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## Membrane toxicity of the protein kinase C inhibitor calphostin A by a free-radical mechanism

Samuel S.-H. Wang<sup>a</sup>, Chris Mathes<sup>b</sup> and Stuart H. Thompson<sup>a</sup>

<sup>a</sup>Neurosciences Program, Department of Biological Sciences, and the Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950 (USA) and

<sup>b</sup>Neurosciences Program, Brain Research Institute, UCLA, Los Angeles, CA (USA)

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The effects of calphostin A on cytoplasmic calcium levels, receptor-mediated calcium release, and membrane input resistance were measured in neuroblastoma cells. Calphostin A is a lipophilic, light-sensitive perylenequinone that generates singlet oxygen when illuminated. It inhibits the activity of protein kinase C ( $IC_{50} = 250$  nM), but only in the presence of light. Phorbol esters normally attenuate carbachol-evoked calcium release. This effect was blocked by simultaneous exposure to light and calphostin A (40 nM) for 30 min. At higher doses (0.5–1  $\mu$ M) calphostin A also approximately doubled the resting calcium level and decreased cell input resistance by 51%. These toxic effects did not occur in the dark or after preincubation with the antioxidant  $\alpha$ -tocopherol. These data support the hypothesis that the calphostins act by partitioning into the membrane and producing singlet oxygen and endoperoxides which then irreversibly modify protein kinase C and other membrane proteins and lipids.

There is great clinical and experimental interest in developing drugs that block protein kinase C with high specificity and freely cross cell membranes. The calphostins provide potential candidates. These lipophilic perylenequinone compounds block protein kinase C (PKC) irreversibly at nanomolar doses [2] but calphostin A and C only block the kinase in the presence of light [M. Stahl, personal communication; 2]. With illumination these compounds also generate singlet oxygen [19] and endoperoxides [18]. This raises the possibility that the generation of reactive oxygen and free radicals causes block of PKC activity. Protein kinase C contains zinc-finger motifs in its diacylglycerol-binding domains [13], and zinc fingers are known to be sensitive to oxidative damage [C. Gitler, personal communication; 6]. The catalytic subunit of PKC translocates to membranes upon activation [12] and the lipophilic calphostins readily accumulate in the same compartments. It is possible, therefore, that the calphostins block PKC by generating non-specifically reactive species in or near membranes and not by binding directly to the kinase.

We tested this hypothesis by applying fura-2 imaging and patch clamp methods to murine N1E-115 neuroblas-

toma cells. These cells express M1 muscarinic receptors and respond to agonist by releasing calcium (Ca) from internal stores after the production of  $IP_3$  [11]. Agonist-evoked Ca release is strongly inhibited by treatment with phorbol esters that activate PKC [9, 16]. The ability of calphostins to prevent inhibition of Ca release by phorbol esters therefore provides a ready assay for the effectiveness of calphostins as blockers of PKC.

We measured resting Ca levels, Ca release in response to agonist, and membrane input resistance. The evidence suggests that calphostin A generates free radicals by a light-dependent process after partitioning into membranes. This might explain the specificity of the compound in blocking PKC over other kinases not associated with the membrane. A preliminary report of this work has appeared [17].

**Cell culture.** N1E-115 neuroblastoma cells were maintained in DMEM plus 10% FBS in 10%  $CO_2$ , plated on cover slips, and grown to 60–80% confluence. Cells were used after 6–20 days of differentiation in 2% dimethyl sulfoxide [7]. The experimental saline contained (mM): NaCl 137, KCl 5.4,  $CaCl_2$  1.8,  $MgSO_4$  0.8,  $KH_2PO_4$  0.4,  $Na_2HPO_4$  0.3, glucose 23, and NaHEPES 20 (pH 7.4,  $T = 27$ – $30^\circ C$ ). Zero Ca saline was made by replacing  $CaCl_2$  with  $MgCl_2$  and adding 2 mM EGTA. A 15–30 min recovery period between agonist applications ensured the reproducibility of the response.

*Correspondence:* S.H. Thompson, Department of Biological Sciences and the Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950, USA.

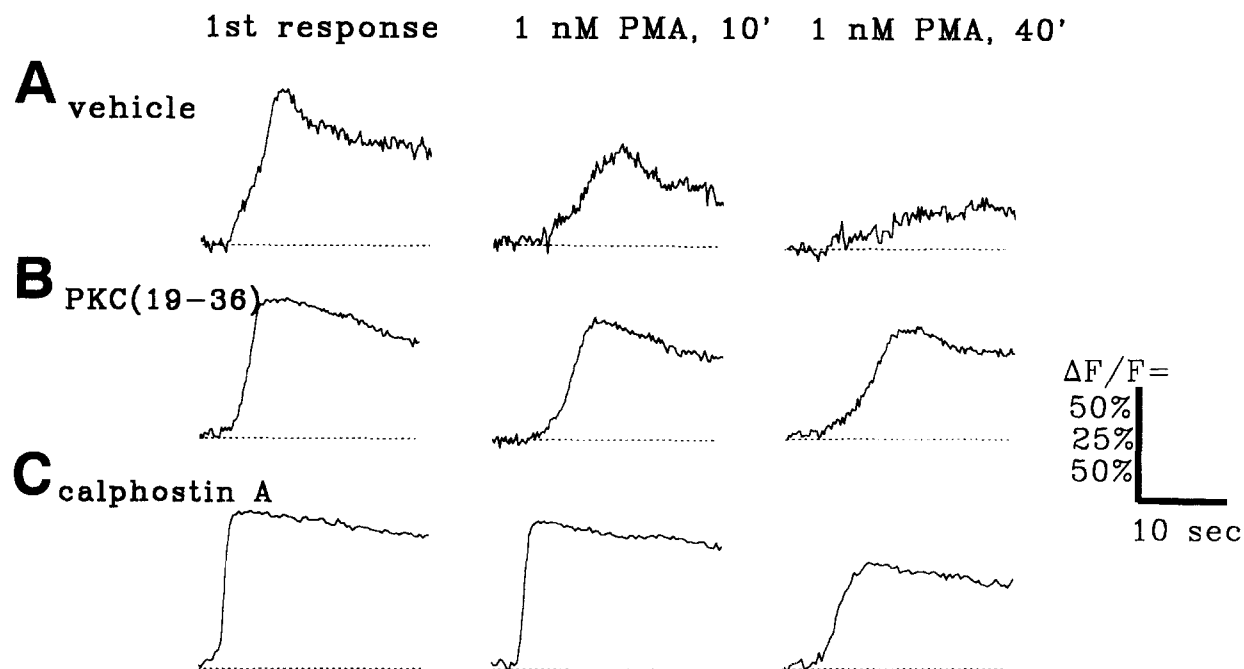


Fig. 1. Suppression of carbachol-evoked Ca release is blocked by PKC inhibitors. Cells loaded with fura-2/AM (A,C) or microinjected with fura-2 free acid (B) were repeatedly challenged with 1 mM carbachol. Traces show inverted  $F_{380}$  fluorescence. At the left are the initial responses in three cells (A-C). A: 10 min treatment with 1 nM PMA caused a large reduction in calcium release (center); the response was nearly gone 30 min later (right). The second cell (B) was microinjected with approximately 1  $\mu$ M PKC(19-36), which prevented PMA from decrementing the calcium response. C: a cell preincubated in 30 nM calphostin A and exposed to brightfield illumination for 30 min also was not affected by PMA.

**Calcium imaging.** Cells were loaded in a solution containing 5  $\mu$ M fura-2/AM and 0.025% Pluronic F-127 (Molecular Probes) for 60 min at 20°C and viewed on a Nikon Diaphot equipped with quartz optics and xenon arc lamp illumination filtered through 10 nm bandpass interference filters. Fluorescence signals were calibrated to  $[Ca]_i$  from the fluorescence ratio  $F_{340}/F_{380}$  [3]. Then, for calibrations from single-wavelength fluorescence ( $F_{380}$ ),  $[Ca]_i$  was calculated by extrapolating values for  $F_{max}$  and  $F_{min}$  at 380 nm illumination and using the formula:

$$[Ca]_i = K_D (F_{min} - F_{380}) / (F_{380} - F_{max}).$$

**Drug application.** Drug solutions were perfused directly into the chamber, except for the membrane-impermeant peptide PKC(19-36). PKC(19-36) was dissolved in microinjection saline and co-injected in a 1:10 or 1:100 ratio with fura-2 free acid, using an Eppendorf microinjector. Microinjection saline contained (mM): NaCl 5, KCl 140, MgCl<sub>2</sub> 2, HEPES 10, pH 7.2 at 20°C.

**Patch clamp.** Cells were voltage-clamped using the nystatin-permeabilized patch technique and whole-cell currents were measured [4, 10]. The pipette solution contained (in mM): KCl 16, K<sub>2</sub>SO<sub>4</sub> 70, MgSO<sub>4</sub> 5, HEPES 10, and sucrose 100 (~320 mOsm) at pH 7.2. At the beginning of each experiment, 0.05% Pluronic F-127 and 100-200  $\mu$ g/ml nystatin were added in sequence to filtered

pipette solution by vortexing. Patch pipettes had resistances of 2-5 M $\Omega$ . Cells were voltage clamped using a List EPC-7 amplifier and PCLAMP software. Holding current and series resistance were determined by applying 30 msec steps to -70 mV from a holding potential of -80 mV. Data were accepted only if the series resistance remained relatively constant during the course of the experiment and did not exceed 20 M $\Omega$ . The voltage control error was always less than 10 mV.

**Lighting.** Illumination intensity was controlled by shuttering the 100 W halogen transmitted light source on the microscope and was 200-500  $\mu$ E m<sup>-2</sup> s<sup>-1</sup>, as measured with a Biospherical Instruments QSL-100 (San Diego, CA) visible light meter. This is typical for light microscopes and is 2-40 times more intense than natural or artificial interior lighting in our laboratory.

Calcium signals measured in response to carbachol in cells loaded with the Ca indicator dye fura-2 are shown in Fig. 1. The column at the left illustrates the initial responses in three cells. Response amplitude is defined as the change in  $[Ca]_i$  from baseline 15 seconds after application of 1 mM carbachol. In the first row, it is seen that a 10 min pretreatment with phorbol ester (phorbol 13-myristate 14-acetate, PMA; 1 nM) caused a large reduction in the Ca response (average change  $-36.7 \pm 9.7\%$  mean  $\pm$  SEM, 11 cells). Thirty min later, the response changed by  $-58.4 \pm 8.1\%$  from its original amplitude.

The cell in the second row was microinjected with the pseudosubstrate inhibitor peptide PKC(19–36) [5]. PMA caused no change in the Ca response ( $+8.9 \pm 31.5\%$ , 5 cells) at 10 min and a small decrease after 30 min ( $-31.1 \pm 20.8\%$ ). The cell in the third row was treated with 30 nM calphostin A and exposed to natural daytime illumination for 30 min at the beginning of the experiment. Like the peptide inhibitor, calphostin A blocked the phorbol ester effect, with no significant change in Ca release at 10 min ( $+3.1 \pm 5.4\%$ ) or at 30 min ( $+18.7 \pm 14.6\%$ ; 27 cells). This shows that calphostin A and illumination together block protein kinase C.

At higher doses of calphostin A we began to observe a progressive failure of Ca homeostasis. Fig. 2 shows  $[Ca]_i$  in a cell after treatment with 1  $\mu$ M calphostin A and brightfield illumination. In this cell,  $[Ca]_i$  rose from about 40 nM to over 100 nM within 10 min of drug and light treatment. By one hour,  $[Ca]_i$  had risen to over 300 nM. At this time the chamber was perfused with zero-Ca saline and  $[Ca]_i$  dropped approximately to the pre-drug level.  $[Ca]_i$  rose again as soon as normal saline was reintroduced into the chamber. Cells injected with pseudosubstrate peptide did not have elevated  $[Ca]_i$ , showing that the toxic effect of calphostin A does not arise from block of protein kinase C. Rather, calphostin A appears to disrupt the normal balance between membrane Ca leak and Ca removal by sequestration or pumping. Treatment of cells with 1  $\mu$ M calphostin A and 4–10 min of tungsten illumination caused the average  $[Ca]_i$  to rise from 137 nM to 187 nM, a change of  $+43.3 \pm 4.4\%$  (85 cells in 3 experiments). This level of drug and illumination also reduced the amplitude of the agonist-evoked Ca release ( $-44.5 \pm 3.8\%$ , 26 cells). Thirty min later, the average change in resting  $[Ca]_i$  was even higher ( $+93.9 \pm 20.1\%$ , 6 cells). Without illumination the same dose of calphostin A was without significant effect on

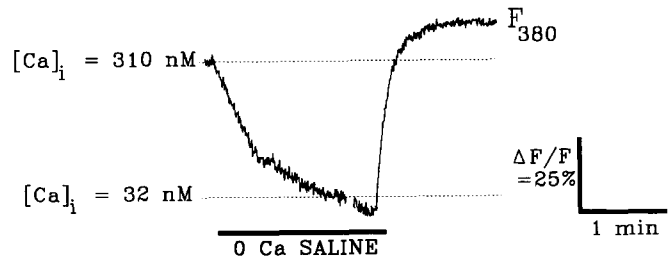


Fig. 2. Failure of Ca homeostasis in a cell bathed in calphostin A. A cell loaded with fura-2/AM was exposed to 1  $\mu$ M calphostin A. Resting  $[Ca]_i$  was originally approximately 40 nM. The recording was taken 1 h after 10 min of brightfield illumination. Normal saline was replaced with 0 Ca saline as indicated by the horizontal bar. Fluorescence at 380 nm illumination is plotted with upward deflection indicating decreased fluorescence and increased  $[Ca]_i$ .

resting calcium ( $+7.2 \pm 5.9\%$ , 119 cells) or Ca release ( $+7.2 \pm 29.1\%$ , 28 cells) for up to 30 min exposure.

If calphostin A elevates calcium by making the membrane more permeable, then this should be visible under voltage clamp as an increase in leak current. We tested this idea in whole-cell voltage clamp experiments (Fig. 3A). Addition of calphostin A (1  $\mu$ M) to the bath in darkness caused the holding current needed to maintain the voltage at  $-80$  mV to increase a small amount and then stabilize. However, when the brightfield illumination was turned on the inward holding current increased steadily until it saturated the amplifier (average rate of rise  $47 \pm 19$  pA/min, 5 cells). At the same time we also observed a progressive removal of sodium current inactivation ( $n = 2$ , not shown), consistent with previous observations with photodynamic drugs in squid axon [14].

If the effect of calphostin A on leak current results from free radical or singlet oxygen generation, it should be reduced by preincubating cells with an antioxidant. In the presence of 100  $\mu$ M  $\alpha$ -tocopherol (Fig. 3B), cal-

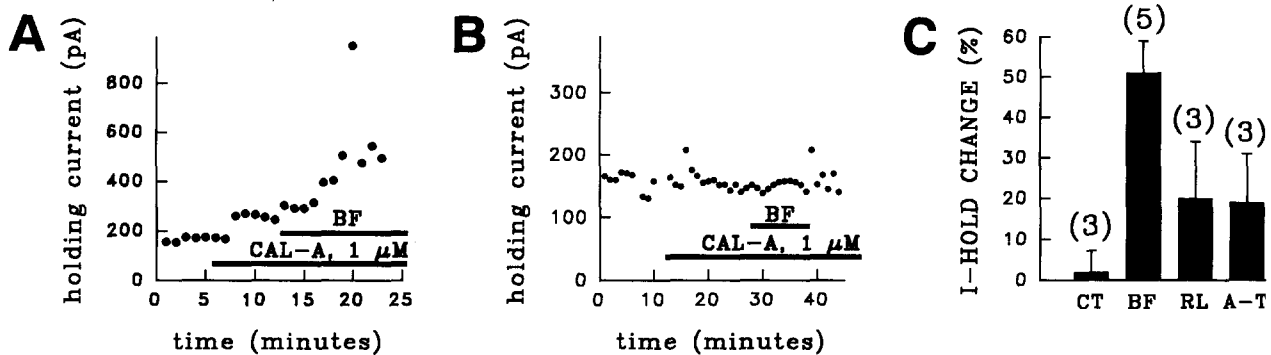


Fig. 3. Free radical-dependent changes in holding current in cells exposed to calphostin A. Cells were patch clamped with the nystatin method and the holding current was measured at  $-80$  mV membrane potential. Holding current in cells exposed to calphostin A (1  $\mu$ M) (A) alone and (B) after preincubation in the free radical scavenger  $\alpha$ -tocopherol (100  $\mu$ M) for 15 min. C: summarized average changes in holding current after 5 min exposure to brightfield illumination or calphostin A alone (control, CT); and 1  $\mu$ M calphostin A plus brightfield illumination (BF), artificial room light (RL), or brightfield illumination and 100  $\mu$ M  $\alpha$ -tocopherol (A-T).

phostin A and light had no effect on holding current after 5 min (change in current =  $+19 \pm 12\%$ ;  $n = 3$ ). Results from 14 patch experiments are summarized in Fig. 3C. In contrast, in fura-2 experiments we were unable to prevent the PKC-blocking activity of calphostin A with antioxidants (0.6% 2-mercaptoethanol or 0.3 mM  $\alpha$ -tocopherol). Consistent with this, 2-mercaptoethanol blocks calphostin A inhibition of PKC $\beta$ 1 activity by only 20% in a lysed-cell assay [M. Stahl, personal communication]. This evidence suggests that preincubation of cells in antioxidants will prevent the toxic effects of free radical generation while preserving the PKC-blocking ability of the calphostins. This implies that calphostins can be used as specific PKC inhibitors in the presence of antioxidants. However, this must be tested on a case-by-case basis.

We have shown that light and calphostin A together irreversibly inhibit PKC, but at higher light or drug levels there is damage to calcium homeostasis and membrane integrity. Past studies have established that perylenequinone compounds such as the calphostins generate singlet oxygen upon illumination [19], and that free radicals and singlet oxygen can disturb intracellular Ca homeostasis [1, 15]. It is likely that the damaging effects of calphostin A result in large part from singlet oxygen or free radical production [2].

Interestingly, antioxidants prevent damage to cells but are not very effective at preventing the PKC-blocking action of calphostins. One possibility is that light converts calphostin to an endoperoxide that interacts covalently and irreversibly with PKC [2]. The other is that calphostin produces reactive species in the membrane in such close proximity to the kinase that antioxidants are unable to stop its action. Free radicals and singlet oxygen will attack proteins that contain cysteine residues as structural elements, such as PKC; plasma membrane Ca-ATPase; and diacylglycerol kinase, which has structural homology to PKC and is also inhibited by calphostin C [8].

We conclude that when a cellular signal is blocked by a calphostin, this is consistent with the hypothesis that the signal can be blocked by free radicals or singlet oxygen. Unfortunately, this does not necessarily mean that the site of action is protein kinase C, or even a similar protein.

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