Response of Cardiac Myocytes to a Ramp Increase of Diacylglycerol Generated by Photolysis of a Novel Caged Diacylglycerol

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ABSTRACT To test the responsiveness of living cells to the intracellular messenger diacylglycerol, we developed a prototype caged diacylglycerol compound, 3-*O*-(α-carboxyl-2,4-dinitrobenzyl)-1,2-dioctanoyl-*rac*-glycerol (designated α-carboxyl caged diC_8), that produces dioctanoylglycerol (diC_8) on photolysis. α-Carboxyl caged diC_8 is biologically inert toward diacylglycerol kinase and protein kinase C in vitro and is readily incorporated into cardiac myocyte membranes, where it has no effect before irradiation. Exposure to near-UV light releases biologically active diC_8 in good yield (quantum efficiency = 0.2). Here we examine a cellular response to controlled elevation of diC_8 within single cardiac myocytes. Twitch amplitude was monitored in electrically stimulated myocytes, and a ramp increase in the concentration of diC_8 was generated by continuous irradiation of cells loaded with the caged compound. The myocyte response was biphasic with a positive inotropic phase (39% increase in twitch amplitude), followed by a large negative inotropic phase (>80% decrease). The time to peak inotropy for both phases depended on the light intensity, decreasing from 376 ± 51 s to 44 ± 5 s (positive phase) and 422 ± 118 s to 51 ± 9 s (negative phase) as the light intensity was increased eightfold. Both phases were inhibited by the protein kinase C inhibitor chelethyrine chloride. An increase in extracellular K^+ from 5 mM to 20 mM to partially depolarize the cell membrane eliminated the positive inotropic phase, but the negative inotropic response was largely unaltered. The results reveal new features in the response of cardiac muscle to diacylglycerol, including a positive inotropic phase and a complex responsiveness to a simple linear increase in diacylglycerol. The effects of photoreleased diC_8 were similar to the effects of opiate agonists selective for kappa receptors, consistent with a major role for diacylglycerol in these responses.

INTRODUCTION

Diacylglycerols represent a major class of intracellular messengers thought to regulate many different cellular processes (Berridge, 1987; Nishizuka, 1988). Diacylglycerols are liberated from membrane phospholipids by phospholipases, which in many cases are controlled by cell surface receptors. The functional role(s) of diacylglycerol in cells is often addressed by the use of phorbol esters, which mimic at least some of the actions of diacylglycerol, such as activation of protein kinase C (Davis et al., 1985; Ebeling et al., 1985). However, phorbol esters typically activate protein kinase C in an irreversible manner, perhaps more strongly than do diacylglycerols, which may mask important features of diacylglycerol action. In addition, diacylglycerols have been shown to elicit biological effects that are independent of protein kinase C (Hockberger, 1989). Previous studies with cardiac myocytes have shown that phorbol esters can induce either an increase (positive inotropy) or decrease (negative inotropy) in twitch amplitude, depending upon the concentration (MacLeod and Harding, 1991; Ward and Moffat, 1992; reviewed in Puceat and Brown, 1994). It has been difficult to examine a range of diacylglycerol concentrations, in part because of poor access to the cellular site of action and uncertainties about the efficiency of diacylglycerol incorporation into cell membranes. We describe here an approach involving the use of photolabile caged forms of diacylglycerols that circumvents these problems and allows diacylglycerols to be introduced into cellular systems in a controlled way. First, we developed a novel caged derivative of dioctanoylglycerol (diC_8) that is partially soluble in aqueous buffers and characterized its biological properties in vitro. Second, we monitored incorporation into cells with radiolabeled forms and showed that this process was rapid and efficient. Third, we demonstrated the utility of the compound by creating a ramp increase of diacylglycerol concentration within individual cardiac myocytes using continuous near-UV irradiation. The myocyte response was complex, displaying both positive and negative inotropic phases, and was dependent upon several factors, including protein kinase C, light intensity, and extracellular K^+. Calibration of the photolysis source permitted estimation of intracellular diacylglycerol levels that elicited positive and negative inotropic phases. The response at an intermediate light intensity matched the response of the myoctes to the kappa opiate agonist U50,488H, and we propose that the complex response to this agonist reflects a complex response of myocytes to a steadily increasing concentration of diacylglycerol. Some of this work has been presented in preliminary form (Huang and Walker, 1994; Sreekumar et al., 1995).

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MATERIALS AND METHODS

Chemicals for synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Lipids were from Avanti Polar Lipids (Alabaster, AL), and biochemicals were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Thin-layer

chromatography was carried out on silica gel K60 F_{254} plates (Merck, Darmstadt, Germany). High-performance liquid chromatography (HPLC) was performed with a pair of Beckman 110B pumps, a 210A sample injector, and a 160 absorbance detector at 254 nm or 280 nm. Analytical HPLC was carried out with a 4.6 mm × 250 mm ODS-3 column (Whatman, Clifton, NJ) and eluted with acetonitrile in water. Preparative HPLC was carried out with a 25-cm Magnum 9 semipreparative ODS-3 column (Whatman) eluted with acetonitrile in water. Solvent mixtures and data acquisition were controlled by Beckman System Gold software.

Synthesis

Details of chemical synthesis and photochemistry will be given elsewhere. Briefly, the caged group was coupled to the glycerol backbone by treatment of isopropylidene glycerol (also called solketal or *rac*-2,2-dimethyl-1,3-dioxolane-4-methanol) with diazo-[2,4-dinitrophenyl]-acetic acid methyl ester. Diazo-[2,4-dinitrophenyl]-acetic acid methyl ester was synthesized by the method of Regitz (1965). The isopropylidene protecting group was removed by acid hydrolysis to give caged glycerol, then acyl chains were added by treatment with octanoyl chloride in pyridine. The resulting α-carboxyl caged dioctanoylglycerol methyl ester was obtained in 40% overall yield. UV-Vis spectrum (ethanol): λ_{max} = 245 nm, ϵ = 10,400 M^{-1} cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 8.9 (s, 1H), 8.5 (d, 1H), 8.1(d, 1H), 5.75 (s, 1H), 5.35 (m, 1H), 3.8–4.4 (m, 4H), 3.7 (s, 3H), 2.3 (t, 4H), 1.6 (m, 4H), 1.25 (m, 16H), 0.85 (t, 6H). FAB mass spectrometry: *m/z* = 583.2 (protonated molecular ion), calculated for $[C_{28}H_{42}O_{11}N_2^+]$ 582.6. The final product, α-carboxyl caged diC_8, was prepared by demethylation using LiI in anhydrous pyridine. Analysis of aliquots of the reaction at various times showed a smooth conversion of a 12.3 min peak to a 10.7 min peak on reverse-phase HPLC in 70% acetonitrile. ^{1}H-NMR in d_6-DMSO was identical to the precursor, except that the singlet at δ 3.7 was absent. Flash photolysis of α-carboxyl caged diC_8 in 50% ethanol, 50 mM Tris (pH 7) showed a presumed *aci*-nitro intermediate species in the photolysis reaction with a peak absorbance at 500 nm and a decay half-time of 1.7 ms. This was taken as the rate-limiting dark reaction for the release of diC_8 from the caged compound.

Radiolabeled α-carboxyl caged diC_8 was prepared by conversion of [1-^{14}C]octanoic acid (NEN DuPont; 10 μCi in ethanol) to octanoyl chloride using oxylyl chloride. Caged glycerol and dry pyridine were added under nitrogen. After demethylation and purification, the specific activity was determined to be 10–100 μCi/nmol.

Protein kinase C assay

Protein kinase C was purified from rat brain according to published procedures (Da Silva et al., 1990; Huang et al., 1993). Enzyme activity was assayed as described (Huang et al., 1993) with some modifications. One hundred twenty-five microliters of reaction mixture contained 20 mM Tris-HCl buffer (pH 7.5), 4.8 mM magnesium acetate, 15 μg histone type III-S, 10–100 μM phosphatidylserine (PS), 1–10 μM $CaCl_2$, 20 μM [γ-^{32}P]ATP with a specific activity of 150–200 cpm/pmol, and 15 ng of enzyme diluted in distilled water. PS and diC_8 were dried from chloroform under N_2 and sonicated on ice for 5 min in 20 mM Tris-HCl (pH 7.5). Caged diC_8 was solubilized in 20 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol. Phosphorylation was carried out at 30°C for 5 min. To stop the reaction, 50-μl aliquots of the incubation mixture were loaded onto squares of Whatman P81 paper and then washed with 1% phosphoric acid five times. Radioactivity was quantified by liquid scintillation counting using a Packard Tri-Carb 4640 counter. Enzyme activity was expressed as picomoles of ^{32}P transferred to histone. Protein kinase C autophosphorylation was carried out under the same conditions described above, except that 150 ng of enzyme was added and histone was omitted. The enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7.5% acrylamide, and ^{32}P labeling was detected by autoradiography.

Diacylglycerol kinase assay

A standard procedure was used to measure the metabolism of caged diC_8 and diC_8 by diacylglycerol kinase (Preiss et al., 1986). Under the conditions of the assay, the kinase quantitatively converts biologically active 1,2-*sn*-diacylglycerol to [^{32}P]PA in the presence of [γ-^{32}P]ATP. A standard curve was linear between 0 and 1000 pmol/tube of 1,2-*sn*-diC_8. Standard samples and caged diC_8 samples before and after photolysis were dried by evaporation with a stream of N_2, then mixed with 20 μl of detergent solution containing 7.5% *n*-octyl-β-glucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid. The lipid/detergent mixtures were vortexed and sonicated for 2 min. To each tube was added 70 μl of 1 mg/ml diacylglycerol kinase from *Escherichia coli* membranes (Calbiochem, La Jolla, CA), and the reaction was initiated by the addition of 10 μl of 5 mM ATP containing 1 μCi of [γ-^{32}P]ATP to each tube. After 30 min of incubation at 25°C the reaction was stopped by the addition of 20 μl of 1% (v/v) perchloric acid. PA was extracted by the addition of 450 μl of chloroform:methanol (1:2, v/v) to each tube and quantified by scintillation counting of the organic phase or, in some cases, after thin-layer chromatography followed by scraping and counting the PA region.

Cardiac myocytes

Ventricular myocytes were isolated from 200-g adult female Sprague-Dawley rats by retrograde perfusion of the heart with collagenase and hyaluronidase as described (Haworth et al., 1989). For physiological measurements, myocytes were allowed to settle onto the glass surface of a perfusion chamber in 1 mM Ca^{2+}, 5 mM K^+ Ringer's (1 mM $CaCl_2$, 118 mM NaCl, 5 mM KCl, 2 mM Na_2HPO_4, 1.2 mM $MgCl_2$, 5 mM pyruvate, 1 mM insulin, 11 mM glucose, 25 mM HEPES adjusted to pH 7.4 with NaOH). The composition of 20 mM K^+ Ringer's was identical, except that 15 mM NaCl was replaced with KCl. The myocyte chamber consisted of a plexiglass plate containing a 1 cm × 1.5 cm well with a glass floor, and inlet and outlet stainless steel tubes for perfusion. A two-channel peristaltic pump was used to perfuse the chamber at 0.1 to 3 ml/min. Platinum electrodes 1.5 cm in length were located along opposite walls of the chamber and wired to a Grass SD9 stimulator (Quincy, MA). For electrical field stimulation the protocol was typically 30–60 V (twice threshold), 1 ms duration, 0.4 Hz at 21–23°C. The chamber assembly was mounted on a Nikon Diphot-TMD inverted microscope, which in turn was mounted on a TMC vibration isolation table (1-Hz cutoff frequency; Peabody, MA). For photolysis, the field of view was irradiated by a 450-W xenon arc lamp through a light port, which contained an epifluorescence arm, a 400-nm dichroic mirror, and a filter wheel that alternated the wavelength between 340 nm and 380 nm at 500 Hz. Cells were observed through a Nikon Fluo APO UV objective (40×, 0.7 NA) and displayed on a video monitor with a Panasonic charge-coupled device/TV video camera. The analog signal from the camera was fed into a JVC Super VHS video cassette recorder and then into a 486 computer for digitization by a Matrox MVP-AT frame grabber controlled by Image 1 software (Universal Imaging Corp, West Chester, PA). For some experiments an analog video edge detector was used (model VED104; Crescent Electronics, Sandy, UT). Caged diC_8 was loaded into 1–5 × 10^5 cells/ml by incubation at a final concentration of 250–500 μM in 0.1–0.2% dimethylsulfoxide (DMSO) for >10 min at room temperature. Myocytes were in 0.5 mM Ca^{2+} Ringer's solution. Cells were then diluted fourfold into 1 mM Ca^{2+} Ringer's in the stimulation chamber and then perfused for 1–2 min to remove DMSO and unincorporated caged diC_8. Typically, a 1-min control period was recorded, then the photolysis light (alternating 340 nm and 380 nm) was introduced by opening a shutter. When the response was at maximum, the shutter was closed to terminate irradiation and the chamber was perfused with 1 mM Ca^{2+} Ringer's.

Calibration of photolysis on the microscope

The efficiency of caged diC_8 photolysis in cells under the microscope was determined by comparison with caged ATP (Walker et al., 1988). Forma-

tion of ATP was quantified in a 10-μl droplet on the microscope stage with a luciferin-luciferase based assay (catalog no. 2005; Analytical Luminescence Laboratory, San Diego, CA). Emission of photons at 562 nm was detected with a photomultiplier tube (SLM Model JD490) and related to ATP concentration by using standard ATP solutions. The standard curve was linear between 0.1 and 10 μM ATP. Irradiation was carried out for 10 to 120 s followed by a 150-s period to detect light output from luciferin-luciferase. During this detection period, the elevated light signal decayed exponentially back to the original level with a half-time of 59 ± 4 s. We attribute this decay to diffusion of ATP out of the region that was photolyzed. The peak light output was converted to ATP concentration and plotted versus irradiation time. The data were fit to an equation that describes a first-order formation of ATP combined with a first-order diffusion out of the irradiated region (1):

$$pA_o e^{-pt}(1 - e^{(p-d)t})/(d - p), \quad (1)$$

where p is the apparent rate constant for caged ATP photolysis (i.e., fraction of caged ATP photolyzed per min), A_o is the initial concentration of caged ATP, d is the rate constant for diffusion, and t is the irradiation time. The best fit was obtained for $p = 6 \times 10^{-3}$ min^{-1} and $d = 1.1$ min^{-1}, consistent with the independently measured diffusion half-time of 59 s. The low apparent rate constant for photolysis, equivalent to 0.6% min^{-1}, confirms that the rate of ATP formation was essentially linear in the time domain used. Factoring in the difference in quantum yield between α-carboxyl caged diC$_8$ and caged ATP (0.2/0.6; assuming photolysis action spectra for caged ATP and α-carboxyl caged diC$_8$ are similar), caged diC$_8$ would photolyze at 0.2% min^{-1}, or essentially linearly over at least 50 min of irradiation.

RESULTS

α-Carboxyl caged diC$_8$ and its presumed photolysis products are illustrated in Fig. 1. The photosensitivity of α-carboxyl caged diC$_8$ was first characterized by HPLC analysis. Exposure of solutions of α-carboxyl caged diC$_8$ to near-UV light (300–360 nm) decreased the area of a 10.7 min HPLC peak in a time-dependent manner (Fig. 2). Using a continuous 75-W xenon arc lamp, the half-time for photolysis was about 1 min (Fig. 2 *C*). It should be recognized that this half-time does not reflect the intrinsic rate of photo-cleavage of the compound, which has been measured by flash

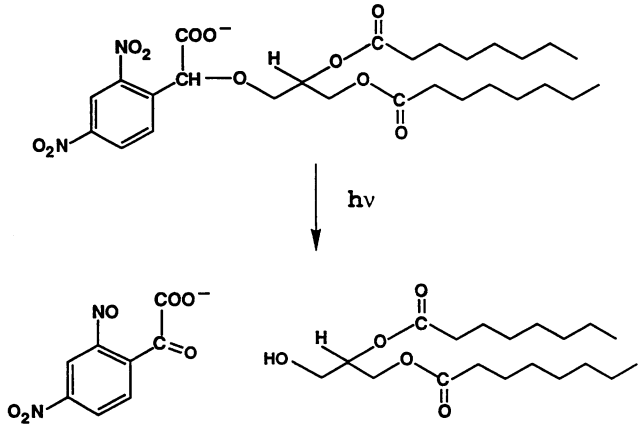

FIGURE 1 Structure and proposed photolysis reaction for α-carboxyl caged diC$_8$.

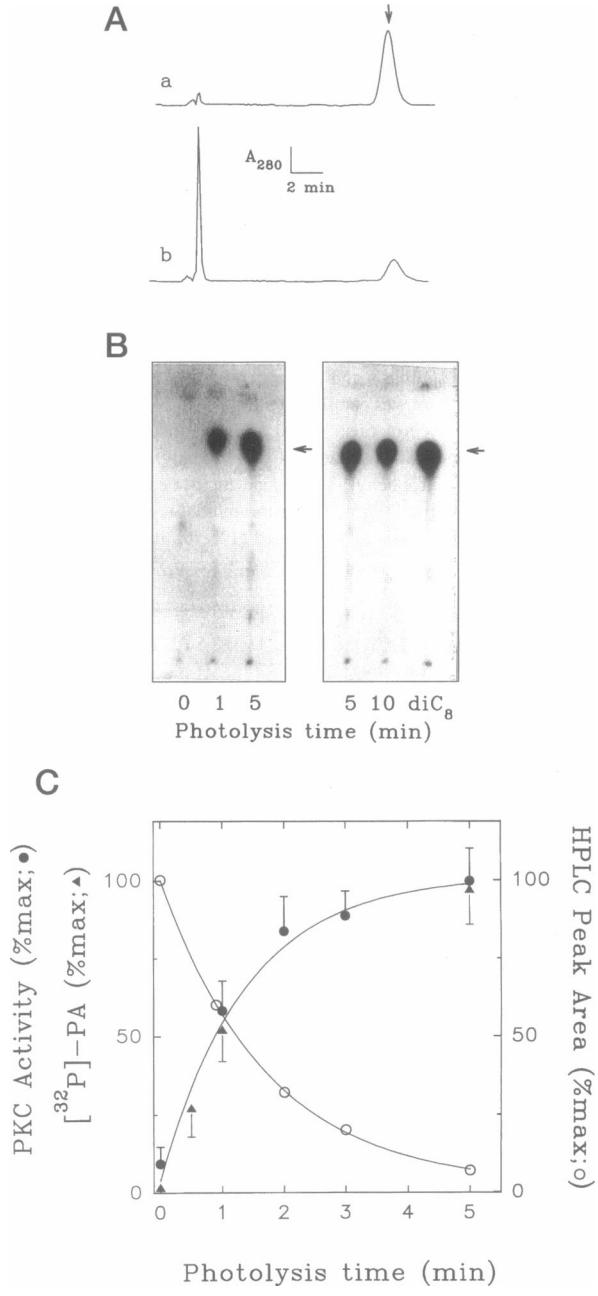

FIGURE 2 Photolysis of α-carboxyl caged diC$_8$. (*A*) HPLC analysis. α-Carboxyl caged diC$_8$ ran as a single peak with a retention time of 10.7 min on an analytical reverse-phase column in 75% acetonitrile 1.5 ml/min (*trace a*). Exposure to a 75-W xenon lamp decreased this peak, and new peaks (presumably photolysis by-products) appeared in the column void volume (*trace b*). Uncaged diC$_8$ was not detectable with the $A_{280\ nm}$ detector used. (*B*) Bioassay of photoreleased diC$_8$ by use of diacylglycerol kinase in vitro. Samples containing 500 pmol of α-carboxyl caged diC$_8$ were irradiated for the indicated times then assayed (see Materials and Methods). Thin-layer chromatograms show [^{32}P]PA (*arrows*) derived from diC$_8$. Exhaustively photolyzed α-carboxyl caged diC$_8$ produced approximately 60% of labeled PA as produced by authentic S-diC$_8$. (*C*) Time course of photolysis of α-carboxyl caged diC$_8$ detected by HPLC peak area (○), by conversion of diC$_8$ to [^{32}P]PA (▲), and by activation of protein kinase C (●). Conditions: 50 μM α-carboxyl caged diC$_8$ in 50 mM Tris, pH 7.5, was photolyzed in a 1-cm quartz cuvette through a UG11 filter, and 25-μl aliquots were injected onto the HPLC or analyzed by the diacylglycerol kinase or protein kinase C assays.

photolysis to be 400 s^{-1} (see Materials and Methods). The uncaging process in Fig. 2 is limited by the low photon density of the lamp. The photosensitivity of α-carboxyl caged diC_8 was not significantly different when measured in 100% ethanol, in 50% ethanol in water, or in 50 mM Tris (pH 7.0). The quantum yield for α-carboxyl caged diC_8 was measured in 50% ethanol, 50 mM Tris (pH 7), 22°C, by relating its photosensitivity to 1(2-nitrophenyl)ethyl phosphate as described (Walker et al., 1993). The quantum yield was 0.2 under these conditions.

Biological properties in vitro

Photolysis of α-carboxyl caged diC_8 produced free diC_8, as measured by the formation of ^{32}P-labeled phosphatidic acid (PA) in the presence of diacylglycerol kinase and [γ-^{32}P]ATP (Fig. 2, *B* and *C*). Before photolysis, PA formation was low, indicating that caged diacylglycerol is not a substrate for diacylglycerol kinase. This is expected and confirms that the caged moiety blocks the free hydroxyl on diacylglycerol. It also shows that contamination by free diacylglycerol was low. Complete photolysis of α-carboxyl caged diC_8 gave about 50–60% of the [^{32}P]PA of authentic S-diC_8, presumably because the caged compound releases racemic diC_8, of which only the S-enantiomer is recognized by the kinase.

Protein kinase C, a major target of diC_8 action, was activated after photolysis of α-carboxyl caged diC_8, further indicating that biologically active diC_8 was released from the compound (Figs. 2 *C* and 3). The time course of photolysis assessed by the activation of protein kinase C matched the time course measured by HPLC analysis and by the formation of [^{32}P]PA (Fig. 2 *C*). Protein kinase C activity before photolysis was low, if care was taken to purify the final product away from contaminating free diC_8. Activation of protein kinase C by α-carboxyl caged diC_8 photolysis displayed many of the properties expected of protein kinase C activation by authentic diC_8 (Nishizuka, 1988; Huang et al., 1993; Hannun et al., 1985). The dose dependence of protein kinase C activation was normal, showing a K_d in the low micromolar range and only a modest degree of cooperativity (Fig. 3). Photoreleased diC_8 activated protein kinase C less than authentic S-diC_8 and with a slightly higher K_d, presumably because only the S-enantiomer of the racemic photoreleased diC_8 potently stimulates protein kinase C activity (Rando and Young, 1984). There was no indication that unphotolyzed α-carboxyl caged diC_8 inhibited protein kinase C activity. The dose-response relationship for diC_8 was unaltered in the presence of 50 μM α-carboxyl caged diC_8 (Fig. 3). Autophosphorylation, a characteristic of protein kinase C activation by phorbol esters and diacylglycerols (Nishizuka, 1988), also occurred after photolysis of α-carboxyl caged diC_8 (Fig. 3, *inset*). Overall, these measurements indicate that authentic diC_8 is released from α-carboxyl caged diC_8 in good yield, and that the photolysis procedure or by-products do not interfere with protein kinase C activation in a detectable way.

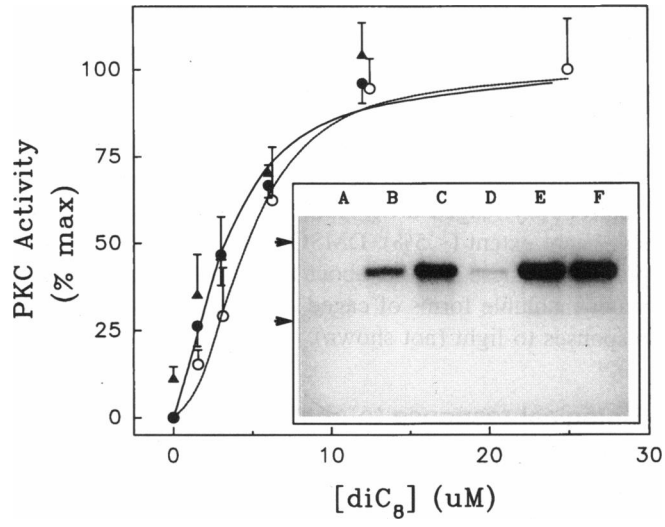

FIGURE 3 Dose-response curves for activation of protein kinase C in vitro. Indicated concentrations of diC_8 were obtained by the addition of exhaustively photolyzed α-carboxyl caged diC_8 (5–10-min irradiation) then tested for protein kinase C activity (○). Line shows the fit to a Hill equation with K_d = 4.8 μM and n_H = 1.9. The dose-response curve for authentic S-diC_8 is shown for comparison in both the absence (●) and presence (▲) of 50 μM unphotolyzed α-carboxyl caged diC_8. Line shows the fit to a Hill equation with K_d = 3.2 μM and n_H = 1.5. Data are mean ± SD for three independent assays. (*Inset*) Autoradiogram of a gel to monitor autophosphorylation of protein kinase C (M_r = 80,000) in vitro. Arrows show positions of molecular weight standards of M_r = 97,000 and 66,000. (*A*) No kinase; (*B*) PS plus 25 μM caged diC8 before photolysis; (*C*) PS plus caged diC_8 after photolysis; (*D*) PS alone; (*E*) PS plus authentic S-diC_8; (*F*) PS plus phorbol myristate acetate.

Incorporation into cells

Incorporation of caged diC_8 compounds into cells was monitored by scintillation counting of ^{14}C-labeled compounds. Cells (10^4-10^8 cells/ml) were incubated at 25°C or 37°C with caged [^{14}C]diC_8 for various times and then centrifuged. The cell pellet was then washed and counted. The α-carboxyl form of caged diC_8 was readily taken up by cells at ≥10^6 cells/ml; rat ventricular myocytes took up 70 ± 2% of the label and rabbit platelets took up 66 ± 5% of the label in a 5-min incubation. Longer incubations did not result in more uptake of counts into cells, suggesting that incorporation of α-carboxyl caged diC_8 was rapid under the conditions used. In the range examined, temperature had little effect on uptake. Because uptake of α-carboxyl caged diC_8 appeared to depend upon cell density, at cell densities ≤ 10^6 cells/ml, DMSO (0.1–0.2%) improved the level and consistency of caged compound incorporation to 50–70%. For this reason DMSO was used to load the compound for physiological measurements. [^{14}C]α-Carboxyl caged diC_8 could be extracted from myoyctes 1 h after loading with little indication of breakdown, showing that it was metabolically stable within the cells. Further evidence for incorpo-

ration and metabolic stability of α-carboxyl caged diC_8 was obtained when myocytes loaded with α-carboxyl caged diC_8 were exposed to UV-light. Characteristic physiological responses (described below) were observed for at least 2 h after loading. By contrast, two water-insoluble forms of caged diC_8, the methyl ester of α-carboxyl caged diC_8 and 2-nitrobenzyl caged diC_8, were not taken up by cells to a signficant extent (<5%). DMSO (1%) increased the incorporation of these forms to about 15%. Loading cells with the water-insoluble forms of caged diC_8 gave no or very weak responses to light (not shown).

Biological response to photoreleased diacylglycerol

Continuous illumination of cardiac myocytes containing α-carboxyl caged diC_8 displayed complex changes in twitch amplitude. Under near-physiological conditions of 5 mM KCl, the response was characterized by an increase in twitch amplitude followed by an abrupt decrease in twitch amplitude (Fig. 4 *A*). The positive inotropic phase averaged a 39 ± 9% ($n = 21$) increase above the control twitch amplitude, and the negative phase was always greater than an 80% decrease below the control amplitude. Some myocytes in the negative inotropic phase displayed intermittant strong twitches, as in Figs. 4 *A* and 5 *A*. The negative inotropy was reversed when illumination was terminated, and the myocyte was perfused with Ringer's solution. Perfusion of cells to wash away unincorporated caged diC_8 before the illumination period did not alter the subsequent response to illumination, nor did perfusion during illumination, suggesting that the physiological response was due to photolysis of caged diC_8 within the cells. Recovery of cells occurred without perfusion but was faster with perfusion, suggesting that washing diC_8 away from the cell surface facilitated recovery.

A series of experiments was carried out to determine the effects of varying the rate of change of diC_8 on the cellular response. This was accomplished by varying the intensity of light illumination by opening or closing exit slits on the monochromator. Variation in the light intensity by eightfold altered the time to peak inotropy for both phases by about eightfold (Table 1). The fastest responses observed were 44 ± 9 s (positive) and 51 ± 9 s (negative) at 32-nm slit width, and the slowest responses were 376 ± 51 s (positive) and 422 ± 118 s (negative) at 4-nm slit width. Regardless of the light intensity, the negative phase always developed rather abruptly and immediately after the positive phase. The time to half-maximum negative inotropy varied approximately proportionally with the light intensity (i.e., the time to half-maximum inotropy multiplied by the light intensity (1/slit width) was approximately constant; Table 1). In this sense, the negative inotropic process behaved as though it had been initiated at a specific threshold concentration of diC_8. The amplitudes of the phases appeared to be independent of the rate of change of diC_8, although small differences could have been masked by cell-to-cell variability.

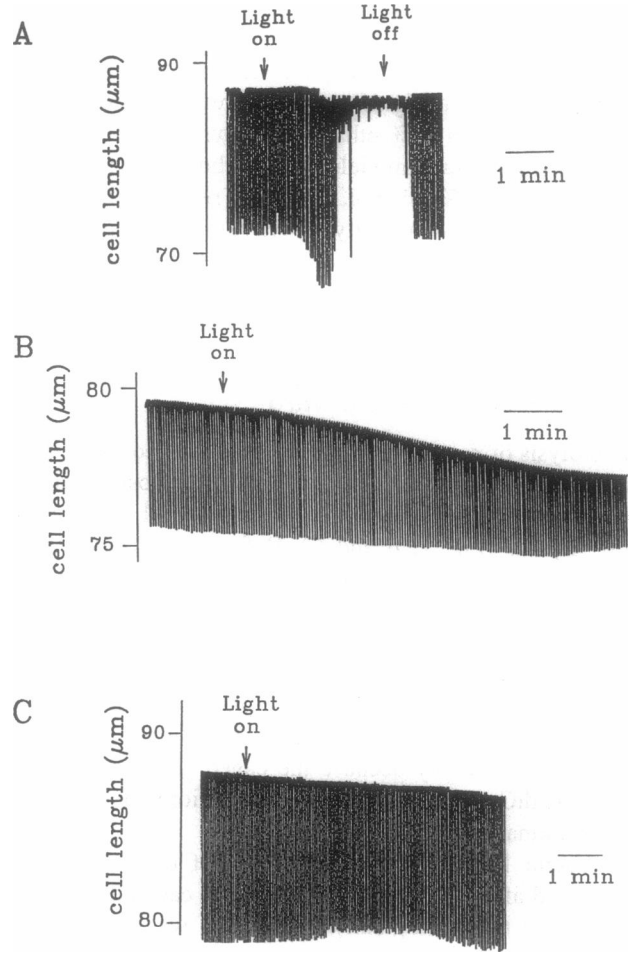

FIGURE 4 Biological effects of α-carboxyl caged diC_8 on twitch shortening of an isolated cardiac myocyte. (*A*) Cells in 5 mM K^+ Ringer's were incubated with 250 μM α-carboxyl caged diC_8 in 0.1% DMSO for 10 min and then stimulated electrically to induce twitch shortening at 22°C. The behavior of individual cells was analyzed using an analog video-based edge detector, with each vertical line in the record representing a single twitch. Arrows indicate the times when the cell was exposed to near-UV light. (*B*) Effects of near-UV illumination in the presence of α-carboxyl caged diC_8 and 10 μM chelethyrine chloride. (*C*) Effects of near-UV illumination in the presence of 250 μM α-carboxyl caged butanol loaded under the same conditions as α-carboxyl caged diC_8. Conditions: 1 mM Ca^{2+}; 0.4-Hz stimulation frequency; 40-V, 1-ms pulse duration; 22°C.

Both phases of the response to illumination were eliminated after incubation of myocytes with the protein kinase C inhibitor chelethyrine chloride (Fig. 4 *B*); similar results were obtained with the less specific protein kinase inhibitor H-7 (not shown). The positive effect appeared to be more sensitive to inhibition than the negative effect. In 12 out of 12 cells the positive phase was blocked, but in 5 out of 12 cells the negative phase developed but only after an exposure of more than 10 min. Control experiments showed that near-UV illumination by itself (without caged diC_8) for up to 30 min had no effect on twitches (not shown). Photolysis of α-carboxyl caged butanol at the same concentration was also without effect (Fig. 4 *C*), showing that by-products did not cause the observed responses.

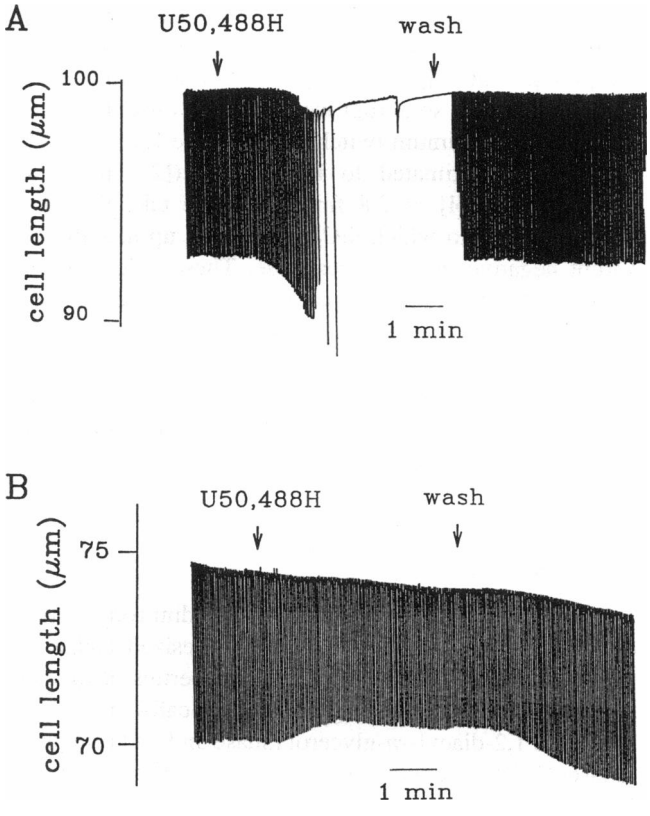

FIGURE 5 Effects of 10 μM U50,488H on twitch shortening of single cardiac myocytes in 5 mM K^+ and in the absence (*A*) and presence (*B*) of 10 μM chelethyrine chloride. The cell was perfused with U50,488H at 1 ml/min. The first arrow indicates the time the drug reached the cell; the second arrow is the time U50,488H-free Ringer's solution reached the cell. Conditions as in Fig. 4.

Response to kappa opiates and effects of extracellular K^+

To address the physiological significance of our observations with photoreleased diC_8 we examined myoycte responses to agonists known to induce strong negative inotropy. Fig. 5 *A* shows a typical response of a single myocyte to one such agonist, the kappa opiate receptor agonist U50,488H. Conditions were the same as in the caged diC_8 experiments, including temperature (21–23°C), stimulation frequency (0.4 Hz), and extracellular Ca^{2+} concentration (1 mM). Treatment with 10 μM U50,488H caused electrically stimulated twitch amplitude first to increase and then to decrease (Fig. 5 *A*). The amplitude of the positive phase was 29 ± 6% (n = 9). The time course of U50,488H action was similar to that observed with photorelease of diC_8 by an intermediate light intensity (compare U50, 488H with 16-nm slit width in Table 1). Moreover, the action of U50,488H was largely blocked by the protein kinase inhibitor chelethyrine chloride (Fig. 5 *B*), consistent with its effects being mediated by diacylglycyerol and protein kinase C.

Variation in extracellular K^+ to partially depolarize the sarcolemma has previously been shown to influence the myocyte response to agonists (Caprogrossi et al., 1991). For this reason, it was of interest to examine the influence of extracellular K^+ on the response to photoreleased diC_8 and to U50,488H. When extracellular K^+ was raised to 20 mM, the positive inotropic phase was no longer observed for either agent, whereas the large decrease in twitch amplitude remained (Fig. 6). The onset of this phase during photorelease of diC_8 still displayed a complex time course and was readily reversed when near-UV illumination was terminated. At 250 μM α-carboxyl caged diC_8 and 16-nm slit width, this large reversible negative inotropic response was observed in 35 of 39 cells. The mean time to half-maximum twitch amplitude was 152 ± 54 s. The time course of the response was dominated by a delay phase of 103 ± 78 s (n = 41), during which twitch amplitude changed by less than 10%. Thus, under conditions where the positive phase was absent, the threshold effect of diC_8 action was readily apparent. Below the threshold concentration of diC_8 there was little or no response; then the response was rapid once the threshold was reached. The time course of onset of the negative inotropic effect appears to be little affected by the level of extracellular K^+ (Table 1). Responses to U50,488H and caged diC_8 photolysis under identical conditions are summarized in Table 1. The time course of the response to 10 μM U50,488H at 20 mM K^+ was also dominated by a substantial delay (137 ± 24 s), followed by a negative phase that was half-maximum at 171 ± 45 s.

TABLE 1 Time course of biological responses in myocytes

	Slit width* (nm)	5 mM K^+ Positive‡ (s)	5 mM K^+ Negative§ (s)	20 mM K^+ Negative§ (s)
Caged diC_8	4	376 ± 51 (n = 6)	422 ± 118 (n = 6)	440 ± 97 (n = 6)
	8	133 ± 33 (n = 3)	140 ± 45 (n = 3)	ND
	16	97 ± 12 (n = 13)	113 ± 19 (n = 13)	152 ± 54 (n = 35)
	32	44 ± 9 (n = 6)	51 ± 9 (n = 6)	ND
U50, 488H	10 μM	101 ± 13 (n = 9)	123 ± 16 (n = 9)	171 ± 34 (n = 14)

Conditions: 1 mM Ca^{2+} Ringer's, 0.4 Hz, 21–23°C. Data presented as mean ± SEM; n is the number of myocytes. ND, not determined.
*Light intensity was varied by opening exits slits on monochromator; caged ATP measurements confirmed a direct relationship between slit width and effective photolysis intensity.
‡Time from start of irradiation to peak positive inotropy in seconds.
§Time from start of irradiation to half-maximum negative inotropy in seconds.

Estimation of diC_8 levels in myocytes

Time courses of cellular responses to photoreleased diC_8 are intriguing because the formation of diC_8 within the cells is expected to be first order or essentially linear with time for low levels of photoconversion. To test this assumption, caged ATP was mixed with a luciferin-luciferase cocktail to directly measure the time course of caged ATP photolysis

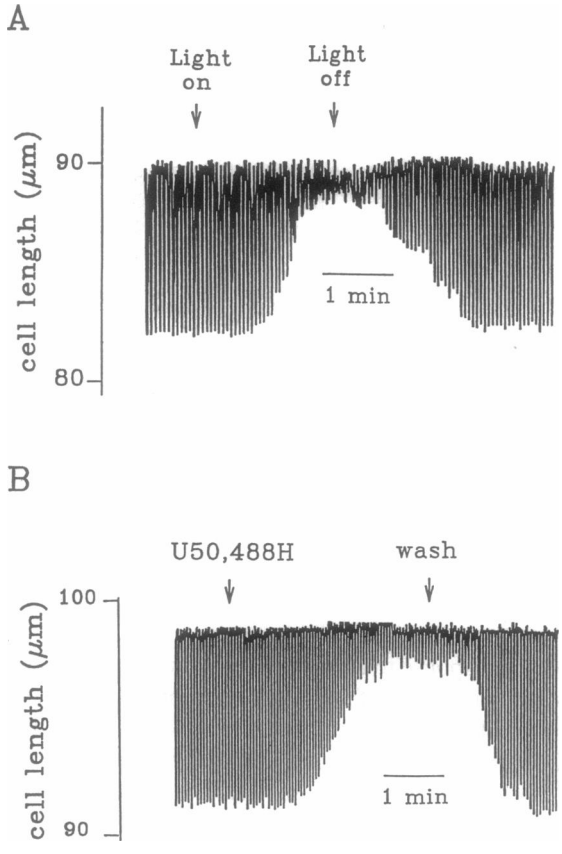

FIGURE 6 Effects of 20 mM K^+ on myocyte responses to photoreleased diC_8 (*A*) and U50,488H (*B*). Conditions and cell treatments were the same as in Figs. 4 *A* and 5 *A*, except that 1 mM Ca^{2+} Ringer's solution contained 20 mM KCl. The digital edge detector and Image 1 software were used to record twitches. The same myocytes gave a biphasic response similar to that shown in Figs. 4 *A* and 5 *A* when 20 mM K^+ was replaced by 5 mM K^+ Ringer's.

on the microscope. Photolysis of 250 μM caged ATP by the 340/380-nm light source with a 16-nm slit produced ATP at 0.6% min^{-1} after correction for diffusion of ATP out of the photolyzed spot (see Materials and Methods). The time course of ATP formation appeared to be linear, as would be expected for photolysis of less than 10% of the caged compound.

This analysis also permitted the concentration of photoreleased diC_8 to be estimated during cell experiments. First, the time course of α-carboxyl caged diC_8 photolysis given in Fig. 2 appears to be first order, and so the assumption of linearity is likely to be valid (see Materials and Methods). Second, quantum yields for caged ATP and α-carboxyl caged diC_8 are known (0.6 and 0.2, respectively). Thus, during continuous irradiation of cells containing α-carboxyl caged diC_8, conversion of the caged compound to free diC_8 should occur linearly at a rate of approximately 0.2% min^{-1}. As a rough estimate, we calculate from 100 nmol α-carboxyl caged $diC_8/10^5$ cells with 50% incorporation, that each cell would contain approximately 0.5 pmol of caged compound. From the physiological responses in Table 1, we calculate the level of diC_8 that gives a peak positive response to be approximately 2 fmol ([1.6 min][0.002 min^{-1}][500 fmol] = 1.6 fmol). Because the negative phase is so abrupt we define the onset time as the time to half-maximum twitch decline. The level of diC_8 at this time is estimated to be 3 fmol ([2.7 min][0.002 min^{-1}][500 fmol] = 2.8 fmol), and we take this as the threshold level to which diC_8 must build up to initiate the abrupt negative inotropic response. These values must be interpreted with caution, in part because the precise location of diC_8 within the cell is unknown. For reference, the amount of phospholipid in a typical cardiac myocyte has been estimated to be 1 pmol (Colvin et al., 1985), and so diC_8 is exerting its biological effects at an approximate ratio of 1 to 300 phospholipids.

DISCUSSION

A new compound, 3-*O*-(α-carboxyl-2,4-dinitrobenzyl)-1,2-dioctanoyl-*rac*-glycerol, has been synthesized and termed α-carboxyl caged diC_8. The unique properties of this compound include the following. It is biologically inactive with respect to 1,2-diacyl-*sn*-glycerol kinase and rat brain protein kinase C. This supports previous work showing that the 3-OH group of diacylglycerol is essential for the stimulation of protein kinase C activity (Ganong et al., 1986). On exposure to near-UV light, biologically active diC_8 is formed with a quantum yield of 0.2. The compound is partially soluble in organic solvents and in aqueous buffers at neutral pH and is photosensitive under both conditions. The compound is readily incorporated into cell suspensions in aqueous media.

Cell-permeable diacylglycerol analogs such as diC_8 are also readily taken up into cell membranes. However, as they are taken up, they initiate biological effects and are subject to metabolism to fatty acids and phosphatidic acids, which may have additional effects. One rationale behind the development of caged diacylglycerols is to permit the incorporation process to be separated from the onset of biological responses. With regard to incorporation, the efficiency of transport of amphipathic lipid molecules (like diacylglycerols and phospholipids) into a membrane is thought to be related to the solubility of monomers in solution (Pownell and Smith, 1989). Compounds with high critical micelle concentrations should be incorporated most efficiently. Although we have not yet measured the critical micelle concentration for α-carboxyl caged diC_8, its solubility in water and its ability to enter cell membranes are likely due to the combination of a negatively charged carboxyl group and short acyl chains. By comparison, caged diC_8 with the conventional 2-nitrobenzyl caged group does not enter cells efficiently, most likely because it forms oil droplets in solution rather than dispersing in such a way that monomers can incorporate into membranes. The commonly used vehicle DMSO improved the incorporation of all forms of caged diC_8 examined, but only α-carboxyl caged diC_8 was

incorporated at a high level (>60%). The α-carboxyl caged form of diC_8 thus represents a significant advance because it is an inactive photosensitive precursor of diC_8, whose uptake into cell membranes is rapid and efficient. We expect this approach to facilitate study of the more physiologically relevant diacylglycerol species with long acyl chains because incorporation into cell membranes can be optimized independently of the onset of biological effects.

Our results with caged diC_8 confirm and extend previous work with uncaged diC_8 that showed a negative inotropic response in cardiac tissues (Caprogrossi et al., 1990; Yuan et al., 1987; Leatherman et al., 1987). Use of caged diC_8 permitted diC_8 to be elevated within cells with a known time course, which is not possible with uncaged diC_8. This revealed that cells responded in a complex manner to diC_8 even when diC_8 was elevated with a simple linear time course. One complexity was the existence of an apparent threshold in the negative inotropic response to diC_8. Under conditions of high extracellular K^+ (20 mM), the negative inotropy only developed after a significant delay. The duration of this delay was approximately inversely proportional to the light intensity, consistent with a set point below which diC_8 had no effect on contractility. Above the threshold the twitch amplitude declined abruptly at all light intensities examined. Under conditions of normal extracellular K^+ (5 mM KCl), the ramp increase of diC_8 revealed a clear positive inotropic response that preceded a similar abrupt negative inotropy. This increase in twitch amplitude in response to photoreleased diC_8 is new and has not been observed by us or by others in perfusion experiments with uncaged diC_8. It is likely that differences in the response of cardiac myocytes to diC_8 reported here are in part the result of overcoming diffusional delays, gaining better access to sites of diC_8 action, and controlling the rate of diC_8 delivery when caged diC_8 is used. Another advantage of caged diC_8 is that the level of diacylglycerol produced within the cells can be calculated from knowledge of the efficiency of incorporation of the caged compound and its rate of photolysis. We calculate that the threshold level of diC_8 for initiation of the negative inotropic phase is about 2–3 fmol per cell. Below this level there was little or no negative inotropy, and above this level the response was quite rapid. It is difficult to compare the effective diC_8 concentration in femtomoles per cell calculated here to that obtained with perfusion of uncaged diC_8, because the rate and efficiency of uncaged diC_8 incorporation are unknown.

The significance of the effects of extracellular K^+ is not clear, but it is probably related to the observation that high extracellular K^+, high extracellular Ca^{2+}, and high stimulation frequency enhance negative inotropy associated with diC_8, phorbol ester, and phenylephrine treatment (Caprogrossi et al., 1990, 1991). It has been suggested that these parameters cause intracellular Ca^{2+} to be elevated at rest, which may influence the isoform of protein kinase C activated (Caprogrossi et al., 1991).

The effects of phorbol esters on cardiac muscle contractility have been controversial. Whereas many groups report negative inotropic responses (reviewed in Puceat and Brown, 1994), two groups have detected increases in contractility under certain conditions (MacLeod and Harding, 1991; Ward and Moffat, 1992). Our results with diC_8 are most compatible with those of Ward and Moffat (1992), who showed positive inotropic responses at low concentrations of phorbol esters (10^{-12} M phorbol myristate acetate, PMA), whereas higher concentrations (10^{-6} M) led to a more readily detectable negative inotropy. However, our positive and negative inotropic responses were not well resolved from one another in time and occurred over a rather narrow range of diacylglycerol concentrations. Our negative inotropic phases were also more sensitive to protein kinase C inhibitors than those reported by Ward and Moffat (1992), but similar to those reported by Caprogrossi et al. (1990).

One important uncertainty in our experimental approach is the cellular distribution of α-carboxyl caged diC_8 and thus the location of diC_8 production on photolysis. We do not believe that this uncertainty has a large effect on the interpretation of time courses of measured physiological responses. For example, if the caged compound is restricted to the outer leaflet of the surface membrane, then photoreleased diC_8 is expected to rapidly equilibrate with both halves of the bilayer on the basis of the measured transbilayer flip-flop rate for short-chain diacylglycerols of 35 s^{-1} ($t_{1/2}$ = 20 ms) at 25°C (Hamilton et al., 1991). A number of potential sites of action of protein kinase C have been identified in cardaic tissues, including voltage-dependent Ca^{2+} channels (Lacerda et al., 1988; Dosemeci et al., 1988; Tseng and Boyden, 1991), K^+ channels (Apkon and Nerbonne, 1988), Na^+/H^+ exchangers (MacLeod and Harding, 1991; Otani et al., 1990), the sarcoplasmic reticulum (Rogers et al., 1990), and myofilament proteins (Venema and Kuo, 1993). Identifying the precise sites of action of photoreleased diC_8 will require further investigation. The responses to photoreleased diC_8 are strikingly similar to responses of the same cells to the kappa-opiate agonist U50,488H. This compound gives a positive inotropic response followed by a large, rapid, and reversible negative inotropic response in cardiac cells (Ventura et al., 1992). We confirmed this response in our myocyte preparation and found that both phases were inhibited by the protein kinase C inhibitor chelethyrine chloride (Fig. 5). The positive inotropic phase of the U50,488H response was also found to be sensitive to extracellular K^+, which strengthens the link between the actions of U50,488H and diC_8. It was speculated by Ventura et al. (1992) that the positive inotropic response to U50,488H was due to formation of inositol 1,4,5-trisphosphate ($InsP_3$) on the basis of its ability to release Ca^{2+} from the sarcoplasmic reticulum. Our results with caged diC_8 show that both the positive and negative inotropic phases can be attributed to activation of protein kinase C by diacylglycerol. These results are consistent with a number of reports that have demonstrated agonist-induced positive inotropic effects that could be blocked by inhibitors of protein kinase C (reviewed by Puceat and Brown, 1994).

On the basis of these results, we propose that an early event after kappa opiate receptor activation is stimulation of a phospholipase C that produces an approximately linear increase in diacylglycerol in the sarcolemma. Our results call into question the role of $InsP_3$ in the adult myocardium, although we cannot rule out the possibility that it contributes to positive inotropy under some conditions (e.g., neonatal cells, specific agonists) or contributes to negative inotropy by depletion of Ca^{2+} from the sarcoplasmic reticulum.

Other agonists also give rise to negative inotropic responses that develop within 1 min at 21°C, including the α-adrenergic agonist phenylephrine (Caprogrossi et al., 1991; Otani et al., 1990), platelet-activating factor (Massey et al., 1991), and angiotensin II (Dosemeci et al., 1988). Photorelease of diC_8 showed that the negative inotropy could develop within 40 s, indicating that diacylglycerols are kinetically competent to mediate such responses. More importantly, the results show that a complex time course of inotropy may be due to the intrinsic responsiveness of the system to diacylglycerol rather than to a complex time course of diacylglycerol formation.

The role played by diC_8 metabolism in the responses to photoreleased diC_8 has not been addressed. The half-life of diC_8 in adult rat myocytes has been shown to be about 10 min (2.5 μM diC_8, 37°C) because of metabolic conversion to monoacylglycerol by diacylglycerol lipase (Chuang and Severson, 1990). Thus, diC_8 breakdown would be too slow to significantly influence the time course under our experimental conditions, except perhaps at the lowest light intensity used. The influence of diC_8 metabolism on both the onset and recovery phases can be tested by the use of specific inhibitors of diacylglycerol kinase and diacylglycerol lipase. If metabolism is a major contributor, then it would be expected to be important in responses to both photoreleased diC_8 and to U50,488H.

In summary, negatively charged α-carboxyl caged groups should be useful for the incorporation and elevation of a variety of diacylglycerol species with defined temporal and spatial characteristics in intact living cells. Use of α-carboxyl caged diC_8 in myocytes has revealed a positive inotropic response to diC_8 and an apparent threshold for the negative inotropic response. This biphasic and nonlinear responsiveness to diC_8 may underlie complex time courses observed for inotropic agents in the mammalian heart.

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