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Photodynamic triggering of calcium oscillation in the isolated rat pancreatic acini

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1. Photodynamic agents, due to their photon-dependent selective activation, can selectively activate a number of physiological processes and may directly modulate signal transduction in a number of cells including pancreatic acinar cells.
2. Activation of the photodynamic agent sulphonated aluminium phthalocyanine (SALPC) triggered recurrent cytosolic calcium ($[Ca^{2+}]_i$) spiking in pancreatic acinar cells.
3. The photodynamically triggered calcium spiking could be blocked by phosphatidylinositol-specific phospholipase C (PI-PLC) inhibitor U73122, but not by phosphatidylcholine-specific phospholipase C inhibitor D609.
4. Removal of extracellular Ca^{2+} abolished spiking, as did 2-aminoethoxydiphenylborate (2-APB), an inhibitory modulator of IP_3-mediated Ca^{2+} release from intracellular stores.
5. These data suggest that SALPC photodynamic action may permanently fix PI-PLC in an active conformation, and this produced recurrent $[Ca^{2+}]_i$ spiking.

Photodynamic action is a process which involves activation of photosensitizing agents by photon energy and subsequent transfer of this photon energy to ground state molecular oxygen through a type II mechanism to generate the excited delta singlet molecular oxygen, and subsequently singlet oxygen-mediated cellular responses (Cui, 1989).

It has been previously found that, after short incubation with cells, the photosensitizer mainly localizes to the plasma membrane (Cui, 1989; Hubmer, Herman, Uberriegler & Krammer, 1996). Upon subsequent light activation, these membrane-bound agents can activate or modulate a number of physiological processes: contraction of smooth muscle cells (Matthews & Mesler, 1984), slow phasic increases of intracellular calcium concentration in fibroblasts, thymocytes, cardiomyocytes (Ben-Hur, Dubbelman & van Steveninck, 1991; Yonuschott, 1991; Yonuschott, Vaugh & Novotny, 1992; Hubmer *et al.* 1996), amylase secretion in pancreatic acinar cells (Matthews & Cui, 1989, 1990*a*, *b*), and inhibition of amylase secretion in AR4-2J cells (Matthews & Cui, 1990*b*; Al-Laith & Matthews, 1994).

The photodynamically induced cellular responses may be due to activation of phospholipase A_2 (PLA_2; Al-Laith, Matthews & Cui, 1993), adenylate cyclase (Penning, van Steveninck & Dubbelman, 1993), P21-RAS and MAP kinase (Rasch, Ouwen & Maassen, 1994), or increase in the expression of immediate early response genes (*fos*, *jun*, *myc*, *egr-1*) (Luna, Wong & Gomer, 1994). Here results are presented showing that brief light activation of sulphonated aluminium phthalocyanine (SALPC) that was bound to the plasma membrane triggered persistant spiking in intracellular calcium concentration ($[Ca^{2+}]_i$) in isolated rat pancreatic acinar cells and this was due to the activation of PI-PLC.

Agonist-induced oscillatory increases in $[Ca^{2+}]_i$ are commonly observed in different cells of both the excitable and the non-excitable type. $[Ca^{2+}]_i$ oscillations in excitable cells may be related to oscillatory electrical activities (this typically includes the pancreatic β cells), which involves cyclic opening of the voltage-operated calcium channels (VOCCs; Larsson, Kindmark, Branstrom, Fredholm & Berggren, 1996). In the non-excitable cell type, this oscillatory pattern has been shown to be intimately connected to agonist-induced hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2; Fewtrell, 1993; Tsunoda, 1993). In the pancreatic acinar cells, the two major calcium-mobilizing receptors, M_3 muscarinic receptors and cholecystokinin A (CCK_A) receptors, are both coupled through G_q protein to phosphatidylinositol-specific phospholipase C (PI-PLC), to generate inositol-1,4,5-trisphosphate (IP_3) which through IP_3 receptors induces Ca^{2+} release from internal stores (Zeng, Xu & Muallem, 1996; Hwang, Jhon, Bae, Kim & Rhee, 1996). Consequently, CCK at appropriate physiological concentrations (< 20 pM) induces recurrent $[Ca^{2+}]_i$ spikes.

As in other non-excitable cells, there has not been a consensus model to account for the detailed mechanisms mediating calcium oscillations in pancreatic acinar cells. The existing models can be categorized as IP_3-oscillating and IP_3-steady models, and typically this includes IP_3–Ca^{2+}

cross-coupling (the ICC model) and calcium-induced calcium release (the CICR model; Shen & Larter, 1995). In the present work, it was found that the leading event in photodynamically induced persistant calcium oscillations was the activation of PI-PLC. The nature of photodynamic action dictates that, after permanent activation of PI-PLC, its activity may not be cyclic or oscillatory. This aspect of the present work is put into the context of present models and new insights into calcium oscillations are discussed.

METHODS

Chemicals

Sulphonated aluminium phthalocyanine (SALPC; tetrasulphonate) was from Porphyrin Products (Salt Lake City, UT, USA). U73122, U73343, D609 (BiomolR) and fura-2 AM (Dojindo) were from Wako Pure Chemicals (Osaka, Japan). Collagenase (type CLSPA, Worthington Biochemicals Co.) was purchased from Funakoshi (Tokyo). CCK octapeptide was from Research plus (Nayonne, NJ, USA). 2-Aminoethoxydiphenylborate (2-APB) was synthesized (Maruyama, Kanaji, Nakade, Kanno & Mikoshiba, 1997*b*).

Isolation of rat pancreatic acini

Male Sprague–Dawley rats of 250–450 g were killed by cervical dislocation and a portion of the pancreas was used. The pancreas was infiltrated with collagenase solution (90 U ml^{-1}), and incubated in this solution in a shaking water bath (37 °C, 120 cycles min^{-1}) for 4 × 10 min periods. Fresh collagenase solution was used from the third digestion. The tissue was airated with plenty of 100% O_2 before each digestion. After the fourth digestion, the pancreas was dissociated with a pipette and filtered through a nylon mesh (150 μm). Filtered acini were first centrifuged through buffer containing 4% bovine serum albumin, then washed three times with normal buffer. Buffer used in this work had the following composition (mM): 118 NaCl, 4·7 KCl, 2·5 $CaCl_2$, 1·13 $MgCl_2$, 1 NaH_2PO_4, 5·5 D-glucose, 10 Hepes, 2 L-glutamine, plus 2 mg ml^{-1} bovine serum albumin, 2 ml amino acid mixture (Gibco BRL), and 0·1 mg ml^{-1} soybean trypsin inhibitor; pH adjusted to 7·4 with 4 N NaOH. Buffer was oxygenated with 100% O_2 before use (for the original method, see Habara & Kanno, 1991).

Measurement of $[Ca^{2+}]_i$ changes

Freshly isolated pancreatic acini were resuspended in buffer, 100 μl of which was diluted to 980 μl with buffer, to which was added 20 μl fura-2 AM (1 mM in DMSO), to get a final fura-2 AM concentration of 20 μM. Fura-2 loading proceeded for 40 min in a shaking water bath (37 °C, 50 cycles min^{-1}), with gentle shaking by hand every 10 min. Fura-2-loaded acini were diluted with buffer and 2 ml aliquots were dispensed to each of several Sykus-Moore chambers with the bottom coverslip treated with Cell-Tak (Becton Dickinson, Bedford, MA, USA). Acini were allowed to attach for at least 15 min before perifusion with buffer was started at 1 ml min^{-1}. All buffer was pre-warmed in a water bath to 37 °C, and buffer containing a relevant drug was delivered to the cell through perifusion (dead time was corrected in all recordings shown). The Sykus-Moore chamber was positioned on the microscope stage of a Nikon P1 calcium measurement system. For $[Ca^{2+}]_i$ measurement, the apical portion of a single acinar cell within acinar formation or formations was optically isolated with a pin-hole device. In all the experiments described in this work, a pin-hole size of 0·5 was used, and a Nikon neutral density filter no. 8 was placed in the excitation light path. $[Ca^{2+}]_i$ changes were recorded as changes in the fluorescence (510 nm) ratio for excitation at 340 and 380 nm (F_{340}/F_{380}).

Light illumination

An HL100R Hoya-Schott (Tokyo) cold light equipped with a focusing lens (HLL201) and a sharp-cut filter (R60; wavelength, λ > 580 nm) was used for illumination. In this work, all illumination was at 55000 lux for 1 min.

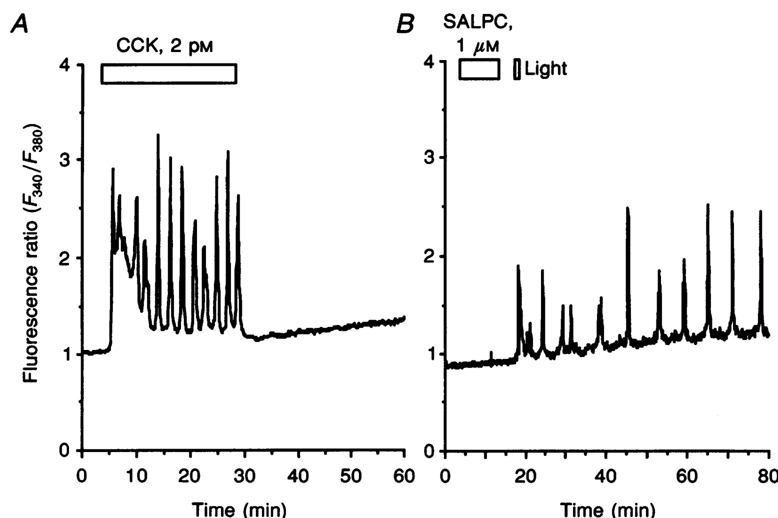


Figure 1. Recurrent $[Ca^{2+}]_i$ oscillations induced by photodynamic action of SALPC

A, CCK-induced $[Ca^{2+}]_i$ spikes. Note the immediate cessation of spiking activities and return to basal levels of $[Ca^{2+}]_i$ after wash-out of CCK. $n = 3$. *B*, $[Ca^{2+}]_i$ spikes induced by photodynamic action of SALPC. Note that in the absence of light illumination (ambient light, < 20 lux) SALPC had no effect on $[Ca^{2+}]_i$. A subsequent brief period of light illumination (55000 lux, 1 min) induced $[Ca^{2+}]_i$ spikes. $[Ca^{2+}]_i$ spikes persisted after light illumination (photodynamic action) had stopped. $n = 19$. In all experiments cells were continuously perifused with incubation buffer. Buffer containing either CCK or SALPC, or light illumination, was applied as indicated by the horizontal bars.

RESULTS

Photodynamic action of SALPC triggers recurrent spikes in $[Ca^{2+}]_i$

Isolated pancreatic acini were routinely tested for functional responsiveness to CCK (2 pM). The apical portion of a single acinar cell within acinar formation(s) was monitored by optical isolation through a pin-hole device. CCK (2 pM) usually induced an immediate rise in $[Ca^{2+}]_i$, followed by gradual decay superimposed with oscillatory increases (phase I), and steady recurrent $[Ca^{2+}]_i$ spikes (phase II), which were maintained for as long as CCK was present in the perifusion solution. After washing out of CCK, the phase II response ceased immediately (Fig. 1*A*). To examine the effects of photodynamic action on $[Ca^{2+}]_i$, 1 μM SALPC was added to the solution superfusing the attached acini for 10 min before washing out with the standard solution for 5 min, and then light was delivered to the acini at an illuminance of 55000 lux (focused light with $\lambda > 580$ nm) through a fibre optic bundle. The photosensitizer SALPC had no effect on $[Ca^{2+}]_i$ in the dark (ambient light, < 20 lux), having the same effect as light alone in the absence of SALPC (data not shown). After washing out SALPC from the perifusion chamber, photosensitizer molecules that remained bound to the cell were activated by light. Light activation (for 60 s) was immediately followed by increases in $[Ca^{2+}]_i$. The initial $[Ca^{2+}]_i$ spike induced during light illumination was followed by more and regularly appearing spikes once the cell was re-exposed to ambient room light. This pattern of regularly occuring spikes continued during the whole period of $[Ca^{2+}]_i$ monitoring (Fig. 1*B*), up to 3 h (not shown).

Inhibitory effects of the PI-PLC inhibitor U73122 on photodynamically evoked oscillatory $[Ca^{2+}]_i$ dynamics

A large number of stimuli could trigger recurrent $[Ca^{2+}]_i$ spikes in the pancreatic acini. To investigate the possible site of photodynamic action, U73122, a PI-PLC inhibitor, was used (Fig. 2). $[Ca^{2+}]_i$ spikes were induced by photodynamic action as before. The $[Ca^{2+}]_i$ spikes were completely inhibited when 10 μM U73122 was added to the solution perifusing the oscillating cells; this complete inhibition was achieved within 2 min of addition of U73122 (Fig. 2*A*). In control experiments, it was confirmed that U73122 at

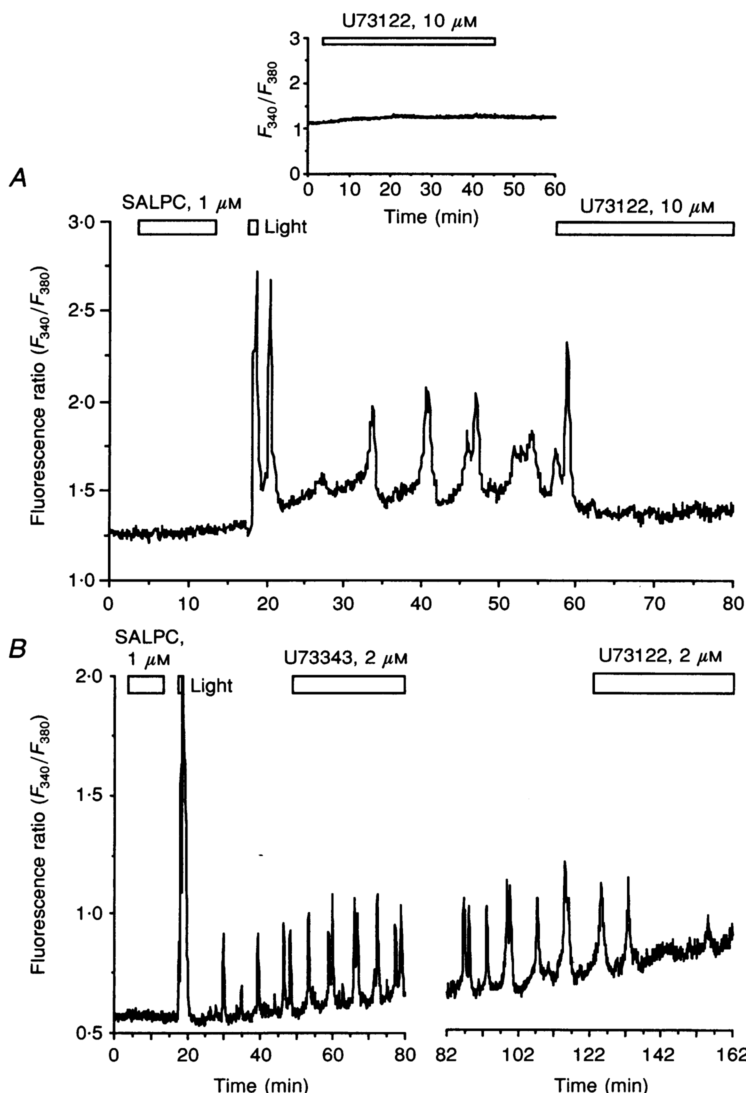


Figure 2. U73122 blocked $[Ca^{2+}]_i$ spikes induced by photodynamic action of SALPC

A, abolition by 10 μM U73122 of $[Ca^{2+}]_i$ spikes induced by photodynamic action. U73122 was added as indicated. $n = 5$. *B*, lack of effect of U73343 on $[Ca^{2+}]_i$ spikes induced by photodynamic action. U73343 (2 μM) and U73122 (2 μM) were applied sequentially to the same cell as indicated by the horizontal bars. $n = 5$. Inset, this indicates the lack of effect of 10 μM U73122 on basal $[Ca^{2+}]_i$ levels. U73122 (10 μM) was added to perifused cell at time indicated by the horizontal bar. $n = 3$.

10 μM had no effect on $[Ca^{2+}]_i$ dynamics in resting cells exposed neither to SALPC nor to light (Fig. 2, inset). To confirm the specificity of U73122 action, the effects of its inactive structural analogue, U73343, were also investigated, at a slightly lower concentration of 2 μM. Just as expected, the photodynamically induced $[Ca^{2+}]_i$ spikes were not affected by U73343 (Fig. 2*B*); as the recording continued in the same cell after wash-out of U73343, an identical concentration of U73122 dramatically inhibited photodynamically induced $[Ca^{2+}]_i$ spikes (Fig. 2*B*). The inhibitory effect of U73122 at this concentration had a longer latency (10 min) than that at 10 μM (Fig. 2*A* and *B*). From these experiments it is clear that SALPC photodynamic action triggers recurrent $[Ca^{2+}]_i$ spikes through activation of PI-PLC.

Photodynamically induced $[Ca^{2+}]_i$ oscillations were not inhibited by the phosphatidylcholine-specific phospholipase C inhibitor D609

It should be born in mind that phosphatidylinositol (PI) is only a minor component in the plasma membrane phospholipid, whereas phosphatidylcholine (PC) is a major component. To examine the potential effects of photodynamic activation by SALPC of PC-specific phospholipase C (PC-PLC) on photodynamically induced $[Ca^{2+}]_i$ dynamics, the effect of a PC-PLC inhibitor, D609, was analysed. In Fig. 3*A*, CCK-induced $[Ca^{2+}]_i$ dynamics were recorded in acinar cells as before; washing out of CCK caused an immediate cessation of Ca^{2+} spikes. To the same cell, D609 was added at 1, 10, and 100 μM in tandem. D609 at the lower concentrations of 1 and 10 μM had no effect by itself, but a higher concentration of 100 μM caused an immediate phasic increase in $[Ca^{2+}]_i$. Therefore in our later experiments only 10 μM was used instead of the usual 100 μM that has been used by others (Schutze, Potthoff, Machleidt, Berkovic, Wiegmann & Kronke, 1992; Dong Sohn, Han, Tashjian, Behar & Biancani, 1995). As shown in Fig. 3*B*, SALPC photodynamic action-induced $[Ca^{2+}]_i$ spikes were not inhibited by 10 μM D609. On the contrary, and in contrast with lack of effect on its own, after photodynamic action D609 appeared to have increased basal calcium concentration, resulting in a slightly increased amplitude of $[Ca^{2+}]_i$ spikes (Fig. 3*B*). It is concluded therefore that PC-PLC does not appear to be involved in the generation of photodynamically induced $[Ca^{2+}]_i$ spikes.

Inhibition of photodynamically induced $[Ca^{2+}]_i$ oscillations in calcium-deficient medium

$[Ca^{2+}]_i$ spikes induced by physiological concentrations of CCK were dependent on extracellular Ca^{2+} concentration. As shown in Fig. 4, 2 pM CCK induced regular $[Ca^{2+}]_i$ spikes over an elevated basal $[Ca^{2+}]_i$ level. Upon removal of extracellular Ca^{2+}, basal $[Ca^{2+}]_i$ decreased within a few minutes; both the frequency and amplitude of CCK-induced $[Ca^{2+}]_i$ spikes were reduced gradually (Fig. 4*A*); and the spikes disappeared completely 30 min after removal of extracellular Ca^{2+}. Upon reintroduction of Ca^{2+} to the perifusion buffer, $[Ca^{2+}]_i$ rose immediately and was followed by a decay on which spikes of higher frequency were superimposed (Fig. 4*A*). Note that reintroduction of

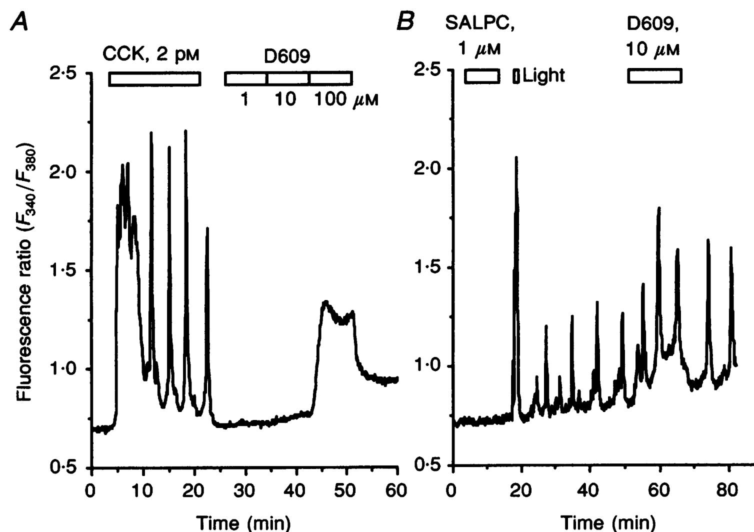


Figure 3. Lack of inhibitory effects of D609 on $[Ca^{2+}]_i$ spikes induced by photodynamic action of SALPC

A, effects of D609 on basal $[Ca^{2+}]_i$ levels. Cell was exposed to 2 pM CCK, which induced immediate $[Ca^{2+}]_i$ spikes, the spikes stopped after wash-out of CCK. D609 was then added at 1, 10, and 100 μM. Note that 100 μM D609-induced phasic increase in $[Ca^{2+}]_i$ was reversed immediately after wash-out of D609. $n = 3$. *B*, lack of inhibition by D609 of $[Ca^{2+}]_i$ spikes induced by photodynamic action. Photodynamic action with SALPC was started as before, and 10 μM D609 was added as indicated. $n = 3$.

extracellular Ca^{2+} resulted in the appearance of the phase I $[Ca^{2+}]_i$ dynamics referred to earlier (Fig. 1*A*). Similar to the CCK-induced $[Ca^{2+}]_i$ spikes, photodynamically induced $[Ca^{2+}]_i$ spikes were also dependent on extracellular Ca^{2+}. Perifusion with the calcium-deficient medium led to the disappearance of Ca^{2+} spikes 7 min later. Reintroduction of extracellular Ca^{2+} led to an immediate re-emergence of $[Ca^{2+}]_i$ spikes of similar frequency and amplitude (Fig. 4*B*). Therefore as with physiological stimulation, SALPC photodynamic action-induced $[Ca^{2+}]_i$ spikes were dependent on extracellular Ca^{2+}.

Inhibition of photodynamically induced $[Ca^{2+}]_i$ dynamics by 2-APB, the plasma membrane-permeant modulator of IP_3-mediated Ca^{2+} release from internal stores

2-APB, the newly discovered plasma membrane-permeant IP_3 receptor modulator (Maruyama, Cui, Kanaji, Mikoshiba & Kanno, 1997*a*; Maruyama *et al.* 1997*b*), had two effects on the recurrent $[Ca^{2+}]_i$ dynamics induced by CCK (Fig. 5*A*). It decreased the interspike $[Ca^{2+}]_i$ to basal level and inhibited completely the recurrent $[Ca^{2+}]_i$ spikes within 2 min of addition. The inhibition was maintained for the 10 min 2-APB application. Upon removal of 2-APB, $[Ca^{2+}]_i$ spikes re-emerged, with enhanced amplitude for the first spike. A longer period of incubation (30 min) of the oscillating cells with 2-APB also decreased the interspike $[Ca^{2+}]_i$ to basal level and inhibited completely the recurrent $[Ca^{2+}]_i$ spikes; but in this case, $[Ca^{2+}]_i$ level gradually increased from the basal level to the preinhibition interspike level on which a few small $[Ca^{2+}]_i$ spikes sometimes emerged. Removal of 2-APB then led to the immediate reappearance of $[Ca^{2+}]_i$ spikes, with the amplitude of the first spike being enhanced. Perifusion with 100 μM 2-APB for 30 min also inhibited the photodynamically evoked recurrent $[Ca^{2+}]_i$ spikes, with a latency of inhibition of interspike $[Ca^{2+}]_i$ level of < 2 min, followed by complete inhibition of the $[Ca^{2+}]_i$ spikes. The decreased interspike $[Ca^{2+}]_i$ level here also gradually recovered to the preinhibition level; this was followed by the emergence of new $[Ca^{2+}]_i$ spikes. Therefore, prolonged perifusion with 2-APB led to gradual dis-inhibition, but the inhibitory effect was still present, because subsequent removal of 2-APB led to the appearence of spikes of higher frequency (Fig. 5*B*). Therefore removal of 2-APB inhibition both in CCK- and photodynamically stimulated $[Ca^{2+}]_i$ dynamics resulted in a transient potentiation. This seems to be a hallmark of this particular IP_3 receptor modulator.

The presence of small $[Ca^{2+}]_i$ spikes during prolonged incubation with 2-APB was unlikely to be due to any net effect by 2-APB itself (Fig. 5*C*). In cells responding to 2 pM CCK by $[Ca^{2+}]_i$ spiking, removal of CCK led to cessation of $[Ca^{2+}]_i$ spikes. Then prolonged incubation (35 min) with 100 μM 2-APB in the absence of CCK or photodynamic action did not induce any $[Ca^{2+}]_i$ spikes. These experiments indicate that any $[Ca^{2+}]_i$ spikes that existed during incubation with 2-APB in CCK- or photodynamically stimulated cells must be due to incomplete inhibition by 2-APB.

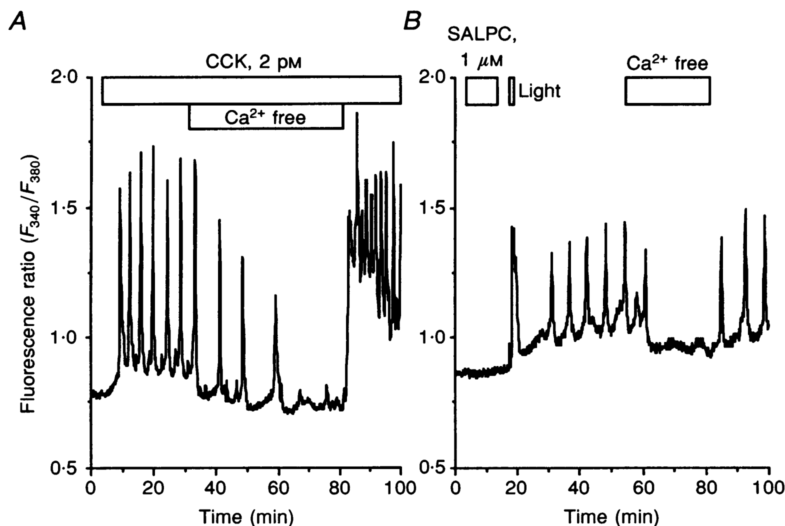


Figure 4. $[Ca^{2+}]_i$ spikes induced by photodynamic action of SALPC disappeared when perifusion was switched to Ca^{2+}-deficient buffer

A, CCK-induced $[Ca^{2+}]_i$ spikes gradually disappeared in Ca^{2+}-deficient buffer. CCK addition and change to Ca^{2+}-deficient buffer is indicated by the horizontal bars. Note the spurry of $[Ca^{2+}]_i$ increasing activities after re-addition of Ca^{2+} to perifusion buffer. $n = 4$. *B*, $[Ca^{2+}]_i$ spikes induced by photodynamic action were abolished when perifusion was switched to Ca^{2+}-deficient buffer. Note the rapid return of $[Ca^{2+}]_i$ spikes after re-addition of Ca^{2+} to the perifusion buffer. $n = 6$.

DISCUSSION

In the present work, we have found that: (1) SALPC photodynamic action of short duration (1 min) stimulated persistant or recurrent $[Ca^{2+}]_i$ spiking in perifused rat pancreatic acini; (2) photodynamically induced recurrent $[Ca^{2+}]_i$ spikes were completely inhibited by the PI-PLC inhibitor U73122 whereas the inactive structural analogue U73343 was ineffective; (3) the $[Ca^{2+}]_i$ spikes were not inhibited by D609, the PC-PLC inhibitor; (4) the $[Ca^{2+}]_i$ spikes were inhibited in a Ca^{2+}-deficient environment with a certain delay; and (5) the $[Ca^{2+}]_i$ spikes were inhibited by 2-APB, the membrane-permeant modulator of IP_3-mediated calcium release. These results are consistent with our previous studies, in which photodynamic action of SALPC stimulated amylase secretion in isolated rat pancreatic acini without significant changes in plasma membrane permeability (Matthews & Cui, 1990*a*,*b*). The photodynamically induced amylase secretion in many respects was similar to secretagogue-induced amylase secretion, including a similar $^{86}Rb^+$ efflux, neglegible lactate dehydrogenase release, significant amylase secretion and a comparable time course for amylase secretion. We therefore postulated that secretagogue receptors or their coupled effector systems must be major targets for the SALPC photodynamic action. In addition, the fact that in AR4-2J cells, photodynamic action generally led to inhibition of amylase secretion instead of stimulation of secretion that was observed in normal pancreatic acinar cells led us to propose that in AR4-2J cells, the major target of photodynamic action might be a selective effect on the membrane proteins involved in transport vesicle exocytosis (Matthews & Cui, 1990*b*). These are all consistent with the observation that SALPC after a short incubation is mainly localized to the plasma membrane (Cui, 1989; Hubmer *et al.* 1996).

In the current study, we present substantial evidence that photodynamic action of SALPC directly activated PI-PLC and its associated $[Ca^{2+}]_i$ dynamics. This conclusion is based on the specific effect of U73122. The PI-PLC inhibitor U73122 both at a commonly used concentration of 10 μM and at a lower concentration of 2 μM completely abolished photodynamically induced recurrent $[Ca^{2+}]_i$ spikes, with a certain delay in the latter case. U73343, in contrast, was completely ineffective. U73122 at higher concentrations may have some unspecific effects as a PI-PLC inhibitor. Notably, at 25 μM it could inhibit store-operated calcium influx in hepatocytes (Berven & Barritt, 1995). In fibroblasts, at 5 μM it also inhibited thapsigargin-induced calcium increases both from intracellular stores and from calcium influx

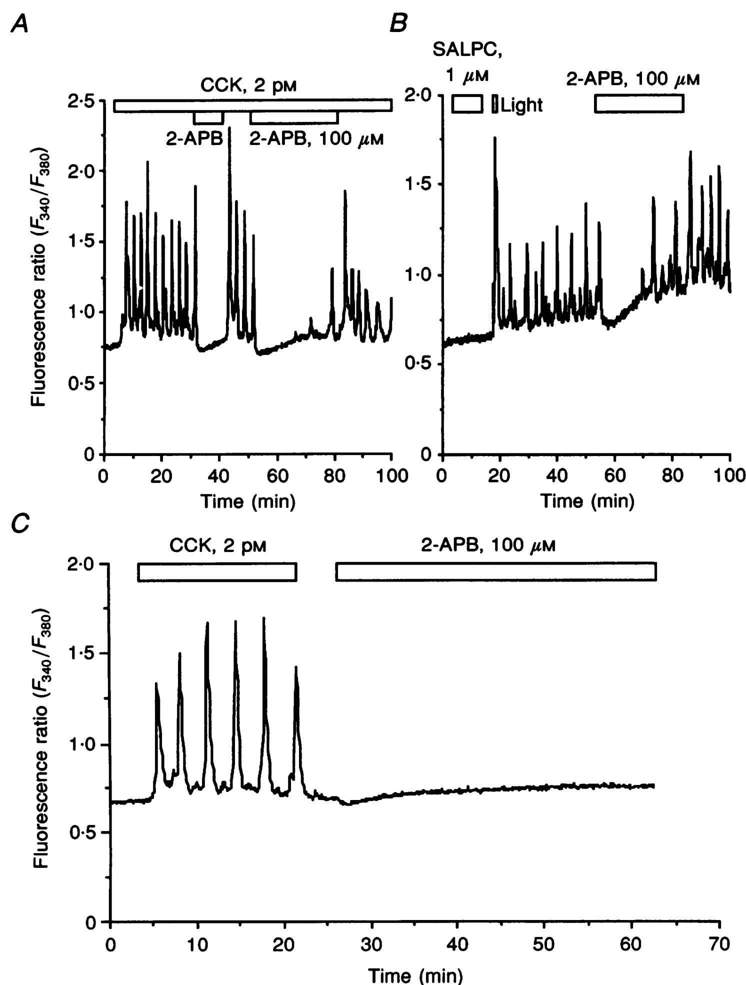


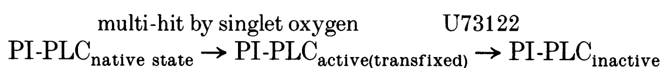
Figure 5. Inhibition by 2-APB of $[Ca^{2+}]_i$ spikes induced by photodynamic action of SALPC

A, reversible inhibition of CCK-induced $[Ca^{2+}]_i$ spikes. CCK (2 pM) was added as indicated. Ten min exposure to 100 μM 2-APB effected a complete and reversible inhibition. Longer period of exposure to 100 μM 2-APB (30 min) also exerted complete inhibition, but note the re-emergence of smaller spike(s) near the end of the 30 min exposure. $n = 5$. *B*, inhibition by 2-APB of $[Ca^{2+}]_i$ spikes induced by photodynamic action. $[Ca^{2+}]_i$ spikes were generated by SALPC photodynamic action, and 2-APB inhibition was complete, but note the gradual reappearance of $[Ca^{2+}]_i$ spikes in the presence of 2-APB after the initial inhibition. $n = 8$. *C*, lack of effects on $[Ca^{2+}]_i$ by 2-APB in the absence of CCK. A cell was exposed to 2 pM CCK, which induced regular spiking activity in $[Ca^{2+}]_i$. Spiking stopped after wash-out of CCK. 2-APB (100 μM) was then added to the perifusion buffer as indicated. $n = 3$.

(Grierson & Meldolesi, 1995). In rabbit pancreatic acinar cells, U73122 alone induced calcium spikes in some of the cells; this was probably due to its inhibitory effect on Ca^{2+}-ATPase of the intracellular stores (Willems, Van de Put, Engbergen, Bosch, van Hoof & de Pont, 1994; De Moel, van de Put, de Pont & Willems, 1995). However, in rat pancreatic acinar cells, U73122 at 10 μM had no effect on $[Ca^{2+}]_i$ by itself (Fig. 2 inset). Therefore in our study, as a specific inhibitor for PI-PLC, U73122 inhibited photodynamically induced $[Ca^{2+}]_i$ spikes.

In rat pancreatic acinar cells, D609 at 100 μM may not be a specific inhibitor for PC-PLC. It had the additional effect of being capable of inducing a phasic increase in $[Ca^{2+}]_i$; this phasic increase was immediately reversed upon wash-out of D609. At lower concentrations such as 10 μM, D609 had a slightly enhancing effect on photodynamically induced calcium spikes, manifesting as a slight increase in basal $[Ca^{2+}]_i$ concentration (Fig. 3*B*). Therefore we conclude that PC-PLC may not be involved in photodynamic generation of recurrent $[Ca^{2+}]_i$ spikes.

PI-PLC activation seems to be absolutely required in photodynamically induced $[Ca^{2+}]_i$ spikes in rat pancreatic acinar cells. Whether it is involved as a trigger in a 'chain reaction' type process, or rather its sustained activity is required for spiking, is not clear. However, it is known that delta singlet oxygen because of its high excitation energy state (94·1 kJ mol^{-1}; see Schaap, 1976) reacts covalently with amino acid residues of membrane protein to modify its conformation, permanently (Spikes & MacKnight, 1970). Also since the photodynamic action was a brief process in comparison with the induced continuous $[Ca^{2+}]_i$ spiking, it would be rather unlikely for PI-PLC activity to fluctuate after photodynamic action. It is all the more noteworthy in the face of this activation of PI-PLC that U73122 could still exert its inhibitory effect. The obvious speculation would be that singlet oxygen generated by photodynamic action of SALPC acts on PI-PLC in a multi-hit process (Cui, 1989), leading to conformational change to an active form, but this active form keeps open the U73122 binding site and upon U73122 binding leads to additional conformational change to an inactive form:



$$\text{PI-PLC}_{\text{native state}} \xrightarrow{\text{multi-hit by singlet oxygen}} \text{PI-PLC}_{\text{active(transfixed)}} \xrightarrow{\text{U73122}} \text{PI-PLC}_{\text{inactive}}$$

This scheme would be consistent with earlier works on conformational changes induced by delta singlet oxygen (Spikes & MacKnight, 1970). Although PC-PLC activity is also likely to generate the protein kinase C activator diacylglycerol, our present study does not suggest a major role for PC-PLC activation in the generation of $[Ca^{2+}]_i$ spikes.

The fact that removal of extracellular calcium led to abolition of photodynamically induced $[Ca^{2+}]_i$ spikes suggests that sustained or periodic Ca^{2+} entry may be important in maintaining the oscillatory activity. Since pancreatic acinar cells lack VOCCs, the stores-operated calcium channels (SOCCs) may play a major role in this process (Friel, 1996). This view is corroborated by data obtained with 2-APB, the novel inhibitory IP_3 receptor modulator. After photodynamic activation of PI-PLC, a certain level of IP_3 may be generated and this level may be maintained in the cytosol. IP_3 binding to IP_3 receptors on the membrane of intracellular Ca^{2+} stores would release Ca^{2+} into the cytosol. Thus the Ca^{2+} release process should be dependent upon both $[Ca^{2+}]_i$ and Ca^{2+} concentration inside the Ca^{2+} stores ($[Ca^{2+}]_s$). Calcium release from the internal stores has been shown by others to be dependent on $[Ca^{2+}]_i$, with a bell-shaped response curve: low but above resting level $[Ca^{2+}]_i$ stimulates the activity of IP_3 receptors, while high $[Ca^{2+}]_i$ has an inhibitory effect (Bezprozvanny, Watras & Ehrlich, 1991; Marshall & Taylor, 1993, 1994). The sensitivity of IP_3 receptors is also dependent on $[Ca^{2+}]_s$: full or saturated stores sensitize IP_3 receptors, whereas empty stores reduce IP_3 sensitivity (Nunn & Taylor, 1992; Oldenshaw & Taylor, 1993; Combettes, Cheek & Taylor, 1996). This together with recent evidence obtained with caged IP_3 in intact leukaemia cells (Oancea & Meyer, 1996) further supports the view that $[Ca^{2+}]_i$ and $[Ca^{2+}]_s$ regulate in concert how much Ca^{2+} is released from the internal stores, and in a cyclic fasion. The only additional input to keep this cascade going may be the opening of SOCCs, to refill from outside of the cell the calcium stores in correspondence to the refill status of Ca^{2+} stores. This view is supported in our present work by the fact that both photodynamically and CCK-induced $[Ca^{2+}]_i$ spikes were abolished in a Ca^{2+}-deficient environment (Fig. 4).

IP_3 receptors are essential constituents in the process of the above-mentioned cyclic releases from internal stores and the resultant cyclic increases in $[Ca^{2+}]_i$. This view is corroborated by data obtained with 2-APB. The inhibitory effects of 2-APB seemed to wear off after about 10 min both in photodynamically induced and CCK-stimulated $[Ca^{2+}]_i$ spikes. The small spikes in the continuous presence of 2-APB were unlikely to be due to any net stimulatory effect by 2-APB, because when CCK was not present, such spikes did not exist. These observations (initially complete, then partial inhibition) are consistent with the mechanism of action of this novel inhibitory IP_3 receptor modulator. In our previous study, we have already shown that 2-APB could enhance IP_3 receptor binding, but with an inhibitory effect on IP_3 receptor activation, and therefore sustained complete inhibition is generally not expected (Maruyama *et al.* 1997*a*, *b*; Cui, Habara, Wang & Kanno, 1997); complete inhibition is seen only at the initial stage.

In summary, we have reached the following conclusions regarding photodynamic action-induced recurrent $[Ca^{2+}]_i$ spikes. When SALPC was activated by photon energy ($h\nu$), its photodynamic action induced a permanent fixation of PI-PLC in an active conformation, resulting in a steady level of PIP_2 hydrolysis and subsequently a raised level of

IP_3 sufficient to release Ca^{2+} from intracellular stores, triggering increases in $[Ca^{2+}]_i$. Increase in $[Ca^{2+}]_i$ and decrease in $[Ca^{2+}]_s$ led to the cessation of Ca^{2+} release from the stores. The partly or completely empty Ca^{2+} stores caused calcium entry through the SOCC to refilled the stores. When stores refill to a set level, IP_3 resumed its effectiveness in releasing calcium into the cytoplasm, therefore the cycle of calcium spiking continued. The compound U73122 by blocking the action of PI-PLC, Ca^{2+}-deficient medium by depriving the Ca^{2+} source for Ca^{2+} entry through SOCC, and 2-APB by inhibitory modulation of IP_3 receptors could all block photodynamically induced recurrent $[Ca^{2+}]_i$ spikes effectively.

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