Reversible Phosphorylation as a Controlling Factor for Sustaining Calcium Oscillations in HeLa Cells: Involvement of Calmodulin-Dependent Kinase II and a Calyculin A-Inhibitable Phosphatase

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ABSTRACT: The role of reversible phosphorylation in histamine-induced Ca^{2+} oscillations in HeLa cells has been investigated by using various activators and inhibitors of protein kinases and phosphatases. Electroporation was employed to introduce impermeable materials into single cells, which proved to be a useful and convenient tool. Of the kinases examined, cAMP-dependent kinase, protein kinase C, and calmodulin-dependent kinase II (CaMK II), only CaMK II was essential. When added during oscillations, both W-7, a calmodulin antagonist, and KN-62, a specific CaMK II inhibitor, caused one large Ca^{2+} spike before halting the process. Introduction of the Ca^{2+}/calmodulin-independent catalytic domain of CaMK II into the cells forestalled their response to histamine. These results show that intracellular Ca^{2+} cannot oscillate when CaMK II is locked in either the inactive or the stimulated state. External Ca^{2+} electroporated into cells preloaded with the catalytic domains was quickly removed (but not when the cells were pretreated with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, tapsigargin), indicating that the ATP-driven Ca^{2+} pump was somehow activated by CaMK II. Protein phosphatase inhibitors calyculin A and okadaic acid abolished ongoing oscillations and, when added at low concentrations, prolonged the interspike interval. Immunoprecipitation experiments with $^{32}P_i$-labeled cells provided the first evidence that inositol 1,4,5-trisphosphate receptor (IP_3R) was phosphorylated by CaMK II *in vivo*. The extent of phosphorylation was increased in the presence of histamine, significantly enhanced by calyculin A, and greatly reduced by W-7. Our observations are consistent with the concept that repetitive phosphorylation-dephosphorylation cycles regulating IP_3R and Ca^{2+} pumps are a controlling factor for sustained Ca^{2+} oscillations in HeLa, and possibly other, cells.

Calcium oscillations, or spikes, observed in many eukaryotic cells signify the regulation of cellular processes by external stimuli like hormones, neurotransmitters, and growth factors in a frequency-modulated manner. Many an excellent review on this topic has appeared [e.g. Berridge (1993), Meyer and Stryer (1991), and Tsunoda (1991)], and several models based on positive or negative feedback of Ca^{2+} have been proposed (Cuthbertson & Chay, 1991; Goldbeter *et al.*, 1990; Harootunian *et al.*, 1991a; Keiser & Young, 1992; Meyer & Stryer, 1988; Parker & Ivorra, 1990; Payne *et al.*, 1990; Somogyi & Stucki, 1991; Woods *et al.*, 1987). Although the mechanisms involved are not wholly understood and seem to vary with cell types, a generally accepted sequence of events leading to Ca^{2+} oscillations has emerged, namely, the binding of an agonist to the membrane receptor, the ensuing activation of phospholipase C (PLC)[1] by GTP-binding protein (G-protein) or tyrosine kinase to produce the inositol 1,4,5-trisphosphate (IP_3) messenger, and the release of Ca^{2+} from, and its sequestration into, intracellular stores involving the IP_3 receptor (IP_3R) and the Ca^{2+}-ATPase pumps. How the oscillations are sustained remains unclear. In view of the rather invariant amplitude of Ca^{2+} transients, however, the essence of the regulatory mechanism must reside in the control of frequency. Presumably, holding the released Ca^{2+} at a constant level is necessary in order to elicit responses from various cooperative Ca^{2+}-binding components.

We set out to examine the role of reversible phosphorylation in the histamine-stimulated HeLa cells because one way to regulate the frequency of spiking or to achieve sustained oscillations is through repetitive phosphorylation−dephosphorylation cycles. IP_3R can be stoichiometrically phosphorylated *in vitro* by cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase, protein kinase C (PKC), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) (Ferris *et al.*, 1991; Komalavilas & Lincoln, 1994; Supattapone *et al.*, 1988; Volpe & Alderson-Lang, 1990), or undergo autophosphorylation (Ferris *et al.*, 1992). Phosphorylation of IP_3R by PKA had been reported to impair Ca^{2+} release from reconstituted vesicles (Supattapone *et al.*,

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[1] Abbreviations: CICR, calcium-induced calcium release; G-protein, GTP-binding protein; IP_3, inositol 1,4,5-trisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; IP_2, inositol 1,4-bisphosphate; IP_3R, IP_3 receptor; CaM, calmodulin; CaMK II, calmodulin-dependent kinase II; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; cAMP, adenosine-3′,5′-monophosphate; PMA, phorbol 12-myristate 13-acetate; STA, staurosporin; TG, tapsigargin; $(C_8)_2$cAMP, N6,O2′-dioctanoyladenosine 3′,5′-monophosphate; PLC, phospholipase C; DOG, *sn*-1,2-dioctanoylglycerol; DAG, diacylglycerol; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; AEBSF, 4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride.

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1988) or enhance Ca^{2+} release from membrane vesicles (Volpe & Alderson-Lang, 1990). Ca^{2+}-ATPase associated with sarcoplasmic reticulum is known to be activated when phospholamban is phosphorylated by CaMK II or PKA (Inui *et al.*, 1986; James *et al.*, 1989). Furthermore, various kinase activators and inhibitors such as forskolin, adenylate cyclase, phorbol ester, the calmodulin (CaM) antagonist W-7, etc., reportedly abolished ongoing Ca^{2+} oscillations (Hill *et al.*, 1988; Tsunoda *et al.*, 1990; Uneyama *et al.*, 1993; Zhao *et al.*, 1990). Ca^{2+}/CaM-dependent kinase and phosphatase also had been reported to control the release of Ca^{2+} from intact (Hill *et al.*, 1988) or permeabilized cells (Zhang *et al.*, 1993). It is important to identify the key players and to find out where and how they exert their effects. HeLa cells were chosen as the system for investigation for the following reasons. First, more than 80% of the cells consistently exhibited Ca^{2+} oscillations in response to the stimulation of histamine. Second, as we shall show later, oscillations in HeLa cells can be studied by focusing on the IP_3-sensitive stores, independent of considerations about the influx of extracellular Ca^{2+} and the ryanodine-sensitive Ca^{2+} stores (i.e. the Ca^{2+}-induced Ca^{2+} release, CICR).

To introduce compounds that cannot penetrate the cell membrane, we used the method of electroporation. Cells subjected to properly controlled electroporation, when resealed, were capable of normal oscillations. Electroporation also permits the introduction of materials into a cell population instead of single cells. We found it to be a useful alternative to the more commonly used microinjection.

We report here that periodic phosphorylation-dephosphorylation involving CaM-dependent kinase II and a calyculin A and okadaic acid-inhibitable protein phosphatase is vital to sustaining Ca^{2+} oscillations in HeLa cells. These enzymes also seem to participate in the regulation of the interval between spikes. It is possible that such reversible phosphorylation coordinating the functions of IP_3R and the Ca^{2+}-ATPase pump is a common mechanism by which oscillations in different cells are maintained.

EXPERIMENTAL PROCEDURES

Materials. PMA, STA, TG, forskolin, ryanodine, calmodulin binding domain from CaMK II (LKKFNARRKLKGAILTTMLA), and $(C_8)_2$cAMP were purchased from Calbiochem. H-89, KN-62, calyculin A, and okadaic acid were obtained from Biomal Research Lab, Inc., and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was purchased from Sigma. Polyclonal anti-IP_3R antibody was kindly provided by Dr. K. P. Campbell, University of Iowa College of Medicine, Iowa City; PKA inhibitor protein by Dr. S. Taylor, University of California, San Diego; the cloned monomeric CaMK II by Dr. J. Hurley, NIDDK, NIH; FK506 by Dr. C. Klee, NCI, NIH; and Ro 31-7549 by Dr. M. A. Beavens, NHLBI, NIH.

Culture of HeLa Cells. HeLa cells seeded on plastic dishes were grown in 90% minimum essential medium (Gibco) with nonessential amino acids, Earle's Salts, and L-glutamine, with 10% fetal bovine serum, at 37 °C, and in a humidified atmosphere of 5% CO_2 and 95% air.

Loading with Fluo-3 and Measurement of Ca^{2+} Signal. HeLa cells were detached from dishes by 0.25% trypsin (Gibco) with 1 mM EDTA for 2 min and then washed with and resuspended in buffer A [10 mM Hepes buffer (pH 7.4) containing 121 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5 mM D-glucose]. The cells were washed three times after 0.5 h of incubation at 37 °C with 2 μM fluorescent Ca^{2+} indicator, Fluo-3/AM (Molecular Probes, Inc.), and resuspended in fresh buffer. They were either used immediately or kept at 4 °C for later use. A fluoromicroscope linked to an image analysis setup was used for monitoring Ca^{2+} signals in single HeLa cells at excitation and emission wavelengths of 480 and longer than 510 nm, respectively. Cell images were recorded on a videotape and then transferred to a computer for data processing; the light intensity of each frame was calculated by the computer. Because of dye bleaching by light, the fluorescence intensity of Fluo-3 decreased with time. The decrease, however, followed a precise first-order kinetic process. Thus, all the fluorescence intensities in the figures have been corrected for this time-dependent loss using the experimentally determined first-order rate constants.

Electroporation. The cell-impermeable materials like cAMP, PKA inhibitor protein, CaM binding domain, CaMK II catalytic domain, and Ca^{2+} were electroporated into HeLa cells [equipment described in Tekle *et al.* (1991)]. The lengths of the square pulses with a constant electric field strength of 1.1 kV/cm were 200 μs, except that 500 μs was used for PKA inhibitor protein and CaMK II catalytic domain. The efficiency of electroporation varies with the size, charge, and structure of the material. For small molecules, using varying concentrations of [^{3}H]IP_3 up to 40 μM, the amount electroporated into the cells was about 10% of that present in the bath at 1.2 kV/cm, 200 μs. Using fluorescein-labeled IgG (1 mg/mL, MW = 150K) as a sample of a large protein molecule, we found the efficiency to be ~0.3% at 1.2 kV/cm, 500 μs. Since it is not feasible to determine the efficiency of electroporation for every compound, only the bath concentration is given for each experiment.

Preparation of CaMK II Catalytic Domains. Preparation and assay for CaMK II catalytic domains were performed as described by Kwiatkowski *et al.* (1990). Briefly, the cloned monomeric CaMK II (147 μg/mL) (Takeuchi-Suzuki *et al.*, 1992) was autophosphorylated at 0 °C for 1 h in 50 mM Hepes (pH 7.5) containing 0.1 mM dithiothreitol (DTT), 1mM ATP, 3.7 μM CaM, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM EDTA, 9% glycerol, and 0.2% Tween 40. Trypsin (type I, 2 μg/mL, Sigma) was then added to proteolyze CaMK II at room temperature. After proteolysis was terminated by the addition of trypsin inhibitor-agarose (Sigma), centrifugation (5000*g*, 30 min) was applied to remove trypsin and its inhibitor. The activities of CaMK II catalytic domains obtained at different proteolysis times were tested with syntide and [γ-^{32}P]ATP in Ca^{2+} free buffer with 0.1 mM EDTA. Due to the 10−60 min limited proteolysis, the total activity was about 2.5-fold of that of intact enzyme, which was in good agreement with the results of Kwiatkowski and King (1989).

Preparation and Solubilization of IP_3R-Associated Microsomes. After 4 h of incubation with 0.5 mCi [^{32}P]-orthophosphate at 37 °C, the HeLa cells were washed three times and resuspended in buffer A, plus protease inhibitors [8 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM leupeptin, and 0.1 mM 4-(2-aminoethyl)-benzenesufonyl fluoride (AEBSF)], 1 mM DTT, and 20 mM sodium orthophosphate. The cells were then treated with

various compounds like histamine, calyculin A, and W-7. After 5 min of incubation, the cells were homogenized, and 5 mM EDTA was added to the homogenate immediately. The homogenate was spun at 5000*g* for 20 min. The supernatant was then centrifuged at 300000*g* for 1 h, and microsomal membranes were obtained from the pellet. Solubilization of the microsomal membranes was carried out in buffer A with 1% Triton X-100 (Joseph & Samanta, 1993). The mixture was incubated on ice for 1 h, followed by an additional centrifugation at 300000*g* for 1 h. The final supernatant was saved and used for immunoprecipitation of IP_3R.

Immunoprecipitation of IP_3R. The ^{32}P-labeled IP_3R-containing extracts (50 μL) were first absorbed by 50 μL of rabbit serum−agarose (Sigma) through a 1.5 h incubation in 400 μL of immunoprecipitation buffer [10 mM Tris (pH 7.8), 0.32 M sucrose, 1 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 8 mM benzamidine, 0.5 mM PMSF, 0.1 mM leupeptin, 0.1 mM AEBSF, 0.4 mg/mL soybean trypsin inhibitor, 0.9 mg/mL pepstatin A, 0.9 mg/mL antipain, and 1.2 mg/mL aprotinin]. After removal of rabbit serum-agarose, 20 μL of anti-IP_3R (Parys *et al.*, 1992) or preimmune serum was added and the mixture rotated for 2 h at 4 °C. The samples were then mixed with 50 μL of protein A-agarose (Sigma) which had been prewashed with immunoprecipitation buffer three times and incubated for an additional 2 h at 4 °C. The supernatants were removed by centrifugation, and the pellets were washed with cold immunoprecipitation buffer six times, dissolved in 40 μL of SDS gel sample buffer, and boiled for 5 min. The final supernatants were used to run SDS-PAGE. The SDS gel was scanned by a phosphoimager to locate the ^{32}P-labeled protein bands.

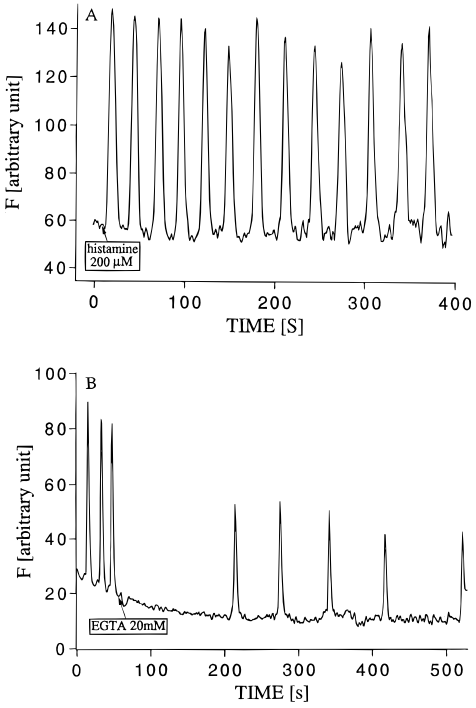


FIGURE 1: Histamine-induced Ca^{2+} oscillations in single HeLa cells. Cell preparation and measurement of fluorescence intensity (*F*) are as described in Experimental Procedures. (A) The arrow indicates when the cells were exposed to 200 μM histamine. This agonist concentration was maintained in the bath throughout the experiment. The zero time refers to the time point at which monitoring of the cells was started. Unless otherwise stated, the buffer used contains 1.8 mM Ca^{2+}. (B) Ca^{2+} oscillation was initiated by 200 μM histamine prior to the addition of 20 mM EGTA (at arrow) to chelate Ca^{2+} (1.8 mM) in the buffer. To avoid pH changes due to the chelation, 50 mM (instead of 10 mM) Hepes buffer was used in this experiment.

RESULTS

Histamine-Induced Ca^{2+} Oscillations in HeLa Cells: Effects of Removal of Extracellular Ca^{2+} and Addition of Ryanodine. In single HeLa cells, histamine induced repetitive Ca^{2+} spikes whose frequency increased with the agonist concentration. Above 50 μM histamine, however, the frequency became histamine dosage-independent. For example, the average frequency (per minute, $n = 10$) for the cell lot used in the experiment shown in Figure 1A was 1 ± 0.4 at 5 μM histamine, 1.4 ± 0.3 at 25 μM, 1.7 ± 0.3 at 50 μM, and 1.7 ± 0.5 at 200 μM. Figure 1A shows representative Ca^{2+} responses of a single HeLa cell stimulated with 200 μM histamine. The amplitudes are quite homogeneous, and each spike rises and falls back to the base line. Upon addition of 20 mM EGTA to the bath to chelate the 1.8 mM Ca^{2+} present in the buffer, the ensuing spike was delayed but the oscillations continued for minutes with smaller amplitudes and a lower frequency (Figure 1B). This observation indicates that, while extracellular Ca^{2+} has an effect on the oscillations, its absence is not critical on a short time scale. Similar observations had been reported for HeLa cells by Bootman *et al.* (1992b). In carbamylcholine-stimulated insulinoma cells, Ca^{2+} oscillations also occurred in the absence of external Ca^{2+} (Prentki *et al.* 1988).

The existence of IP_3-sensitive Ca^{2+} stores in HeLa cells has been reported by Bootman *et al.* (1992a). To examine whether the CICR mechanism also operates in HeLa cells, we checked the effect of 40 μM ryanodine or 10 mM adenine either by incubating it with HeLa cells for over 30 min prior to histamine stimulations or by adding it directly to oscillating cells. In either case, no effect on oscillations was detected. Furthermore, binding experiments according to the method of McPherson and Campbell (1990) showed no binding of [^{3}H]ryanodine to the membranous fractions of HeLa cells.

The above experiments demonstrated that the basic mechanism of Ca^{2+} oscillations in HeLa cells can be studied by concentrating on the IP_3-sensitive pool without considering the ryanodine-sensitive Ca^{2+} pool and influx of external Ca^{2+}.

Role of cAMP-Dependent Protein Kinase. To examine the role of the major kinases known to phosphorylate IP_3R, we employed their activators and inhibitors as probes. The effect of PKA on Ca^{2+} oscillations has been reported for other systems, but its role in HeLa cells is not known. The cell-permeable activators, N6,O2-dioctanoyladenosine 3,5-monophosphate [$(C_8)_2$cAMP, 10 μM], and forskolin (4 μM), an activator of adenylate cyclase, were preincubated with HeLa cells for 10 min before addition of 100 μM histamine. Normal oscillations comparable to those of untreated cells were observed. Forskolin (4 μM) added to the bath during oscillations also produced no effect. H-89, a specific inhibitor of PKA with an IC_{50} of 48 nM (Chijiwa *et al.*, 1990) likewise had no effect on ongoing oscillations at a bath concentration of 1 μM.

The cell-impermeant effectors were electroporated into cells by the procedure described in Experimental Procedures. It should be noted that the efficiency of electroporation varied

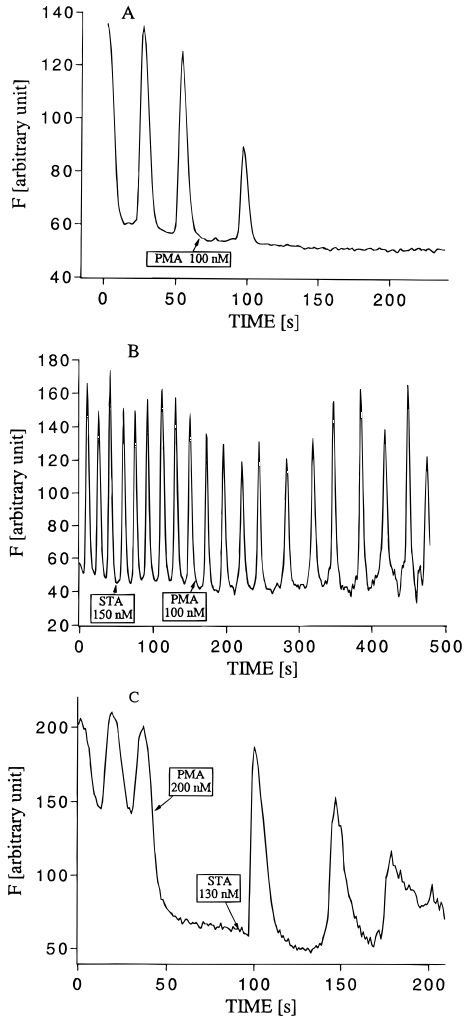


FIGURE 2: Effect of PKC on Ca^{2+} oscillations in HeLa cells. (A) Inhibition of Ca^{2+} oscillations by PMA. Ca^{2+} oscillation was started by 200 μM histamine; 100 nM PMA was then added. (B) Lack of effect of STA and partial inhibition by PMA. Ca^{2+} oscillation was initiated by 200 μM histamine, followed by the addition of 150 nM STA at the first arrow and then followed by the addition of 100 nM PMA at the second arrow. (C) Reversal of PMA inhibition by STA. The HeLa cell was stimulated by 200 μM histamine, followed by the addition of 200 nM PMA and 130 nM STA at different time points (indicated by arrows).

with each material. Cells electroporated with either cAMP (1 mM in cuvette) or the 26K PKA inhibitor protein (40 nM in cuvette, K_i = 0.22 nM) also showed normal Ca^{2+} oscillations in response to stimulation by histamine.

Effect of PKC and Its Specific Inhibition by Staurosporin. PKC has been proposed to play a key role in Ca^{2+} oscillations (Keizer & Young, 1991; Woods *et al.*, 1987) because diacylglycerol (DAG), the coproduct of IP_3, and Ca^{2+} can activate PKC which, directly or indirectly, suppresses the activity of PLC (Cockcroft & Gomperts, 1985; Ryu *et al.*, 1990). In the case of HeLa cells, 100 nM phorbol 12-myristate 13-acetate (PMA), a potent exogenous activator of PKC, generally abolished Ca^{2+} oscillations with one (or two) more delayed spike(s) with a smaller amplitude (Figure 2A). On the other hand, a potent PKC inhibitor, staurosporin (STA, IC_{50} = 0.7 nM), did not inhibit ongoing Ca^{2+} oscillations at 150 nM, and histamine was able to steadily induce normal Ca^{2+} oscillations in HeLa cells preincubated with STA. When 150 nM STA was added during spiking prior to the addition of a suitable concentration of PMA (100 nM), the latter seemed to partially activate PKC; Ca^{2+} oscillations were not inhibited, but the frequency was slightly decreased (Figure 2B). When the order of addition was reversed, oscillations were first abolished by 200 nM PMA and then restored by 130 nM STA with an altered pattern (Figure 2C). Similar observations have been reported by Tsunoda *et al.* (1990), Uneyama *et al.* (1993), and Zhao *et al.* (1990). STA has also been shown to inhibit PKA (K_i = 7 nM) (Tamaoki *et al.*, 1986) and CaMK II (IC_{50} = 20 nM; Yanagihara *etal.*, 1991) *in vitro*. But we found that it does not seem to penetrate beyond the plasma membrane of HeLa cells. Therefore, it is specific for PKC and has no effect on these cytosolic enzymes. Only when STA was electroporated into HeLa cells did it interrupt Ca^{2+} oscillations, presumably by inhibiting CaMK II (see next section).

Another highly specific inhibitor of PKC, Ro31-7549 (K_i = 70 nM) (Elliott *et al.*, 1990; Ozawa *et al.*, 1993), behaved in a manner similar to that of STA. It had no effect on Ca^{2+} oscillations at bath concentrations up to 10 μM.

Dioctanylglycerol (DOG), a DAG-like activator of PKC (Ganong *et al.*, 1986), when used to mimic the endogenous DAG only slightly increased the interval between spikes at concentrations up to 100 μM with little effect on the amplitude. It seems that DOG, unlike PMA, cannot effectively activate PKC in HeLa cells.

Effects of CaMK II on IP_3R and the ER Ca^{2+}-ATPase. Because of its activity dependence on Ca^{2+} and CaM, the broad specificity CaMK II is a likely candidate for feedback regulation of Ca^{2+} oscillations. W-7 (50−200 μM), a Ca^{2+}-dependent antagonist of CaM, when added during oscillations, caused a large burst of Ca^{2+} that was sometimes followed by a smaller, continuous Ca^{2+} release (Figure 3A). Similarly, HeLa cells preloaded with W-7 or the 20-residue CaM binding domain (100 μM) from CaMK II by electroporation produced a large Ca^{2+} burst upon histamine stimulation. To ascertain that the effects of these inhibitors were due to their action on CaMK II and not due to inhibitions of other CaM-dependent enzymes, we used a highly specific inhibitor of CaMK II, KN-62 (bath concentration = 10 μM, K_i = 0.9 μM, K_i for PKA and PKC are all >100 μM) (Tokumitsu *et al.*, 1990), which exhibited identical effects on oscillating cells (Figure 3B). Since W-7 should have inhibited the CaM-dependent phosphatase, calcineurin, and we did not detect any inhibition of calcineurin by KN-62 (up to 10 μM) in assays using *p*-nitrophenyl phosphate as substrate, the fact that W-7 and KN-62 gave the same results also shows that calcineurin played no role in HeLa cell Ca^{2+} oscillations. This observation is supported by our experiment showing the lack of any effect on oscillations by 2 μM FK506 (Liu *et al.*, 1991) which, when complexed with its binding protein, becomes a potent and specific inhibitor of calcineurin.

The effects of W-7, CaM binding domain, and KN-62, however, were different from those of thapsigargin (TG) which selectively and irreversibly inhibits the endoplasmic reticulum (ER) Ca^{2+}-ATPase pump (Thastrup *et al.*, 1990). As can be seen from Figure 3C, TG added during Ca^{2+} oscillations gave rise to a sustained Ca^{2+} release and abolished further Ca^{2+} oscillations. Moreover, neither W-7 nor KN-62 had an effect on resting cells, though upon addition of histamine, these cells produced a large Ca^{2+} peak, demonstrating that the Ca^{2+} release mechanism of IP_3R was not blocked. TG upon addition induced a Ca^{2+} leak in

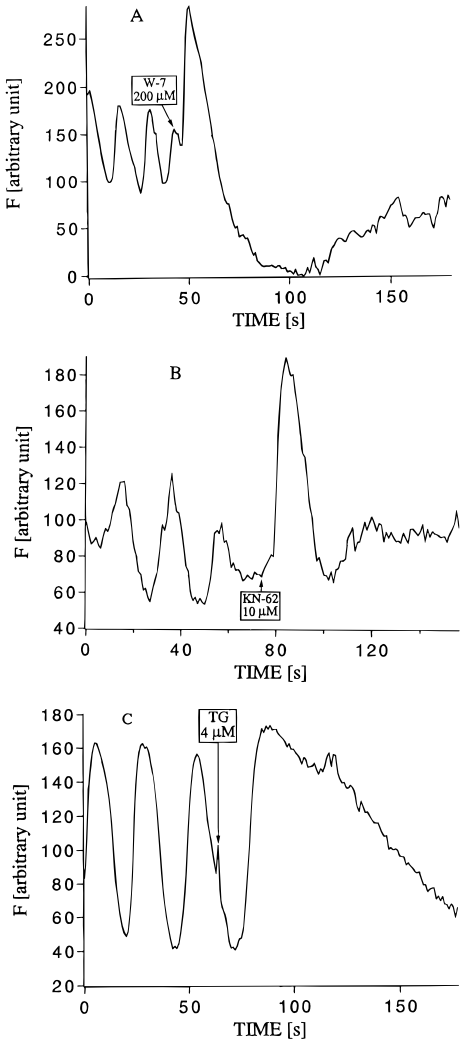


FIGURE 3: Abolishment of ongoing Ca^{2+} oscillations by W-7, KN-62, and TG. Ca^{2+} oscillation was started by 200 μM histamine. (A) 200 μM W-7, (B) 10 μM KN-62, and (C) 4 μM TG were added at points indicated by arrows.

resting cells. The observations are consistent with the idea that W-7 and KN-62 inhibit CaMK II which is largely inactive at the low Ca^{2+} level of resting cells, while TG inhibits the basal activity of the ER Ca^{2+}-ATPase pump.

To verify the involvement of CaMK II in Ca^{2+} oscillations, the HeLa cells were electroporated with CaMK II catalytic domain (4.2 μM in cuvette) prepared by limited proteolysis. The catalytic domain is active in the absence of Ca^{2+}/CaM. Of the cells so treated, 82% ($n = 84$) lost any detectable Ca^{2+} signal when stimulated by 200 μM histamine; meanwhile, 81% of the cells ($n = 42$) used for control (exposed to the same electric field without, or with, boiled CaMK II catalytic domain) gave normal Ca^{2+} spikes in response to the same level of histamine.

CaMK II has been reported to activate the sarcoplasmic reticulum Ca^{2+}-ATPase pump through phosphorylation of phospholamban (Carafoli & Chiesi, 1992). The plasma Ca^{2+}-ATPase pump, on the other hand, is directly activated by CaM (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977). Whether CaM/CaMK II also regulates the Ca^{2+}-ATPase pumps in HeLa cells was examined by direct electroporation of Ca^{2+} into HeLa cells loaded with Fluo-3. In the absence of W-7, the intensity of fluorescence burst immediately upon application of the electrical pulse (Figure

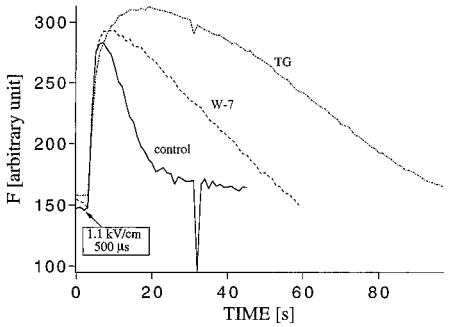


FIGURE 4: Effect of W-7 and TG on Ca^{2+} signals induced by electroporation of external Ca^{2+} into the cell. Electroporation was applied to Fluo-3-loaded cells suspended in buffer A, containing 200 μM $CaCl_2$ in the presence of 100 μM W-7 (dashed line) or 5 μM TG (dotted line). The solid line is the control (in the absence of W-7 and TG). The electroporation procedure is as described in Experimental Procedures.

4, solid line), reaching a maximum in about 3 s and returning to the base line in about 10 s. This pattern indicates that (1) openings for Ca^{2+} influx as a result of the electrical pulse are made and closed at the cell plasma membrane in a few seconds and only a limited amount of Ca^{2+} gets into the cell and (2) Ca^{2+} electroporated into the cytosol is quickly removed, possibly partially into intracellular stores by the ER Ca^{2+} pump and partially out of the cell by the plasma Ca^{2+} pump. In the presence of W-7, removal of Ca^{2+} from the cytosol was considerably slowed as shown in Figure 4 (dashed line), confirming that CaM-dependent processes are essential to the activation of the Ca^{2+}-ATPase pumps in HeLa cells. Participation of the ER Ca^{2+}-ATPase was shown by the experiment in which the cells, after preincubation with TG to inhibit the ER Ca^{2+}-ATPase and deplete the Ca^{2+} stores, were rather slow in removing the electroporated Ca^{2+} (Figure 4, dotted lines).

Similar Ca^{2+} electroporation experiments were carried out with cells that have been preloaded with the Ca^{2+}/CaM-independent CaMK II catalytic domain (4.2 μM). At an external Ca^{2+} level of 200 μM, these cells (23 out of 25) gave little or no Ca^{2+} signals (solid line in Figure 5) while the control cells (24 out of 28) displayed a typical burst (dotted-and-dashed line in Figure 5). Introduction of the active CaMK II domain did not damage the ER Ca^{2+}-ATPase since addition of TG to HeLa cells so-treated resulted in a sustained Ca^{2+} release. Moreover, HeLa cells (10 out of 10) preloaded with CaMK II catalytic domain and pretreated with 5 μM TG for 20 min to inhibit the ER pump were unable to remove electroporated Ca^{2+} quickly (dotted line, Figure 5). These results confirm the idea that the ER Ca^{2+}-ATPase, when preactivated by CaMK II, quickly pumps the influxed Ca^{2+} into intracellular stores. It should be noted that raising the external Ca^{2+} to higher concentrations, as expected, resulted in increasingly larger Ca^{2+} bursts since the pumps, even when activated, have finite capacities.

It is clear from the above experiments that CaMK II is an indispensable link in maintaining repetitive Ca^{2+} spikes in HeLa cells. When it is inhibited by W-7, the CaM binding domain, or KN-62, or when its presence is "over expressed" by the introduction of CaMK II catalytic domain, the delicate regulation of its activity, which in turn regulates the IP_3R and the ER Ca^{2+}-ATPase, is disturbed and Ca^{2+} oscillations are interrupted.

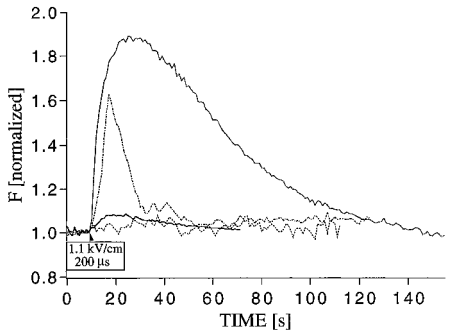


FIGURE 5: Suppression of Ca^{2+} signals by CaMK II catalytic domain in HeLa cells. HeLa cells were first electroporated with 4.2 μM CaMK II catalytic domain according to the method described in Experimental Procedures. The cells were then transferred into buffer A containing 200 μM Ca^{2+}. A second electroporation was applied with a pulse length of 200 μs and a field strength of 1.1 kV/cm to trigger the entry of Ca^{2+}. The dashed-and-dotted line represents the result typical of control with (or without) boiled CaMK II active domain (24 out of 28 cells). The solid line is an example of suppressed Ca^{2+} signals (due to rapid removal) in cells (23 out of 25 cells) preloaded with 4.2 μM CaMK II catalytic domain. The dotted line shows results from cells (10 out of 10) preloaded with CaMK II catalytic domain and treated with 5 μM TG for 20 min to inhibit ER Ca^{2+}-ATPase prior to Ca^{2+} electroporation. The dashed line shows a control electroporation experiment in Ca^{2+} free buffer without CaMK II catalytic domain.

The effects of inhibitors on CaMK II and the CaMK II catalytic domain on histamine-induced Ca^{2+} oscillations are summarized in Table 1.

Effects of Phosphatase Inhibitors Calyculin A and Okadaic Acid. If phosphorylation by CaMK II is vital to maintaining Ca^{2+} oscillations, then dephosphorylation must be equally important. Two cell-permeable inhibitors specific for types 1 and 2A phosphatases, calyculin A and okadaic acid (Cohen *et al.*, 1990), were employed to test this concept. Calyculin A was found to affect Ca^{2+} oscillations in a dosage-dependent manner. At a bath concentration of >200 nM, it generally halted ongoing Ca^{2+} oscillations (Figure 6A), sometimes after a single delayed, smaller spike in a way analogous to that observed with PMA (Figure 2). Interestingly, lower doses of calyculin A led to an altered pattern of Ca^{2+} oscillations, a longer interspike interval and a lower amplitude (Figure 6B). Ca^{2+} oscillations were not perturbed if the bath concentration of calyculin A was lower than 20 nM. When the cells were preincubated with 100 nM calyculin A for 5 min, histamine was unable to elicit any response. Okadaic acid had a similar effect, but higher doses (>2 μM) were needed to abolish ongoing Ca^{2+} oscillations. Addition of 100 nM okadaic acid to ongoing oscillations also led to longer intervals between spikes (data not shown). Although the higher dosage of calyculin A and okadaic acid needed for inhibition may suggest that the phosphatase likely falls in the type 1, rather than the type 2, category (Bialojan & Takai, 1988; Ishihara *et al.*, 1989), our data do not permit a clear distinction between the two.

Phosphorylation of IP_3R by CaMK II In Vivo. To answer the question of whether phosphorylation of IP_3R by CaMK II actually occurred during histamine-induced Ca^{2+} oscillations in HeLa cells, we prepared cells labeled with $^{32}P_i$ under varying experimental conditions. Figure 7 shows the ^{32}P-labeled protein bands from microsomal fractions of HeLa cells that were immunoprecipitated by anti-IP_3R antibody (see Experimental Procedures for details) on a 4 to 12% SDS gradient gel; the location of IP_3R is indicated by an arrow. Compared with the control (lane C, without histamine), histamine clearly enhanced phosphorylation of IP_3R (lane E), indicating that the extent of phosphorylation of IP_3R was increased during Ca^{2+} oscillations. When IP_3R was exposed to W-7, and pretreated with histamine (lane D), the intensities of the IP_3R bands were essentially the same as that of the control. Lane F confirms that the presence of calyculin A significantly increased IP_3R phosphorylation. Addition of okadaic acid gave similar results (data not shown). These findings clearly show that a type 1 (or a type 2A) phosphatase is responsible for dephosphorylation of IP_3R. In a separate experiment, the microsomal fractions of HeLa cells were run on SDS gels and the IP_3R bands localized by Western immunoblotting (data not shown). The results were identical to those shown in Figure 7.

DISCUSSION

After screening several cell types, we chose the HeLa cell for studying Ca^{2+} oscillations because it represents a typical IP_3-induced Ca^{2+} release system. This notion is supported by the insensitivity of HeLa cells to ryanodine and adenine and the lack of binding of ryanodine to the membranous preparations, which indicate that the CICR mechanism is not operative in this cell line. The fact that Ca^{2+} oscillations continued for minutes after removal of external Ca^{2+} also is inconsistent with the CICR model which requires replenishment of the Ca^{2+} stores from external Ca^{2+} sources for sustained oscillations.

To introduce impermeable materials into the cells, we adopted the method of electroporation. With the experimental conditions established by us, 200−500 μs at 1.1 kV/cm, we did not encounter detectable adverse effects. In control experiments, cells that were subjected to electroporation and allowed to reseal gave normal oscillating patterns when stimulated with histamine. The cell membrane also appeared to open and reseal rather quickly as shown by the small amount of Ca^{2+} electroporated into the cell by a 500 μs pulse shown in Figure 4. Thus, electroporation is a versatile alternative to other methods like microinjection for the following reasons. (1) Since the desired components can be delivered into many cells, one can scan the cells and select the representative ones for monitoring. (2) The fate of electroporated compounds that have no readily detectable signals or indicators can be followed as a population by disrupting the cells at different time intervals and analyzing their contents. (3) Injury to the cell in well-controlled electroporation is considerably less than that in microinjection. (4) Technically, the method is easy to use once the device is set up.

To study the role of phosphorylation in histamine-induced Ca^{2+} oscillations, we examined kinases that have been implicated in previous reports, PKA, PKC, and CaMK II. PKA has been reported to promote Ca^{2+} mobilization in some cells but inhibit the process in some others. In a mouse fibroblast cell line (Horn *et al.*, 1991) and rat hepatocytes (Blackmore & Exton, 1986), cAMP raises the IP_3 level which in turn mobilizes Ca^{2+}. In guinea pig hepatocytes (Capiod *et al.*, 1991), cAMP generates Ca^{2+} fluctuation without changing the IP_3 level. However, cAMP inhibits Ca^{2+} release in renal JG cells (Kurts & Penner, 1989) and rat megakaryocytes (Uneyama *et al.*, 1993). In C_6BU1 cells

Table 1: Effects of Inhibitors and the Catalytic Domain of CaM-Dependent Kinase II on Histamine-Induced Ca^{2+} Oscillations in HeLa Cells[a]

addition	concentration[b] (μM)	experiment[c]	result[d]
inhibitors			
W-7 ($K_i = 30\ \mu$M)	50−200	added after stimulation	one Ca^{2+}burst (23/26)
		preincubation before stimulation	one Ca^{2+} burst (14/15)
		electroporation of external Ca^{2+}	removal of Ca^{2+} slower than control (17/20)
KN-62 ($K_i = 0.9\ \mu$M)	10	added after stimulation	one Ca^{2+} burst (10/10)
		preincubation before stimulation	one Ca^{2+} burst (6/7)
CaM binding domain of CaMK II ($K_i = 52$ nM)	100	electroporated before stimulation	one Ca^{2+} burst (12/14)
CaMK II catalytic domain	4.2	electroporated before stimulation	no oscillations or Ca^{2+} signal (69/84)
		catalytic domain electroporated before electroporation of external Ca^{2+}	Ca^{2+} rapidly removed (51/56)
		above experiment performed with cell pretreated with TG	Ca^{2+} slowly removed (20/20)

[a] Experimental conditions are as described in Experimental Procedures. [b] Refers to the concentration in the bath or cuvette. [c] Stimulation means addition of histamine. [d] Number of responses are given in parentheses. The total number of experiments were samples taken from hundreds of video recordings.

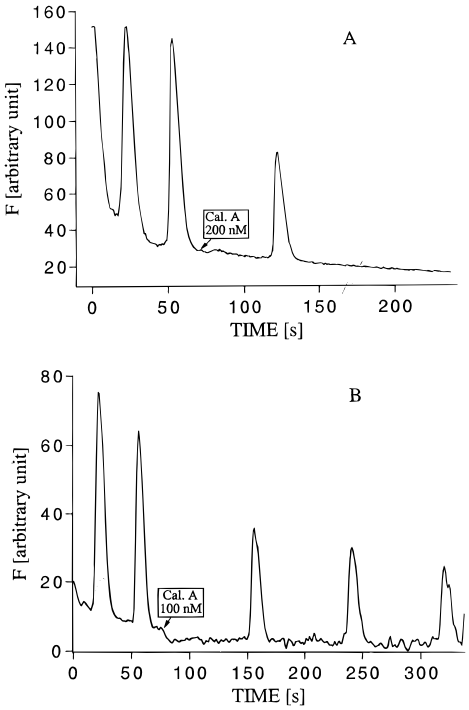


FIGURE 6: Effect of calyculin A on Ca^{2+} oscillations. Ca^{2+} oscillations were initiated by 200 μM histamine prior to the addition of 200 nM (A) or 100 nM (B) calyculin A at points indicated by arrows.

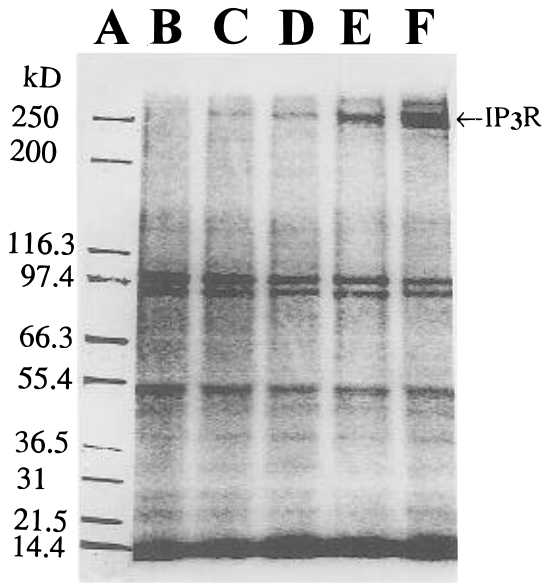


FIGURE 7: ^{32}P-labeled phosphorylation of IP_3R in intact cells treated with different effectors. The IP_3R-containing extracts from HeLa cells and the immunoprecipitation of IP_3R with polyclonal anti-IP_3R were described in Experimental Procedures. The precipitates were boiled for 5 min in SDS sample buffer, run on 4−12% SDS gels, and scanned on a phosphoimager: lane A, protein markers; and lane B, control cells (no addition of any effector) with preimmune serum. Lanes C−F are extracts immunoprecipitated with anti-IP_3R: lane C, control; lane D, cells incubated with 400 μM histamine for 5 min followed by 5 min of incubation with 200 μM W-7; lane E, cells incubated with 400 μM histamine for 5 min; and lane F, cells incubated with 200 nM calyculin A for 5 min.

(Kim *et al.*, 1989) and Jerkat cells (Park, *et.al.*, 1992), PKA phosphorylates PLC, thereby inhibiting IP_3 production. With HeLa cells, we used cAMP and forskolin as activators and H-89 and the 26K PKA inhibitor protein as inhibitors. We found no effect of these compounds on the histamine-induced Ca^{2+} oscillations. Therefore, we conclude that PKA does not take part in Ca^{2+} spiking in this cell line. Tsunoda (1990) likewise did not observe any PKA effect in pancreatic acinar cells.

Contrary to the diverse effects of PKA, PKC always decreases Ca^{2+} release in various cell types when activated by PMA (Tsunoda *et al.*, 1990; Uneyama *et al.*, 1993; Zhao *et al.*, 1990). But inhibition or down regulation of PKC does not hinder normal oscillations, indicating that PKC is nonessential (Harootunian *et al.*, 1991b). Our data confirm these observations and reaffirm the concept that PKC is not required for sustaining Ca^{2+} oscillations. PMA-activated PKC probably abolishes oscillations or lowers the frequency by inhibiting PLC through phosphorylation of the phospholipase and thereby altering its interaction with G-protein (Ryu *et al.*, 1990). Activation of the plasma Ca^{2+}-ATPase by PKC may also be the cause (Smallwood *et al.*, 1988). The inhibitory effect is not due to phosphorylation of IP_3R by PKC because (1) our data clearly showed that IP_3R phosphorylation was suppressed in the presence of W-7, a CaM antagonist that has no effect on PKC, and (2) a microsomal fraction of HeLa cells pretreated with staurosporin and histamine showed the normal extent of phosphorylation of IP_3R.[2]

[2] D. M. Zhu, P. B. Chock, and C. Y. Huang, unpublished data.

Our experimental results (Table 1) strongly suggest that CaMK II is the kinase actively involved in the control of Ca^{2+} oscillations in HeLa cells. Insertion of either inhibitors of CaMK II or its catalytic domain leads to abolition of oscillations; i.e. oscillations cease to operate when the kinase is locked in the inactive or stimulated state. Of the inhibitors used, W-7 combines with CaM in a Ca^{2+}-dependent manner; the CaM binding domain from CaMK II is specific for CaM. KN-62, on the other hand, at a bath concentration of 10 μM should be specific for CaMK II ($K_i = 0.9$ μM) since K_i values for PKA and PKC are greater than 100 μM. The fact that the catalytic domain of CaMK II was able to block Ca^{2+} oscillations further demonstrates that this CaM-dependent kinase likely is involved. CaMK II's role in activating the ER ATPase pump is shown by the ability of the CAMK II catalytic domains to quicken the removal of Ca^{2+} electroporated into the cell. Steinhardt *et al.* (1994) reported that CaMK II may function in cell membrane resealing. One may wonder whether the quick removal of Ca^{2+} was actually the result of rapid membrane sealing, i.e. little or no Ca^{2+} getting into the cell. This possibility is eliminated by our experiments showing that cells loaded with CaMK II catalytic domain were incapable of quick removal of electroporated Ca^{2+} if the ER ATPase had been inhibited by TG (Figure 5). All of our observations are consistent with the idea that, when Ca^{2+} is released, CaMK II is activated; phosphorylation of IP_3R then shuts off the Ca^{2+} channel, and the phosphorylation of a phospholamban-like component activates the ER Ca^{2+}-ATPase pump to refill the Ca^{2+} stores. Our experiments with IP_3R immunoprecipitated from cells labeled with $^{32}P_i$ (Figure 7) under stimulative and inhibitory conditions provided the first evidence that IP_3R is phosphorylated by CaMK II *in vivo* during oscillations. Such phosphorylation apparently impairs IP_3R's ability to extrude Ca^{2+} since cells preloaded with CaMK II active domain were unable to respond to histamine stimulation. This view is supported by the fact that addition of histamine to HeLa cells pretreated with calyculin A, a condition leading to phosphorylation of IP_3R, did not evoke any response at all. If the phosphorylation did not close the Ca^{2+} channel, one would expect to see a single Ca^{2+} spike. Also, calyculin A added to ongoing Ca^{2+} either interrupted oscillations (blocked any further Ca^{2+} release) or generated a single, smaller spike. Furthermore, the large Ca^{2+} burst seen on addition of CaMK II inhibitors (Figure 3A,B) is consistent with unphosphoryated IP_3R failing to curtail Ca^{2+} release. Although the Ca^{2+} burst can also be explained by lack of activation of the ER and plasma membrane Ca^{2+}-ATPase pumps by CaM, it is rather unlikely. The rate of Ca^{2+} removal was not seriously reduced in the presence of W-7 (Figure 4). The basal activity of ER Ca^{2+}-ATPase must be quite high because the irreversible inhibitor of the pump, TG, caused a rather rapid Ca^{2+} release in the absence of stimuli; the basal activity presumably is necessary to counteract this considerable Ca^{2+} "leakage" from the stores even when the cells are at rest. However, the ATPase activity can be stimulated many-fold by CaMK II as shown by the swift removal of electroporated Ca^{2+} in cells preloaded with the active domain of CaMK II (Figure 5). Our finding agrees with the *in vitro* studies of Supattapone *et al.* (1988) which held that phosphorylation results in inhibition of Ca^{2+} release from IP_3R, though Volpe and Alderson-Lang (1990) reported enhancement.

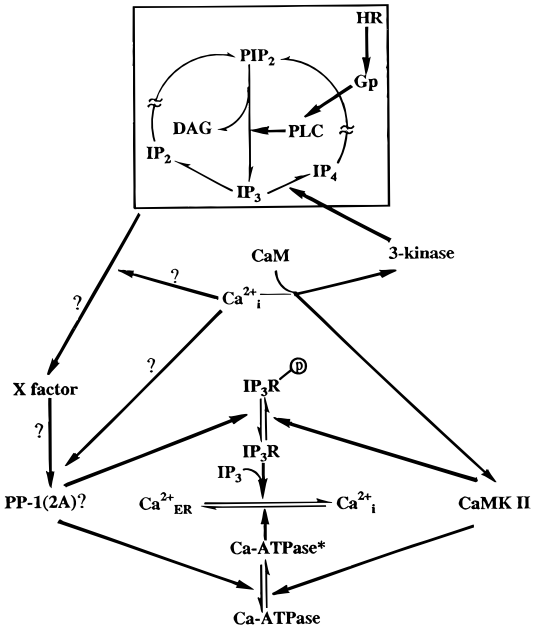
The concept that reversible phosphorylation cycles mediate Ca^{2+} oscillations in HeLa cells is strengthened by experiments using phosphatase inhibitors calyculin A and okadaic acid. The presence of these compounds clearly enhanced IP_3R phosphorylation (Figure 7) and abolished oscillations or prolonged the period between spikes (Figure 6). The role of the phosphatase (or phosphatases since the deactivation of ER Ca^{2+}-ATPase may require a different phosphatase), as we shall discuss later, may be pivotal in the modulation of frequency.

A salient feature of sustained Ca^{2+} oscillations is the rather constant amplitude originally noted by Woods *et al.* (1986). This phenomenon is not surprising since the Ca^{2+} concentration must be maintained at a certain threshold level in order to activate various highly cooperative Ca^{2+} binding processes. It has been recognized that cooperativity is required to generate the threshold (Meyer & Stryer, 1991). For example, the Ca^{2+} concentration dependence of the CaM-stimulated cyclic nucleotide phosphodiesterase is very sharp (Huang *et al.*, 1981). At a CaM concentration of ~ 2 μM, the enzyme is basically inactive at Ca^{2+} concentrations below 3×10^{-7} M, but rises to half-maximal activation at 6×10^{-7} M and becoming fully active at $>10^{-6}$ M. The Ca^{2+} concentration required for half-maximal activation decreases with increasing CaM concentrations. Thus, the amplitude of Ca^{2+} oscillation may vary in different cells, depending on the concentration and type of Ca^{2+}-binding proteins present. Given the requirement that the amplitude must be maintained at or above a functional level, the only way to regulate cellular activities in a certain time period is to adjust the frequency.

It can be envisioned in a simplistic way that keeping the Ca^{2+} spikes at a constant level can also be accomplished through the highly cooperative CaM-dependent reactions. For instance, in a system like the HeLa cells (cf. Scheme 1), when cytosolic Ca^{2+} reaches a "threshold" level, CaMK II will be activated, resulting in the shutting off of the Ca^{2+} channel activity of IP_3R and the activation of the ER Ca^{2+} pump. The plasma membrane Ca^{2+} pump will also be activated by Ca^{2+}/CaM. Such a mechanism will automatically maintain the amplitude of the Ca^{2+} spikes near the cell's threshold level.

How can the frequency be regulated, or how is the interspike interval controlled? The sharp rising phase of a Ca^{2+} spike is often considered to be due partly to the highly cooperative nature of IP_3 binding to IP_3R (Meyer & Stryer, 1988). Assuming the binding process is rapid, the period between transients can be the time required to accumulate a threshold concentration of IP_3. Oscillation of *free* IP_3 is a requirement of such a mechanism. But IP_3 need not be generated anew for each spike; it only needs to oscillate between free and bound forms. With other mechanisms, IP_3 oscillation is not a requisite (Goldbeter *et al.*, 1990; Somogyi & Stucki, 1991; Wakui *et al.*, 1989). In a system like the HeLa cells, the lull between spikes can be the time required to reactivate the IP_3R for IP_3 binding or Ca^{2+} release. Since IP_3R is a tetramer with two phosphorylation sites per subunit (Mikoshiba, 1993), the dephosphorylation (reactivation) process may be a highly cooperative one if only the totally dephosphorylated IP_3R is capable of releasing Ca^{2+}. In this sense, the interspike interval would be the time required for dephosphorylation of IP_3R and/or activation of the phosphatase. This concept is in agreement with our observation

Scheme 1: Proposed Mechanism of Ca^{2+} Oscillations in HeLa cells[a]



[a] HR, histamine receptor; Gp, G-protein; PIP_2, phosphatidylinositol bisphosphate; IP_2, inositol 1,4-bisphosphate; Ca^{2+}_{ER}, Ca^{2+} in ER stores; Ca^{2+}_i, cytosolic Ca^{2+}; IP_3R-Ⓟ, phosphorylated form of IP_3R; Ca-ATPase*, activated form of Ca-ATPase; PP, protein phosphatase. Other abbreviations are as defined in the text. The rectangle contains reactions that may generate the proposed X factor. Potential interaction pathways are indicated by question marks (?).

that calyculin A can prolong the intervals between Ca^{2+} transients (Figure 6B).

It is well-established that the frequency of spiking is agonist concentration-dependent. To account for this dependency, if dephosphorylation of IP_3R in HeLa cells is a crucial event, the phosphatase activity must be regulated directly or indirectly by a factor that is generated by a reaction related to the agonist-receptor interaction. This "X factor" can be a product or its derivatives of the PLC-catalyzed reaction, such as inositol 1,3,4,5-tetrakisphosphate (IP_4) or a lipid. It should be of interest to note that inositol phosphates have been reported to stimulate the activity of a type 1 phosphatase (Zwiller *et al.*, 1988) and IP_4 production is catalyzed by IP_3 3-kinase, a CaM-dependent enzyme (Johanson *et.al.*, 1988; Lee *et al.*, 1990; Ryu *et al.*, 1987). Alternately, it can be a G-protein (cf. reaction pathways in the rectangle in Scheme 1). Ca^{2+} can also be involved in the regulation of either the X factor or the phosphatase. The fact that removal of external Ca^{2+} led to longer interspike intervals and lower amplitudes (Figure 1B) further indicates that the cytosolic Ca^{2+} level and the replenishment of Ca^{2+} stores are important factors in determining the spatiotemporal patterns of oscillations.

The involvement of kinases in Ca^{2+} oscillations has been reported or suggested by numerous workers. The mechanism proposed by us is different in the sense that we see the phosphorylation-dephosphorylation cycle as the oscillator that participates in the control of the interspike interval. It is also different from the concept of "frequency encoding through protein phosphorylation" of Goldbeter *et al.* (1990), which is concerned with how Ca^{2+} oscillations can be translated into a frequency-dependent physiological response. The role of CaMK II in HeLa cells is further distinct from those reported for CaMK II in other systems (Somogyi & Stucki, 1991; Zhang *et al.*, 1993) in that it is one of negative feedback. Negative feedback by Ca^{2+} or PKC has been invoked to be the cause of oscillations (Parker & Ivorra, 1990; Payne *et al.*, 1990; Woods *et al.*, 1987). However, we do not exclude the possibility that there may be a positive Ca^{2+} feedback in the gating of IP_3R or in the generation or binding of the hypothetical X factor. Albeit the proposed mechanism for Ca^{2+} oscillations presented in Scheme 1 is intended for the HeLa cell system, conceivably reversible phosphorylation steps regulating the release and sequestration of intracellular Ca^{2+} can be a general mechanism for sustaining oscillations in different cells.

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