigargin, and hence displayed no stimulated capacitative Ca\(^{2+}\) entry, showed no difference in basal Ca\(^{2+}\) uptake rates between calyculin A-treated and -untreated groups (Fig. 4C).

To address the possibility that inhibition of capacitative Ca\(^{2+}\) entry by phosphatase inhibitors occurs only in Bapta-loaded conditions, we have conducted experiments in which cells were loaded with indo-1 alone, under conditions in which the intracellular Ca\(^{2+}\) pool was depleted with thapsigargin prior to the addition of calyculin A. Indo-1-loaded cells suspended in Ca\(^{2+}\)-free Na\(^+\) medium were treated with thapsigargin in the presence of 150 nM charybdotoxin. Charybdotoxin was added to eliminate the Ca\(^{2+}\)-dependent hyperpolarization...
brought about by the activation of Ca\(^{2+}\)-activated K\(^+\) channels, known to be present in these cells (18, 34). Following Ca\(^{2+}\) store depletion, cells were exposed to calyculin A for 100 or 500 s prior to the readdition of 1 mM Ca\(^{2+}\). Representative results from such experiments are shown in Fig. 5 and demonstrate a time-dependent inhibition by calyculin A of the increase in [Ca\(^{2+}\)]\(_i\), via capacitative Ca\(^{2+}\) entry. In addition to demonstrating a clear time dependence for the inhibitory effect of calyculin A, these data allow us to rule out any effect of Bapta loading in the interpretation of the present results.

In order to ensure that the inhibitory effect of phosphatase inhibitors was not directly attributable to a collapse of resting \(E_m\), experiments were performed using bis-oxonol to measure \(E_m\) in cells in the presence and absence of phosphatase inhibitors. Resting \(E_m\) in Bapta-loaded cells suspended in Ca\(^{2+}\)-free Na\(^+\) medium was not significantly different in control and inhibitor-treated cells (−59.3 ± 5.5 mV in okadaic acid treated cells and −58.0 ± 1.5 mV in paired control cells \((n = 3)\); −59.5 ± 2.6 mV in calyculin A-treated cells and −54.8 ± 2.1 mV in paired control cells \((n = 4)\)). On the strength of these results it is not possible to attribute the inhibitory effect of either of these agents to differences in \(E_m\).

Experiments were performed to investigate the effect of phosphatase inhibition on the depolarization previously attributed by our laboratory to electrogenic Ca\(^{2+}\) influx via capacitative Ca\(^{2+}\) entry (18, 19, 21). The capacitative Ca\(^{2+}\) entry pathway was activated in control cells and in cells treated with calyculin A (100 nM) for 10 min, and \(E_m\) was monitored fluorometrically using bis-oxonol. Fig. 6 shows representative \(E_m\) changes associated with the addition of 4 mM Ca\(^{2+}\) to control cells suspended in Ca\(^{2+}\)-free medium containing 150 nM charybdotoxin to inhibit Ca\(^{2+}\)-dependent K\(^+\) channel activity. Ca\(^{2+}\) addition to control cells resulted in a depolarization of 30.6 ± 5.5 mV \((n = 7)\). Consistent with the inhibitory effect of calyculin A on changes in [Ca\(^{2+}\)]\(_i\), and unidirectional Ca\(^{2+}\) uptake, the addition of 4 mM Ca\(^{2+}\) to calyculin-treated cells resulted in a significantly smaller depolarization of 10.0 ± 4.2 mV \((n = 4, p \leq 0.05)\) (Fig. 6). Okadaic acid treatment also significantly attenuated the depolarization observed upon readdition of Ca\(^{2+}\) to the external medium although to a lesser degree than that seen following calyculin A treatment, producing a maximum depolarization of 19.7 ± 1.5 mV \((n = 3, p \leq 0.05)\).

**DISCUSSION**

The present results are consistent with the regulation of capacitative Ca\(^{2+}\) entry by a dephosphorylation event in rat thymic lymphocytes. This conclusion is based upon the finding that the structurally distinct type 1/2A serine/threonine phosphatase inhibitors, okadaic acid and calyculin A, dramatically inhibit the Ca\(^{2+}\) influx induced by thapsigargin-mediated release of Ca\(^{2+}\) from intracellular stores. In addition, the \(E_m\) depolarization previously attributed to an increase in plasma membrane Ca\(^{2+}\) conductance following activation of capacitative Ca\(^{2+}\) entry (18, 19, 21) is markedly attenuated when cells are treated with okadaic acid or calyculin A. While it is not possible to completely rule out a direct inhibitory influence of these agents on capacitative Ca\(^{2+}\) entry independent of their
inhibitory effect on type 1/2A phosphatase activities, two pieces of evidence argue against such a postulate. First, nor-okadaone, a compound structurally related to okadaic acid but possessing little or no ability to inhibit type 1/2A phosphatase activity (33) has no effect on Ca\(^{2+}\) influx mediated via depletion of Ca\(^{2+}\) stores. Second, the degree of inhibition produced by calyculin A demonstrates a clear time dependence, inconsistent with a simple blocking effect of this agent on the Ca\(^{2+}\) influx pathway.

Interestingly, activation of phorbol ester-sensitive PKC activity by exposure to PMA had no effect on thapsigargin-mediated Ca\(^{2+}\) influx measured fluorometrically using Mn\(^{2+}\) as a Ca\(^{2+}\) surrogate or directly as unidirectional Ca\(^{2+}\) influx determined isotopically. This lack of effect was found despite the ability of PMA to induce a Na\(^{+}\)-dependent alkalization previously shown to be a result of PKC-stimulated Na\(^{+}/H^{+}\) antiport activity (29). Taken in concert, these data are consistent with the regulation of capacitative Ca\(^{2+}\) entry by phorbol ester-insensitive serine/threonine kinase activity. The marked and relatively rapid onset of inhibition of capacitative Ca\(^{2+}\) entry following the addition of calyculin A highlights a potentially critical point of modulation of this Ca\(^{2+}\) influx pathway. Interestingly, okadaic acid and calyculin A were ineffective in activating capacitative Ca\(^{2+}\) entry on their own. Such a result points to a important modulatory role of capacitative Ca\(^{2+}\) entry by type 1/2A protein phosphatase activity independent of the activation mechanisms. However, modulation of type 1/2A phosphatase activity by events associated with depletion of Ca\(^{2+}\) stores must not be ruled out.

While these data are consistent with an important modulatory role of a phorbol ester-insensitive serine/threonine kinase activity in the modulation of capacitative Ca\(^{2+}\) entry in thymic lymphocytes, alternative interpretations must be entertained.

Experiments were performed to ensure that the inhibitory effect of phosphatase inhibitors was not directly attributable to a collapse of resting \(E_{m}\). This is especially important in the light of the fact that capacitative Ca\(^{2+}\) entry occurs via an inwardly rectifying pathway in the range 0 to −53 mV in this cell type. As a result of this rectification, depolarization will have a marked inhibitory effect on Ca\(^{2+}\) influx. Measurements of \(E_{m}\) rule out this possibility, given that no difference in resting \(E_{m}\) in control or phosphatase inhibitor-treated cells was detected. The \(E_{m}\) values measured in the present experiments are similar to previously reported normal \(E_{m}\) values in rat thymic lymphocytes (18). Furthermore, such normal resting \(E_{m}\) values ensure that the observed inhibition cannot be explained on the basis of diminished cell viability following exposure to either okadaic acid or calyculin A.

The possibility exists that the inhibition of the capacitative Ca\(^{2+}\) entry pathway by phosphatase inhibitors occurs as a result of a direct inhibitory effect of these agents on the ability of thapsigargin to release Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores. Such a phenomenon has previously been reported in rabbit platelets (35). To address this issue, we have conducted experiments in which thapsigargin was added to cells in Ca\(^{2+}\)-free medium in the presence and absence of okadaic acid or calyculin A. In the absence of extracellular Ca\(^{2+}\) the magnitude of the transient rise in [Ca\(^{2+}\)]\(_{i}\) is proportional to the amount of Ca\(^{2+}\) released from the stores. We have found that a 30-min treatment with either calyculin A or okadaic acid failed to alter the magnitude or kinetics of the [Ca\(^{2+}\)]\(_{i}\) changes following the addition of thapsigargin. As a result, the inhibition seen after the addition of phosphatase inhibitors cannot be explained in terms of interference with the ability of thapsigargin to deplete intracellular Ca\(^{2+}\) stores.

Recent work from other laboratories has implicated protein phosphorylation in the control of capacitative Ca\(^{2+}\) entry (for review see Ref. 2). Such regulation may occur either at the level of the communication step between the depleted Ca\(^{2+}\) stores and the plasma membrane or at the Ca\(^{2+}\) entry pathway itself. There is, however, a great deal of discrepancy between the results obtained. For example, two studies have demonstrated an enhancement of capacitative Ca\(^{2+}\) entry following phorbol ester activation of PKC (6, 7). In these studies, phorbol esters stimulated capacitative entry in the pancreatic cell line, RINmF5 (7), while low doses of PMA enhanced Ca\(^{2+}\) influx in Xenopus oocytes (6). However, in the lymphocytic cell line Jurkat (8), human neutrophils (9) and peripheral T-lymphocytes (11), Drosophila photoreceptors (10), and rat basophilic leukaemia cells (12), phorbol ester activation of PKC has been reported to inhibit the capacitative Ca\(^{2+}\) entry pathway. These findings are in contrast to the present study, in which no effect of phorbol ester was found (see Figs. 1 and 2).

Previous work using type 1/2A phosphatase inhibitors has also yielded conflicting results. In accordance with an observed inhibition of the capacitative Ca\(^{2+}\) entry pathway by phorbol ester stimulation of PKC, an inhibition of this pathway by phosphatase inhibitors has been reported in human neutrophils (13). Such a finding is in agreement with the present study in which we demonstrate a dramatic inhibition of the capacitative Ca\(^{2+}\) entry pathway using identical phosphatase inhibitors. In contrast, Ca\(^{2+}\) influx mediated by the putative “Ca\(^{2+}\) influx factor” has been shown to be enhanced following treatment with type 1/2A phosphatase inhibitors that promote serine/threonine phosphorylation (3–5).

The present study indicates the existence of a PP1/2A-mediated dephosphorylation event in the maintenance of the capacitative Ca\(^{2+}\) entry pathway. In contrast to other cell types, this inhibition resulting from enhanced phosphorylation of serine/threonine residues is not subject to exacerbation by phorbol ester-sensitive serine/threonine kinases. These data could be explained by the existence of a phorbol ester-insensitive PKC isotype that exerts an inhibitory effect upon the Ca\(^{2+}\) influx pathway. Interestingly, the dominant isotypes of PKC in rat thymic lymphocyte are the “conventional” isotype \(\alpha\), and the phorbol-insensitive, “atypical,” isotype \(\zeta\), as determined by immunoblot analysis. Given the presence of the \(\zeta\) isotype, it is tempting to speculate on a role for this PMA insensitive kinase in the serine/threonine phosphorylation-mediated inhibition of capacitative Ca\(^{2+}\) entry as evidenced by its susceptibility to PP1/2A inhibitors. As a corollary, such a marked and rapid

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2 I. Marriott and M. J. Mason, unpublished observations.

3 B. S. Beckman, I. Marriott, and M. J. Mason, unpublished observations.
sensitivity of the pathway to phosphatase inhibitors may im-
ipple the control of phosphatase activity as being an impor-
tant site in the overall regulation of capacitative Ca\textsuperscript{2+} entry.

The data presented in this study suggest that modification of
phosphorylation of target protein(s) on serine/threonine amino
acid residues exerts an important modulatory effect upon the
capacitative Ca\textsuperscript{2+} entry pathway but that this phosphorylation
event does not occur as a result of phorbol ester-sensitive PKC
activity. Whether these results point to the existence of a
phorbol ester-insensitive PKC isotype that provides a potent
inhibitory influence on this pathway remains to be resolved,
and further studies are required to address this issue.

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Additions and Corrections

Vol. 271 (1996) 24164–24171

Inhibition of phospholipase D activity by fodrin. An active role for the cytoskeleton.

Sandra Lukowski, Marie-Christine Lecomte, Jean-Paul Mira, Philippe Martin, Huguette Gautero, Françoise Russo-Marie, and Blandine Geny

Page 24169, Table I: This table was printed incorrectly. The correct table is shown below:

| Enzymatic activity | No addition | + Fodrin (3 nM) |
|--------------------|-------------|----------------|
| Phospholipase A₂ (% of ³H-labeled amino acid release of total incorporated) | Basal level | 7.80 ± 0.83 | 2.30 ± 0.82 |
|  | + GTPγS (20 µM) | 12.25 ± 1.51 | 2.20 ± 1.06 |
| Phospholipase C (% of phosphoinositide hydrolysis in IP₂ + IP₃) | Basal level | 0.84 ± 0.17 | 0.64 ± 0.08 |
|  | + GTPγS (20 µM) | 5.43 ± 0.64 | 1.35 ± 0.39 |
| Adenylate cyclase (pmol/mg/min) | Basal level | 44.13 ± 0.26 | 47.77 ± 1.63 |
|  | + Mn²⁺ (1 mM) | 149.65 ± 1.90 | 142.37 ± 11.93 |

* As PLA₂ was almost not detectable in undifferentiated HL-60 cells, fodrin effect on PLA₂ activity was tested in cells previously differentiated for 2 days with dibutyryl cAMP (500 µM). Results are the mean ± S.E.

* Basal levels for PLA₂ and PLC represent the enzyme activity measured in the absence of GTPγS at pCa5 with Mg-ATP (2 mM).

* Results are mean ± S.E. IP₂, inositol 1,4-phosphate; IP₃, inositol 1,4,5-phosphate.

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Evidence for a phorbol ester-insensitive phosphorylation step in capacitative calcium entry in rat thymic lymphocytes.

Ian Marriott and Michael J. Mason

Pages 26734, 26735, and 26736, legends to Figs. 2, 3, and 4: p values quoted should read p ≤ 0.05 and not p ≥ 0.05 as stated in the legend.

Pages 26735 and 26736, legends to Figs. 3 and 4: Sentence beginning “C, unidirectional uptake rates derived from data presented in A” should read: “C, unidirectional uptake rates derived from data presented in B.”

Vol. 271 (1996) 7297–7300

Anti-Ig-induced calcium influx in rat B lymphocytes mediated by cGMP through a dihydropyridine-sensitive channel.

Amir A. Sadighi Akha, Nicholas J. Willmott, Kieran Brickley, Annette C. Dolphin, Antony Galione, and Simon V. Hunt

The first author’s name was listed incorrectly in the author index. The author’s surname is Sadighi Akha and should have been cited as Sadighi Akha, Amir A.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.