Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5199–5203, June 1993
Physiology

"Caged" phenylephrine: Development and application to probe the mechanism of α-receptor-mediated vasoconstriction

(α1-adrenergic receptor/vascular smooth muscle/calcium)

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Communicated by Oliver H. Lowry, February 12, 1993

ABSTRACT **A "caged" analogue of the α-adrenergic receptor agonist phenylephrine (PE) was prepared by exploiting the 2-nitrobenzyl protecting group and using a synthetic procedure developed to permit preferential derivatization at the amino group. On isolated adult rat mesenteric arterioles, caged-PE had no measurable effects at concentrations up to 100 μM; 0.5-ms light flashes in the presence of caged-PE, however, produced marked and dose-dependent vasoconstriction. Flash-induced vasoconstrictions were blocked by the α-receptor antagonist phentolamine and were unaffected by the β-receptor antagonist propranolol, indicating that the light-induced responses reflect the selective activation of α-adrenergic receptors. After a single flash, a large transient decrease in vessel diameter was recorded, and in most vessels, this was followed by a smaller, sustained constriction. The sustained component of the contraction was selectively eliminated when Ca^{2+} was removed from the bath, which suggests that different mechanisms underlie the transient and the sustained responses to PE. The responses to single flashes of varying intensities occurred with a mean latency of 460 ms, which is consistent with the intermediacy of several steps between α-receptor activation and contraction. We anticipate that it will be possible to extend this approach to develop caged analogues of other neurotransmitters for mechanistic and kinetic studies.**

Previously, this laboratory and others (1–3) have exploited the 2-nitrobenzyl protecting group (see Fig. 1) in the development of "caged" analogues of a variety of intracellular second messengers. The rationale for this approach was the anticipated spatial and temporal resolution afforded by controlling the intracellular messenger application with light, as compared to more conventional techniques. There are, however, a number of criteria that a caged compound need fulfill to be useful in biological experiments (1–3). First, the caged analogue should lack biological activity, so that it can be applied under steady-state conditions. Second, photorelease should proceed with reasonable quantum efficiency and be devoid of competing or side reactions. Third, photorelease should be fast and irreversible, preferably being complete in ≤1 ms. Finally, any side products produced on photolysis should be chemically inert, biologically inactive, and nontoxic. Given the success of this approach in providing caged intracellular second messengers (1–3) and our interest in the mechanisms involved in the modulation of voltage-gated ion channels by adrenergic receptor stimulation (4–6), we reasoned that this methodology could be exploited to develop caged adrenergic receptor agonists. In support of the general applicability of this approach, the 2-nitrobenzyl moiety has recently been used in the development of caged analogues of carbamylcholine, a nicotinic cholinergic receptor agonist (7, 8).

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In vascular smooth muscle, stimulation of α-adrenergic receptors produces vasoconstriction that results from an increase in the concentration of intracellular free Ca^{2+} (9). Both increased Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular stores (9–15) appear to be involved (9), although the relative importance of these two pathways varies in different species, as well as in different vessels in the same species (9). The differential involvement of postsynaptic α_1- and α_2-adrenergic receptors could be important in regulating the two pathways for increasing the intracellular free Ca^{2+} concentration, although this idea remains controversial (10, 16, 17). Alternatively, the two pathways may reflect the coupling of α_1 receptors to different intracellular second messengers (10, 16–18).

Although the detailed mechanisms have not been delineated, the α-receptor-stimulated increase in Ca^{2+} influx is believed to be mediated by a direct effect on plasmalemmal Ca^{2+} channels (9–15). Increased Ca^{2+} release from intracellular stores, on the other hand, has been proposed to result from the action of inositol 1,4,5-trisphosphate (IP_3) on intracellular Ca^{2+} channels (9–15); IP_3 is released on stimulation of α_1 receptors (9) by phospholipase C hydrolysis of phosphatidylinositol bisphosphate. Assuming that these putative mechanisms could be analyzed in detail if there was a rapid and reliable method for manipulating the extent of α-receptor activation, we designed, prepared, and characterized a photoactivatable, caged analogue of the α-receptor agonist phenylephrine (PE). Here, we describe the properties of caged-PE and demonstrate the application of this compound to investigate the mechanism(s) involved in α-receptor-mediated vasoconstriction in isolated adult rat mesenteric arterioles.

METHODS

Preparation of Caged-PE. The selective derivatization of the amine functionality of PE was achieved by treating 2-nitrobenzyl bromide with PE in dimethyl sulfoxide (DMSO). After isolation and purification, caged-PE (Fig. 1) was characterized by standard spectroscopic techniques. Details of the chemical synthesis and characterization will be described elsewhere. The site of attachment of the photolabile group on the nitrogen atom was inferred from the ^1H NMR chemical shift of the benzylic hydrogens of the 2-nitrobenzyl moiety, which appeared as a characteristic doublet of a doublet (AB pattern) at a chemical shift of ≈4.0 ppm.

For laser flash photolysis experiments, caged-PE was diluted to a final concentration of 3 mM in either 0.1 M phosphate buffer at pH 7.0 or a 90:10 mixture of 0.1 M phosphate buffer/acetonitrile (or methanol) also at pH 7.0;

Abbreviations: PE, phenylephrine; IP_3, inositol 1,4,5-trisphosphate; DMSO, dimethyl sulfoxide.

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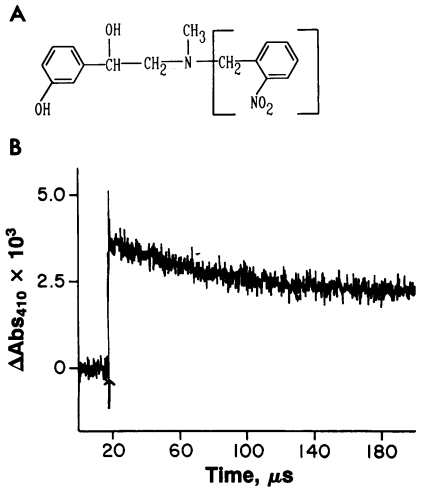

FIG. 1. Caged-PE, prepared by selective derivatization on the amine functionality of PE, undergoes rapid photodecomposition to yield free PE. (*A*) Chemical structure of caged-PE. The 2-nitrobenzyl moiety is indicated by the brackets. (*B*) Flash photolysis of caged-PE was carried out as described in *Methods*. The monitoring wavelength was 410 nm. Flashes were presented at the time indicated by the arrow, and an instantaneous change in optical density was recorded. The increase in optical density was transient; here it decayed with a time constant of 57 ± 5 μs. Similar results were obtained in five separate sets of experiments. The increase in optical density at steady state is due to the fact that the extinction coefficient of the 2-nitrosobenzaldehyde photoproduct at 410 nm is larger than that of the starting material (caged-PE).

experiments were performed at room temperature. A Q-switched neodynium:YAG laser (model YG 481, Quantel, Santa Clara, CA), providing 11-ns, 100-mJ pulses at 355 nm, was employed as the excitation light source. The transient absorbance change at 410 nm, due to the formation and the decay of the acinitro intermediate, was monitored. Results obtained in a typical experiment are illustrated in Fig. 1*B*.

Physiological Studies. Experiments were conducted on isolated adult rat mesenteric arterioles, 100–200 μm in diameter. Arterioles, free of branches, were dissected, cut into 3- to 5-mm lengths, and placed into a water-jacketed chamber (Living Systems Instruments, Burlington, VT). Glass pipettes, fabricated from 1.2-mm (outer diameter) borosilicate glass (Sutter Instruments, Novato, CA) and heat-polished to an inner tip diameter of 50–75 μm, were used to cannulate the vessels at both ends; vessels were secured to the pipettes with 11-0 nylon suture. The bath (surrounding the vessel) was perfused continuously in most experiments with buffered physiological salt solution with the following composition: 119 mM NaCl, 24 mM $NaHCO_3$, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 0.025 mM EDTA, and 5.5 mM glucose. Phenol red was added at 15 mg/liter, and the solution was bubbled with 95% O_2/5% CO_2 to a pH = 7.3–7.4. In the "0" Ca^{2+} bath solution used in the experiment in Fig. 5, the $CaCl_2$ concentration was lowered to 0.18 mM, and 0.25 mM EGTA was added to buffer the free Ca^{2+} concentration to 250 nM. In the experiments here, the luminal solution was not perfused. All experiments were performed at 36 ± 1°C. After cannulation, the pressure inside the vessel lumen was maintained at 60 mm Hg (1 mm Hg = 133 Pa) by using a pressure servo system (Living Systems Instruments). The vessels were allowed to stabilize in the chamber for 1 hr prior to beginning experiments.

Vasoconstriction and vasorelaxation were monitored by a video dimension analysis system in which the image of a vessel was directed through the video port of the microscope to a camera (Dagi–MTI, model CCD72, Michigan City, IN) connected to a video frame grabber board (Turevision Tara-M8) in a 386 25-MHz computer (Northgate) and to a video monitor (Sony model PVM 1343) and recorder (Toshiba DX-900). Simple algorithms were developed to analyze the video images and determine the inside and outside diameters of a vessel; these were measured, recorded, and displayed in real time at video rate (30 Hz). The inside and outside diameter values, displayed and recorded, were averages obtained from analyses of 100 video lines, equal to approximately a 100- to 200-μm length of the vessel.

For experiments, caged compounds were dissolved in DMSO and added to the circulating (extracellular) bath solution to produce the desired final concentration. The resulting concentration of DMSO in the vessel bath varied from 0.1% to 0.5% (vol/vol). Control experiments revealed that this concentration of DMSO had no measurable effects on vessels in the resting state or on vessels precontracted by PE. Recently, we prepared the HCl salt of caged-PE, which is highly water soluble. In future applications, therefore, it will not be necessary to use DMSO. After addition of caged-PE to the bath, vessels were allowed to equilibrate for 2–3 min. The perfusion was stopped 15 s prior to and 30 s after each flash. For photolysis, a xenon arc lamp (Hi-Tech Scientific; model XF-10), rated to deliver 1 J per flash with the capacitors charged to 385 V, was employed. The flash-lamp was mounted at the fluorescence port of the microscope, and light was directed to the vessel through a ×20 lens (numerical aperture = 0.50), using a fluorescence filter cube (Nikon UV-2A, excitation wavelength = 365 ± 10 nm) with the barrier filter removed. In the experiments here, the lamp was routinely operated at ≈350 V, although for the dose–response studies, the lamp voltage was varied over the range of 100–350 V. Flash durations were ≈0.5 ms, and ≈16% of the lamp output was in the 300- to 400-nm region, corresponding to a total intensity of ≈160 mJ over this wavelength range. In experiments conducted on a caged analogue of epinephrine, we found ≈1% conversion of the cage per flash. Given the structural similarity between caged-PE and this caged epinephrine analogue, it seems likely that the percent conversion per flash for caged-PE is similar to this value of 1%.

RESULTS

Properties of Caged-PE. The chemical structure of caged-PE is given in Fig. 1. In principle, the photolabile protecting group (i.e., the cage) could be attached to any of the functional groups of PE. Our interest was to develop a synthetic method to allow the preferential attachment of the photolabile group on the nitrogen atom of the amino group. The rationale for this selection was twofold. First, we hypothesized that blocking the amino group would reduce receptor affinity and, therefore, agonist activity. In addition, by analogy to previous findings in other systems, we speculated that photorelease from a protected amine would be rapid (refs. 1–3; see also *Discussion*), as compared to that expected from an ether oxygen.

As noted above, for a caged compound to be useful in physiological studies, it should not exhibit biological activity. Preliminary experiments, therefore, were directed toward determining whether caged-PE displayed any activity at either α- or β-adrenergic receptors. At 1 μM, PE induces maximal constriction of isolated adult rat mesenteric arterioles (Fig. 2*A*). This effect is blocked completely by the α antagonist phentolamine and is unaffected by the β antagonist propranolol (data not shown), indicating that the PE-induced vasoconstriction is mediated through activation of α-adrenergic receptors. In the same preparation, application of caged-PE at concentrations up to 100 μM, however, did not result in any measurable contraction (Fig. 2*C*), indicating that the addition of the 2-nitrobenzyl moiety eliminated α-agonist

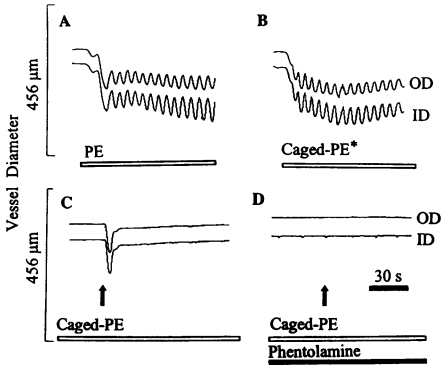

FIG. 2. Flashes in the presence of caged-PE effect transient vasoconstrictions that are blocked by phentolamine. Vessels were mounted, perfused, and allowed to stabilize, as described in *Methods*, before beginning the experiments; all perfusion solutions contained 1 μM propranolol to eliminate β-receptor-mediated effects. (*A*) Contractions produced by PE. When the control perfusion solution was switched to one containing 1 μM PE (indicated by the bar), the vessel constricted maximally. Although the contraction was maintained in the continued presence of PE, returning to the control perfusion solution resulted in relaxation of the vessel to approximately the same diameter measured prior to exposure to PE. (*B*) Bath application of a preirradiated solution of caged-PE at a concentration of 30 μM (indicated by the bar) to the same vessel produced maximal constriction that was indistinguishable from the response to PE in *A*. (*C*) However, the subsequent application of 30 μM caged-PE that had not been irradiated to the same vessel had no measurable effects on vessel diameter. When a single light flash was delivered (at the arrow), the vessel constricted (see text). (*D*) The effects of the flash (at the arrow) were blocked when 10 μM phentolamine was also included in the bath (with the 30 μM caged-PE). The ID and OD labels in this and in all subsequent figures refer to the inside and the outside diameters, respectively, of the vessel.

activity. In addition, in vessels preconstricted with 1 μM PE, no relaxation was observed after the addition of 100 μM caged-PE (data not shown), suggesting that caged-PE is also not a potent α-receptor antagonist. The structural modifications resulting from the addition of the 2-nitrobenzyl moiety, therefore, appear to prevent caged-PE from binding to α receptors. In addition, because β agonists cause relaxation of vessels preconstricted by PE (9), the absence of any effect of caged-PE on the responses to PE also suggests that caged-PE is not an agonist at smooth muscle β-adrenergic receptors.

Light flashes in the presence of caged-PE produce marked vasoconstriction (Fig. 2*C*). After a single flash, a large transient decrease in vessel diameter is recorded, and in most vessels, this is followed by a smaller, sustained constriction (Fig. 2*C*). Interestingly, the waveforms of the measured responses are markedly different than those seen in response to bath application of either PE (Fig. 2*A*) or a preirradiated solution of caged-PE (Fig. 2*B*). In particular, the constriction produced after a flash is largely transient, and the sustained component of the response is relatively small (Fig. 2*C*). Control experiments revealed that flashes in the absence of caged-PE produce no measurable changes in vessel diameter, whether presented in the absence or in the presence of PE. Similar to the vasoconstrictions produced by PE, the (transient and sustained) responses seen after light flashes in the presence of caged-PE are blocked completely when 10 μM phentolamine is included in the bath (Fig. 2*D*), indicating that the flash-induced effects are mediated by activation of α-adrenergic receptors.

In addition to releasing PE, photolysis of caged-PE yields 2-nitrosobenzaldehyde (1–3), and we were interested in determining if there were any direct effects of this photolysis by-product on the properties of isolated vessels and/or on the responses to PE. However, 2-nitrosobenzaldehyde could not be independently prepared under steady-state photolysis conditions, presumably because nitroso compounds rapidly undergo further photochemical/thermal reactions to yield azo derivatives (19). In an alternate approach, therefore, 2-nitrosobenzaldehyde was prepared *in situ* by photolysis of 2-nitrobenzyl alcohol. At a concentration of 30 μM, 2-nitrobenzyl alcohol had no measurable effects when applied either alone or to vessels preconstricted with 10 μM PE. In addition, light flashes presented during exposure to 2-nitrobenzyl alcohol in the absence and in the presence of PE did not alter vessel diameter. Thus, we conclude that the by-product (i.e., nitrosobenzaldehyde) of the photorelease of PE from caged-PE has no measurable effects in this preparation.

Kinetics of Flash-Mediated Effects. In experiments employing bath application of PE, 5–10 sec are generally required after the onset of the perfusion to observe a response, and maximal responses are seen after ≈1 min of continuous perfusion (Fig. 2*A*). Although these long latencies could simply reflect the limitations of the perfusion system and/or diffusional delays, it also seemed possible that the time course of the response could be limited by the cascade of cellular events necessary to initiate smooth muscle contraction following α-receptor stimulation (9). To distinguish between these possibilities, we examined the kinetics of vasoconstriction after single light flashes in the presence of 30 μM caged-PE. Results obtained in a typical experiment are illustrated in Fig. 3. In this example, the onset of the contraction was evident at 0.38 s, and the maximal response occurred 3.14 s after the flash. Similar results were obtained on three different vessels, yielding a mean (± SD) latency to response of 460 ± 64 ms and a mean (± SD) time to peak contraction of 2.94 ± 0.19 s.

Dose–Response Relations. To examine the dose–response relation for PE, the concentration of caged-PE in the bath was varied, and the responses to single light flashes (at 350 V) were recorded. In addition, the effects of single light flashes of varying intensities in the presence of (differing concentrations of) caged-PE were examined. As illustrated in Fig. 4, the magnitude of the peak constriction produced by single light flashes (at a constant voltage of 350 V) was dependent on the concentration of caged-PE in the bath. A saturating response to a single flash was observed at a bath concentration of caged-PE of ≈30 μM (Fig. 4). If we assume that the percent conversion for caged-PE per flash is similar to the 1% conversion observed for caged epinephrine (see *Methods*), this would correspond to a saturating dose of 0.3 μM PE, which is similar to the concentration (of 1 μM) that produces a maximal response when PE is applied directly. At each concentration of caged-PE, the peak amplitude of the contraction also varied with the flash intensity (Fig. 4*A*). The dose–response relation for PE derived from data obtained in two experiments similar to that illustrated in Fig. 4*A* is shown in Fig. 4*B*. The data are well-described by the Hill equation with $n = 0.8$. Again, assuming 1% conversion per flash, these analyses provide an estimated EC_{50} for PE of 30 nM.

Mechanism(s) Underlying the Responses to PE. As noted above, the flash experiments clearly reveal two distinct

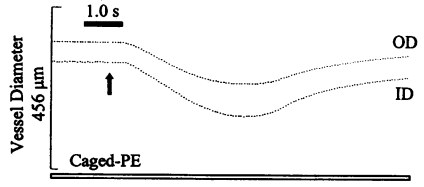

FIG. 3. Time course of PE-induced vasoconstriction after single light flashes. Experiments were performed as described in the legend of Fig. 2. After a single flash presented (at the arrow) in the presence of 30 μM caged-PE, the vessel responded with a latency of 380 ms; the peak contraction was recorded 3.14 s after the flash. Similar results were obtained in two other preparations (see text).

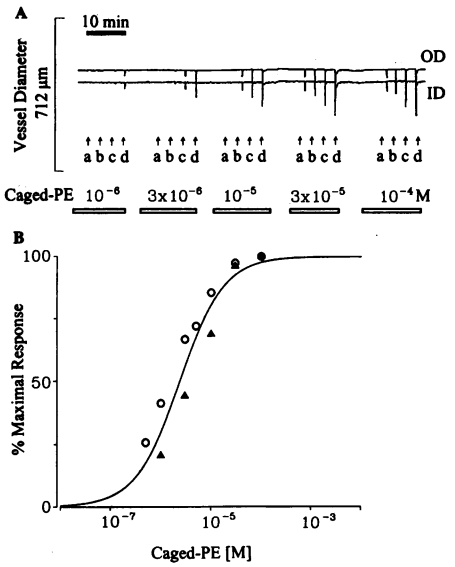

FIG. 4. Dose-dependence of PE-induced vasoconstriction. Experiments were performed as described in the legend of Fig. 2, except that the caged-PE concentration or the flash intensity was varied. (*A*) After recording the control response to PE, the vessel was exposed to increasing concentrations of caged-PE over the range of 1 to 100 μM, and the responses to single flashes (indicated by the arrows) of varying intensities were recorded. Flash intensity was changed by varying the voltage (on the power supply) to which the capacitors were charged. In the experiment illustrated here, flashes were produced with the power supply voltage at 100 V (a), 150 V (b), 200 V (c), and 350 V (d). As is evident, the amplitude of the contraction increased in parallel with the concentration of caged-PE and with the light intensity at a fixed concentration of PE. (*B*) The dose–response curve for contractions recorded in response to flashes (at 350 V) in the presence of caged-PE. The caged-PE concentrations on the abscissa are the bath concentrations of caged-PE used. Data obtained in experiments on two vessels are plotted with different symbols. The solid line is the best-fit Hill equation. Assuming that ≈1% of the cage is converted per flash (see *Methods*), these analyses provide an estimated EC_{50} for PE of 30 nM and an *n* (cooperativity factor) value equal to 0.8.

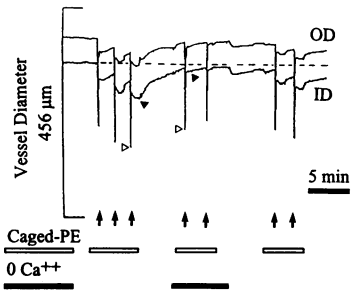

FIG. 5. Flashes in the presence of caged-PE produce transient contractions even in the absence of extracellular Ca^{2+}. Experiments were performed as described in the legend of Fig. 2, except that during the experiment (at the time indicated by the solid bar) the perfusion solution was changed to one containing 0 Ca^{2+} (see *Methods*). In the normal, Ca^{2+}-containing bath solution, the first three flashes in the presence of caged-PE evoked transient vasoconstrictions followed by slower and smaller, more sustained contractions (see also Fig. 2*C*). In each case, the vessel relaxed because the perfusion was started again after the flash, and the PE produced by the flash diffused away from receptors. For the third flash of this series, the transient and sustained components of the contraction are indicated by the open and closed arrowheads, respectively. When extracellular Ca^{2+} was removed, flashes evoked only transient responses (indicated by the open arrowhead after the first flash of this series); the sustained component of the contraction, which should be evident at the point indicated by the closed arrowhead, was selectively eliminated (see text). The loss of the sustained component of the contraction was reversed completely when the Ca^{2+} concentration in the bath solution was returned to the control level (1.8 mM).

phases of the response to PE—i.e., transient and sustained—that are not evident when PE is applied to the bath (compare Fig. 2 *A* and *B* with *C*). Interestingly, these findings suggest that there are two mechanistically distinct components underlying the measured responses to PE (Fig. 2 *A* and *B*) and that it might be possible to use caged-PE to separate these components, thereby allowing each to be analyzed in detail independently. To explore this possibility directly, the responses to single flashes in the presence of caged-PE were also examined in the absence of extracellular Ca^{2+}. As illustrated in Fig. 5, preliminary experiments reveal that the transient component of the contraction is largely unaffected by removal of extracellular Ca^{2+}, whereas the sustained component is selectively eliminated. Experiments exploiting caged-PE to explore the possibility that different receptors and/or second messenger pathways mediate the two components of PE-induced vasoconstriction will be of interest to pursue.

DISCUSSION

Properties of Caged-PE. The results presented here indicate that it is possible to mimic the vasoconstricting effects of PE by presenting light flashes in the presence of caged-PE. Although, in principle, the cage could have been attached to any one of the functional groups of PE, the synthetic strategy developed here allowed preferential derivatization at the amino group. As noted above, we selected the amine functionality based on the hypotheses that derivatizing this group would reduce agonist activity and that photorelease from a protected amine would be rapid (1–3). Although light initiates the process, mechanistic studies suggest the intermediacy of a thermal reaction in the pathway leading to release from caged compounds, and the nature of the heteroatom to which the 2-nitrobenzyl group is attached appears to affect the rate of release (1–3). Preliminary flash photolysis experiments suggest that PE is released from caged-PE rapidly, proceeding with a time constant of ≈50 μs at room temperature (21–22°C) and pH 7.4 (Fig. 1*B*). This finding and the observation that caged-PE has no measurable α- (or β-) receptor activity on isolated adult rat mesenteric arterioles suggest that the synthetic strategy was indeed a reasonable one. It will be of great interest to examine the properties of caged-PE at other α- and β-adrenergic receptors in other preparations to determine if the results obtained on isolated adult rat mesenteric arterioles can be generalized. It is also of interest to note that the synthetic methodology developed has been readily applied to the development of caged analogues of other catecholamines (S.M. and J.M.N., unpublished observations).

Mechanism of PE-Induced Vasoconstriction. Despite the rapid release of PE from caged-PE, the experiments here reveal that there is a latency of several hundred milliseconds after light flashes before a change in vessel diameter can be detected. Interestingly, this value is similar to that reported recently by Somlyo *et al.* (20) for the latency to the onset of vasoconstriction of guinea pig portal vein following light flashes in the presence of another caged-PE analogue that releases PE slowly (with a time constant of ≈300 ms at 21°C). Because our preliminary flash photolysis experiments indicate that PE is released from caged-PE in microseconds (see above), however, we can be certain that the time courses of the responses measured in our experiments are not in any way limited by the (thermal) rate of release of PE. We interpret the observed latency as indicating that there are likely several steps between PE binding to α receptors and vasoconstriction. This is consistent with previous work sug-

gesting that vascular α_1 receptors are coupled to inositol phospholipid metabolism (9). We anticipate that additional studies, using caged-PE in combination with caged analogues of GTP, IP_3, and Ca^{2+} and direct measurements of the intracellular free Ca^{2+} concentration, will delineate the detailed mechanisms and reveal the rate-limiting step in α-receptor-mediated vasoconstriction. Indeed, recent results suggest that the rate-limiting step is between α-receptor occupancy and the production of IP_3 (21).

Initially, we were somewhat surprised by the finding that the contractions recorded after light flashes in the presence of caged-PE are largely transient, lasting only a few seconds (Figs. 2*C* and 3–5), whereas contractions produced by bath application of PE are maintained (Fig. 2 *A* and *B*). Assuming that a single mechanism was involved, we interpreted the largely transient nature of the flash-induced responses as suggesting that PE rapidly dissociates from its receptor. Because the bath is large relative to the volume in which the photolysis occurs, it seemed reasonable to expect that after unbinding, PE diffuses away from the receptors and that the effective PE concentration near the receptors decreases rapidly; few receptors will be reactivated, and the vessel will relax. The sustained component of the contraction, then, would simply reflect the effective rate of PE diffusion away from receptors (i.e., PE binding and unbinding) and/or the rate of perfusion of the bath. The finding that the sustained component of the contraction is selectively eliminated when Ca^{2+} is removed from the bath, however, indicates that diffusion is not the major factor involved in determining the size and duration of the sustained contraction. In fact, this finding suggests the very interesting possibility that different mechanisms and/or different α receptors underlie the transient and sustained components of the responses to single light flashes in the presence of caged-PE. Experiments aimed at exploring the mechanisms mediating these responses in greater detail are needed.

The largely transient responses to flashes (Fig. 5) in the absence of extracellular Ca^{2+} might suggest that PE rapidly dissociates from its receptor or, alternatively, that the stores of intracellular Ca^{2+} are rapidly depleted in such a way that, in the absence of extracellular Ca^{2+}, the vessel relaxes even though the receptors remain occupied. Repeated flashes in the presence of caged-PE, however, produce responses of similar magnitudes, suggesting that the transient nature of the response likely reflects a rapid decrease in the effective PE concentration near receptors. It should be possible by using caged-PE in combination with caged analogues of the intracellular messengers IP_3 and Ca^{2+} to explore these mechanistic issues in more detail. Nevertheless, the fact that the flash-induced vasoconstrictions are largely transient suggests some mechanistic insights into the finding that contractions are maintained in the continued presence of PE. For example, the simplest interpretation of maintained contractions is that these reflect the steady-state level of binding and unbinding of PE. In addition, the experimental observations suggest that receptors must remain available for agonist rebinding in the continued presence of PE and that these receptors also must remain able to couple agonist binding to vasoconstriction. It is possible that spare receptors are involved or that PE activates the same receptors repeatedly, with no apparent desensitization. Further experiments will be needed to distinguish between these possibilities, and we anticipate that caged-PE will be extremely useful in probing the receptor-mediated mechanisms in greater detail.

We thank Dr. Christopher J. Lingle for loaning us a Hi-Tech Scientific flashlamp for some of the experiments described here. We also thank Joel Solomon and Francois Tiaho for many helpful comments and discussions. The flash photolysis experiments and the analyses of the data obtained in these experiments were performed at the Center for Fast Kinetics Research at the University of Texas at Austin. This facility is supported jointly by the Biomedical Research Technology Program of the Division of Research Resources at the National Institutes of Health (RR00886) and by the University of Texas at Austin. This work was supported by the National Heart, Lung and Blood Institute (Grant HL-34161) of the National Institutes of Health.

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