106470-84-2; ApGpCpApUpUp, 106470-85-3; ApCpGpApUpUp, 106470-86-4; ApGpCpApApUp, 106470-87-5; $A(pU)_3$, 74713-41-0; ApUpUp, 6200-33-5; pApUpUp, 106470-88-6; pApApUp, 106470-89-7; pCp, 2922-94-3; ApGpA, 3393-24-6; GpApA, 3308-20-1; pApUpGp, 82604-39-5; ApG, 3352-23-6; GpA, 6554-00-3; ApGpApC, 79507-37-2; GpApApC, 106470-90-0.

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Mobilization of Hepatic Calcium Pools by Platelet Activating Factor†

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ABSTRACT: In the perfused rat liver, platelet activating factor, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC), infusion produces an extensive but transient glycogenolytic response which at low AGEPC concentrations (i.e., 10^{-11} M) is markedly dependent upon the perfusate calcium levels. The role of calcium in the glycogenolytic response of the liver to AGEPC was investigated by assessing the effect of AGEPC on various calcium pools in the intact liver. Livers from fed rats were equilibrated with $^{45}Ca^{2+}$, and the kinetics of $^{45}Ca^{2+}$ efflux were determined in control, AGEPC-stimulated, and phenylephrine-stimulated livers during steady-state washout of $^{45}Ca^{2+}$. AGEPC treatment had only a slight if any effect on the pattern of steady-state calcium efflux from the liver, as opposed to major perturbations in the pattern of calcium efflux effected by the α-adrenergic agonist phenylephrine. Infusion of short pulses of AGEPC during the washout of $^{45}Ca^{2+}$ from labeled livers caused a transient release of $^{45}Ca^{2+}$ which was not abolished at low calcium concentrations in the perfusate. Moreover, there occurred no appreciable increase in the total calcium content in the liver perfusate at either high or low concentrations of calcium in the perfusion fluid. Infusion of latex beads, which are removed by the reticuloendothelial cells, caused the release of hepatic $^{45}Ca^{2+}$ in a fashion similar to the case with AGEPC. Our findings indicate that AGEPC does not perturb a major pool of calcium within the liver as occurs upon α-adrenergic stimulation; it is likely that AGEPC mobilizes calcium from a smaller yet very important pool, very possibly from nonparenchymal cells in the liver.

1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC), a potent phospholipid mediator of biological inflammatory responses in various cell types, e.g., basophils (Hensen & Cochrane, 1971; Pinckard et al., 1979; Benveniste, 1974), mast cells (Camussi et al., 1977; Clark et al., 1980), platelets (Demopoulos et al., 1979; Hanahan et al., 1980), and neutrophils (Clark et al., 1980; O'Flaherty et al., 1981a,b), has been shown to exert several additional biological responses in tissues, such as guinea pig heart (Burke et al., 1982) and guinea pig ileum (Findlay et al., 1981). Recently, we have

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described potent glycogenolytic effects of AGEPC in the perfused rat liver (Buxton et al., 1984a,b) and increased turnover of inositol phospholipids in isolated rat hepatocytes incubated with AGEPC (Shukla et al., 1983; Fisher et al., 1984). Exposure of the perfused rat liver to AGEPC at very low concentrations (ca. 100–1000 pM) causes enhanced glycogenolysis and oxygen consumption (Buxton et al., 1984b). In hepatocytes, AGEPC leads to a rapid, transient breakdown of phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate; however, the glycogenolytic response to AGEPC seen in perfused liver is absent in AGEPC-stimulated hepatocytes (Fisher et al., 1984). The ability of the liver to respond to AGEPC is dependent upon the perfusate calcium concentration (Buxton et al., 1984b). Even so, attempts to demonstrate a net release of calcium into the perfusate from livers stimulated with AGEPC, similar to the release of calcium seen upon stimulation by α-adrenergic agents such as phenylephrine (Blackmore et al., 1979), have been unsuccessful. The precise mechanism by which AGEPC stimulates hepatic glycogenolysis has not been elucidated. The rapid turnover of phosphatidylinositol 4,5-phosphate in hepatocytes suggests formation of inositol phosphate moieties within the cell.

In many systems, such as parotid gland (Irvine et al., 1984), insect salivary gland (Berridge, 1983), pancreatic acinar cells (Streb et al., 1984), and permeabilized guinea pig hepatocytes (Burgess et al., 1983), inositol 1,4,5-triphosphate has been implicated in the release of calcium from internal stores in the cell, possibly the endoplasmic reticulum (O'Rourke et al., 1985; Berridge & Irvine, 1984). However Charest et al. (1985) have provided preliminary evidence that AGEPC stimulation of rat hepatocytes causes no increase in cellular inositol 1,4,5-triphosphate or elevation of cytosolic Ca^{2+} levels, using QUIN-2 to estimate changes in intracellular calcium levels. Since AGEPC has been shown to inhibit purified plasma membrane (Ca^{2+}–Mg^{2+})-ATPase (Chan & Junger, 1983; Mostafa et al., 1983), the effects of AGEPC on cell calcium movements may be somewhat more complicated than those observed with phenylephrine or vasopressin.

We decided to investigate the effect of AGEPC on calcium movements in the intact perfused rat liver. The present study demonstrates that while mobilization of a small pool of calcium in the liver occurs, AGEPC stimulation of the liver is not accompanied by measurable changes in total perfusate calcium, neither demonstrable efflux nor influx. Changes in efflux of preloaded $^{45}Ca^{2+}$ precede the release of glucose from perfused livers following AGEPC stimulation. Infusion of latex beads, which are removed from the perfusate by the reticuloendothelial cells, into perfused livers leads to release of glucose and calcium in a manner similar to that seen with AGEPC infusion, suggesting a role for the reticuloendothelial cells in the response of the liver to AGEPC.

Materials and Methods

Male Sprague-Dawley rats (180–220 g), fed ad libitum, were used for all of the experiments. Livers were perfused by using a nonrecirculating perfusion system (Scholz et al., 1973) with Krebs–Henseleit bicarbonate buffer containing calcium as indicated in the individual figures, pH 7.4, equilibrated with a mixture of oxygen/carbon dioxide (95%:5%) and maintained at 37 °C. Prior to perfusion with $^{45}Ca^{2+}$, livers were perfused for 30 min to ensure the equilibration of the preparation with perfusate calcium and to wash endogenous hormones from the liver. Perfused livers were loaded with $^{45}Ca^{2+}$ for the washout experiments by infusing $^{45}Ca^{2+}$ for 50 min prior to the beginning of the washout period. Effluent perfusate was collected at specified intervals for the determination of glucose, labeled calcium, and total calcium. Glucose was determined in samples of the effluent perfusate by using the method of Bergmeyer (Bergmeyer et al., 1974). $^{45}Ca^{2+}$ was determined by liquid scintillation counting of samples of the effluent perfusate. Total calcium in the effluent perfusate was determined by atomic absorption spectroscopy.

AGEPC (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was obtained from Bachem (Budendorf, Switzerland) and was determined to be of high purity by using methods reported previously (Satouchi et al., 1981). 1-*O*-Hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine [(lyso)GEPC] was purchased from Novabiochem (Laufelfingen, Switzerland). Bovine serum albumin (2.5 mg/mL in isotonic saline) was used to complex the AGEPC and (lyso)GEPC prior to infusion. Unless otherwise indicated, AGEPC was infused into livers at a concentration of 20 nM. Latex beads, 0.913-μm diameter, were purchased from DOW Chemical Co. and were infused at a 1:4000 dilution from stock (approximately 6.6×10^7 beads/mL final concentration). $^{45}Ca^{2+}$ was obtained from ICN. All figures are representative of at least four experiments with essentially identical results.

Results

AGEPC Effects on Efflux Kinetics of $^{45}Ca^{2+}$ from Perfused Rat Liver. To gain insight into whether AGEPC affects the mobilization of calcium in perfused rat liver and to characterize the requirement of calcium for the glycogenolytic response of AGEPC, we performed experiments designed to probe the kinetics of steady-state calcium efflux from perfused liver at several perfusate calcium concentrations. Using livers equilibrated with $^{45}Ca^{2+}$, the washout of $^{45}Ca^{2+}$ from the liver was monitored, and the kinetics of $^{45}Ca^{2+}$ efflux were calculated by using compartmental analysis of the washout data. For each calcium concentration (0.05–1.25 mM), livers were loaded with $^{45}Ca^{2+}$ as described under Materials and Methods. Throughout the experiment, the perfusate calcium concentration was maintained constant. Near the end of the 50-min labeling period, effluent perfusate collection was initiated for determination of released $^{45}Ca^{2+}$ from the liver. The effects of AGEPC on the dynamics of steady-state calcium efflux were determined by initiating an infusion of AGEPC (20 nM) 5 min prior to the beginning of the washout period in order to allow the transient effects of AGEPC, i.e., vasoconstriction or perturbations in oxygen consumption, to subside or stabilize. The infusion of AGEPC continued throughout the washout period. Control livers were equilibrated with $^{45}Ca^{2+}$ in the same manner but with no stimulation applied. For comparison with stimulation by an α-adrenergic agent, an infusion of phenylephrine (10 μM) was given to the $^{45}Ca^{2+}$-labeled livers in a similar manner as with the infusion of AGEPC.

Representative washout profiles for control, AGEPC-stimulated, and phenylephrine-stimulated livers are shown in Figure 1A,B at 0.5 mM Ca^{2+} in the perfusate. Initially, there was a rapid efflux of calcium which represented the clearing of $^{45}Ca^{2+}$ from the vasculature and extracellular space. Comparison of the $^{45}Ca^{2+}$ washout of the AGEPC-treated livers with the $^{45}Ca^{2+}$ washout profile for phenylephrine-stimulated livers indicated that the effect of infusing AGEPC on the washout profile for $^{45}Ca^{2+}$ did not produce the gross changes seen with phenylephrine even though livers were exposed to AGEPC at a concentration which elicits maximal effects on oxygen consumption and glycogenolysis. At different perfusate Ca^{2+} levels (0.05 and 1.25 mM), the effect of AGEPC on the calcium washout profiles was quantitatively similar to the untreated control washouts, whereas phenylephrine always produced gross changes in the washout profile.

Table I: Fractional Transfer Coefficients for Calcium Efflux in Perfused Livers[a]

condition	f_{01}	f_{12}	f_{21}	f_{23}	f_{32}
control (n = 3)	8.725 (1.238)	0.3154 (0.0471)	0.3628 (0.0139)	0.1002 (0.0081)	0.1970 (0.0325)
AGEPC (n = 7) (20 nM)	8.459 (2.39)	0.5480 (0.2358)	0.5335 (0.2969)	0.1053 (0.0329)	0.1687 (0.0568)
phenylephrine (n = 3) (10 μM)	9.227 (1.899)	0.4079[b] (0.0529)	0.7518[b] (0.1811)	0.0314[b] (0.0275)	0.0260[c] (0.0096)

[a] Livers were perfused and samples collected as described in Figure 1. Data were analyzed by using the interactive version of SAAM, running on a VAX 11/780, using a series arrangement of compartments. The fractional transfer coefficient f_{ij} represents the fraction of compartment j transferred to compartment i per minute. Compartment 0 represents loss from the system. Numbers in parentheses represent standard deviations. [Ca^{2+}] = 500 μM. [b] $P < 0.05$. [c] $P < 0.01$.

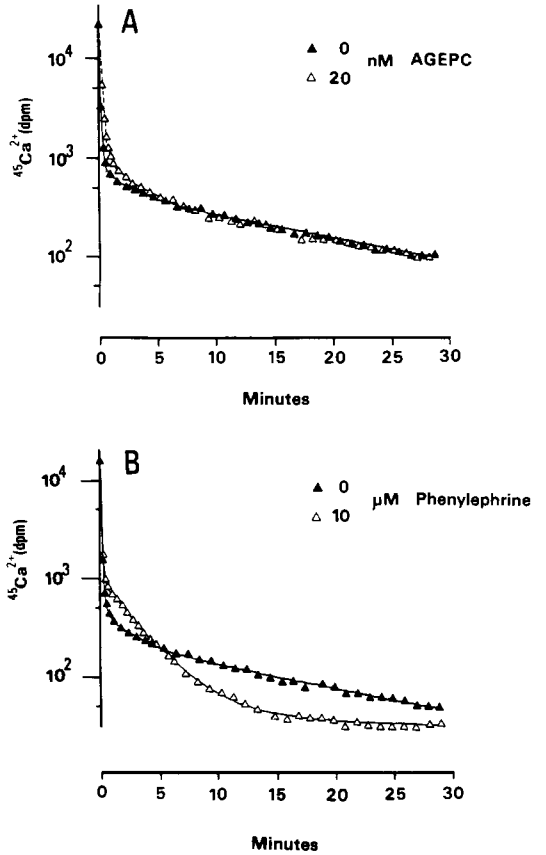

FIGURE 1: Calcium efflux from livers preloaded with $^{45}Ca^{2+}$. Livers were perfused for 30 min prior to being loaded with $^{45}Ca^{2+}$ for 50 min. Following the 50-min loading period, the infusion of $^{45}Ca^{2+}$ was terminated, and samples of the effluent perfusate were collected and analyzed for $^{45}Ca^{2+}$ by using liquid scintillation counting. Infusions of AGEPC (20 nM) or phenylephrine (10 μM) were begun 5 min prior to the commencement of washout and were continued throughout the washout period. The calcium concentration in the perfusate was 0.5 mM throughout the experiment. Samples were collected at 12-s intervals for the first 5 min of washout and thereafter at 30-s intervals. (A) AGEPC (△); control (▲). (B) Phenylephrine (△); control (▲).

The data obtained from the washout of $^{45}Ca^{2+}$ from perfused livers were used to estimate parameters describing a compartmental model of calcium movements within the liver. Using a computer program, SAAM (Berman & Weiss, 1978; Boston et al., 1981), to fit iteratively the washout data to a compartmental model of calcium in the liver, it was determined that the minimal model required three compartments to explain adequately the washout data. Data were analyzed for a three-compartment model with the compartments arranged in series, similar to a model proposed for calcium uptake by hepatocytes (Barritt et al., 1981; Parker et al., 1983). The results of fitting this model to the three sets of data in Figure 1 are shown in Table I. Fitting the data to a three-compartment model demonstrated that the fractional transfer coefficients between compartments for livers exposed to AGEPC were not significantly different from the coefficients obtained from control livers, whereas in the case of phenylephrine significant differences were observed for most of the fractional transfer coefficients. For any of the perfusate Ca^{2+} concentrations used, exposure of livers to AGEPC did not lead to pools of calcium with kinetic behavior different from control livers.

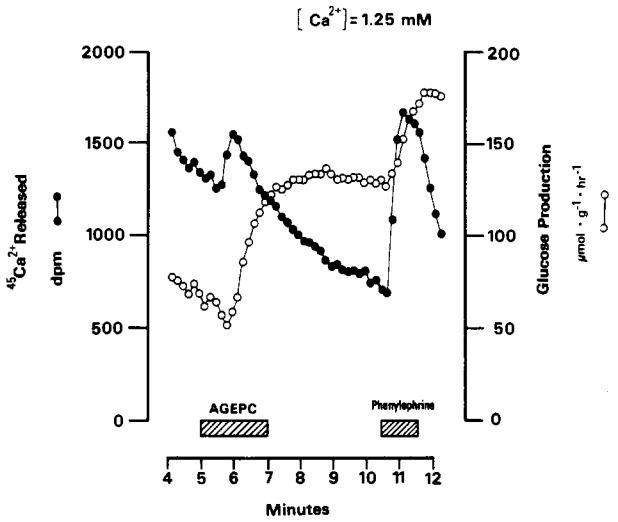

FIGURE 2: AGEPC- and phenylephrine-stimulated release of calcium and glucose from liver preloaded with $^{45}Ca^{2+}$. Livers were loaded with $^{45}Ca^{2+}$ for 50 min as described in Figure 1. After the livers were loaded, washout proceeded for 4 min prior to sample collection. At 5 min into the washout, a 2-min infusion of AGEPC (20 nM) was begun, and at 10.5 min, a 1-min infusion of phenylephrine (10 μM) was started. Samples were collected at 12-s intervals. $^{45}Ca^{2+}$ and glucose concentrations were determined as described under Materials and Methods. [Ca^{2+}] = 1.25 mM.

Transitory Effects of AGEPC on Calcium Mobilization. Since AGEPC exhibits homologous desensitization with respect to its other effects in the liver (Buxton et al., 1984b), we explored the possibility that the effects of AGEPC on cell calcium dynamics were short-lived and hence were not apparent in the steady-state washout experiments. Figure 2 shows the results of short-term exposure of livers to AGEPC and phenylephrine during the washout of $^{45}Ca^{2+}$. Livers were equilibrated with $^{45}Ca^{2+}$ as described above and were allowed to release the labeled calcium with the perfusate calcium concentration maintained constant. In order to have a stable base line from which to observe a transient release of $^{45}Ca^{2+}$, sample collection was initiated at a point 4 min after cessation of the loading period when the initial rapid phase of $^{45}Ca^{2+}$ efflux had ended. At 5 min into the washout, a 2-min infusion of AGEPC (20 nM) was started, and at 10.5 min into the washout, a 1-min infusion of phenylephrine (10 μM) was applied in order to compare the actions of the two agents. Figure 2 shows the resulting transient release of $^{45}Ca^{2+}$ from the liver as a result of the infusion of the two agents. The peak of $^{45}Ca^{2+}$ released, which occurred following infusion of AGEPC, indicated that AGEPC caused the mobilization of a pool of liver calcium, though one which was not as substantial as the release of $^{45}Ca^{2+}$ during the infusion of phenylephrine. The stimulation of glucose release by AGEPC and phenyl-

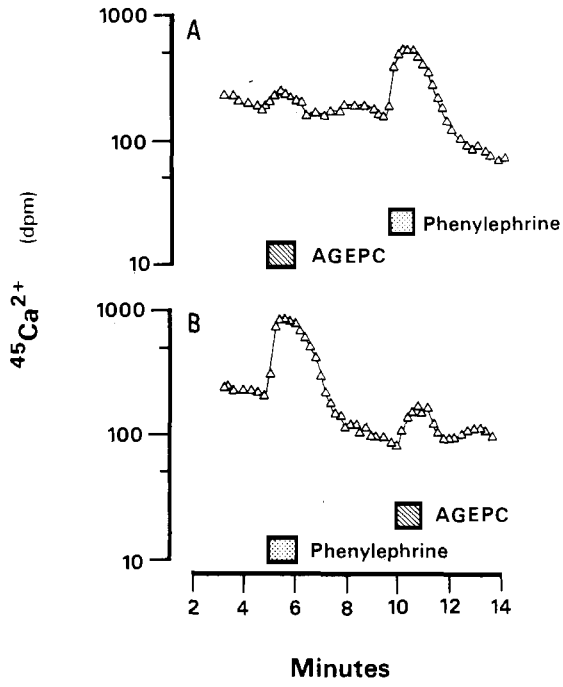

FIGURE 3: AGEPC- and phenylephrine-stimulated release of calcium from liver preloaded with $^{45}Ca^{2+}$. Livers were loaded with $^{45}Ca^{2+}$ for 50 min as described in Figure 1. Following the loading period, a 3-min period of washout occurred prior to sample collection. (A) At 5 min, a 1-min pulse of AGEPC (20 nM) was begun. At 10 min, a 1-min pulse of phenylephrine was begun. (B) The order of the AGEPC and phenylephrine infusions was reversed. Samples were collected at 12-s intervals. $[Ca^{2+}]$ = 0.5 mM.

ephrine indicated an interesting additive effect of the two agonists. To ascertain whether prior infusion of a given agent, AGEPC or phenylephrine, affects the response to a later infusion of the other agent, an experiment similar to that shown in Figure 2 was performed with interchanged infusions of AGEPC and phenylephrine, at 5 and 10 min into the washout, respectively. Figure 3 shows the results of this experiment. After a 50-min labeling period and 5-min washout, a 1-min infusion of AGEPC (20 nM) was administered to the liver followed at 10 min by a 1-min infusion of phenylephrine (10 μM), and the efflux of $^{45}Ca^{2+}$ was measured. Figure 3A illustrates a representative experiment of this type, and a similar experiment is shown in Figure 3B, but with the order of the AGEPC and phenylephrine infusions reversed. The amount of $^{45}Ca^{2+}$ released, measured by calculating the area under the peaks and corrected for liver weight and perfusate specific activity during $^{45}Ca^{2+}$ loading, was approximately 8-fold greater for phenylephrine than for AGEPC. Furthermore, prior exposure to phenylephrine did not alter significantly the subsequent release of $^{45}Ca^{2+}$ by AGEPC. When a pulse of AGEPC was given at a point 10 min into the washout but with no prior phenylephrine exposure, the calculated area of the peak of released $^{45}Ca^{2+}$ was not significantly different from the peak area obtained with prior exposure to phenylephrine at a maximal dose. Consecutive pulses of AGEPC, at 5 and 10 min, for example, demonstrated that desensitization of the transient calcium efflux occurs (data not shown) as with the glycogenolytic response observed previously.

Figure 4 illustrates a washout–pulse experiment done at two levels of calcium in the perfusate. The efflux of calcium is plotted on a linear scale, and the release of glucose from the liver as well as the total calcium in the perfusate is shown for two different concentrations of calcium in the perfusate, 0.05 and 0.5 mM calcium. The amount of AGEPC given to the liver was a relatively high dose and had an effect on the liver measured by the release of both $^{45}Ca^{2+}$ and glucose. Perfusate calcium levels, measured by using atomic absorption spectroscopy, did not change with infusion of AGEPC at either perfusate concentration of calcium, even though there was enhanced transient release of $^{45}Ca^{2+}$. This was likely a result of the small amount of calcium mobilized by AGEPC. A pulse

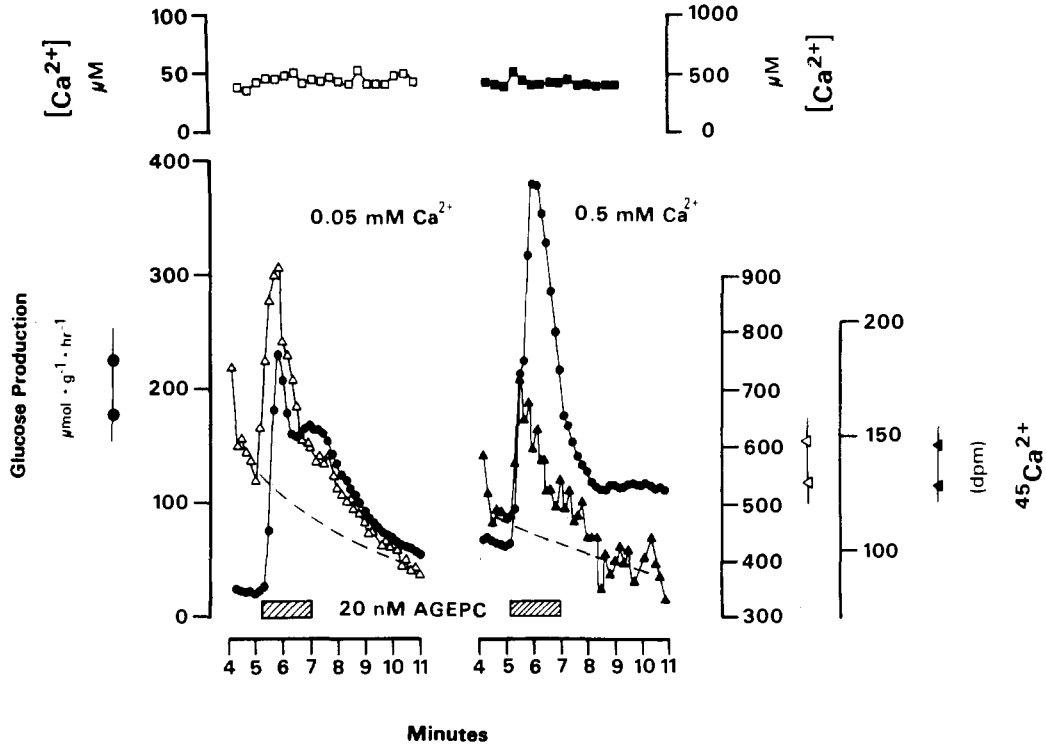

FIGURE 4: AGEPC-stimulated release of calcium from livers preloaded with $^{45}Ca^{2+}$. Livers were loaded with $^{45}Ca^{2+}$ for 50 min as described in Figure 1. Following the loading period, a 4-min period of washout occurred prior to sample collection. At 5 min into the washout, a 2-min infusion of AGEPC (20 nM) was begun. Samples were collected at 12-s intervals. Glucose, $^{45}Ca^{2+}$, and total calcium concentrations in the perfusate were measured as described under Materials and Methods. Perfusate Ca^{2+} concentrations were as indicated.

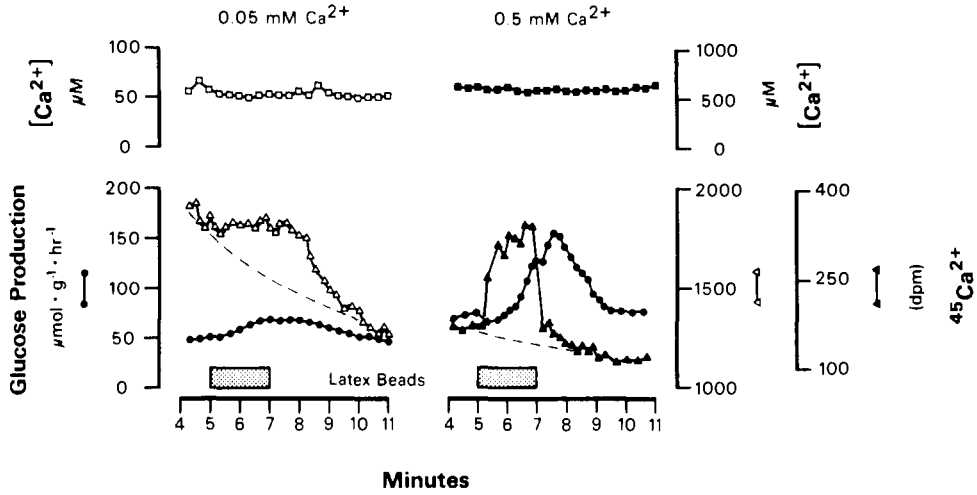

FIGURE 5: Latex bead stimulated release of calcium from livers preloaded with $^{45}Ca^{2+}$. Livers were treated as described in Figure 2. Latex beads (6.6×10^7 beads/mL final concentration) were infused for a 2-min period starting at 5 min into the washout of $^{45}Ca^{2+}$ from preloaded livers. Samples were collected at 12-s intervals. Glucose, $^{45}Ca^{2+}$, and total calcium concentrations in the perfusate were measured as described under Materials and Methods. Perfusate Ca^{2+} concentrations were as indicated.

infusion of phenylephrine under the same conditions results in sizable perturbation of perfusate Ca^{2+} (Buxton et al., 1984b). A pulse infusion of (lyso)GEPC (20 nM), which does not have the biological activity of AGEPC, did not have any effect on the efflux of $^{45}Ca^{2+}$ or the release of glucose in similar experiments, nor did a pulse infusion of the bovine serum albumin (BSA) vehicle.

In order to test the possible involvement of other cell types in the liver, such as Kupffer and sinusoidal cells, we performed the following experiment which parallels the experiments done with AGEPC. Latex beads (0.913-μm diameter) were infused into $^{45}Ca^{2+}$-equilibrated livers in a fashion similar to the experiments performed with the other agonists noted in Figure 4. It was intended to stimulate reticuloendothelial cells to phagocytose the latex particles passing through the liver. Livers were equilibrated with labeled calcium as before, and $^{45}Ca^{2+}$ was allowed to wash out from the liver for 5 min prior to infusion of the suspension of latex beads. Figure 5 shows the response of the liver to latex beads at two different perfusate calcium concentrations. At the lower concentration of perfusate calcium, only a slight, if any, release of $^{45}Ca^{2+}$ was observed, and the stimulation of glucose release was minimal. At the higher concentration of calcium, a much larger release of glucose occurred as well as a substantial release of $^{45}Ca^{2+}$. In similar experiments, infusion of heat-aggregated IgG complex, which is removed also by the reticuloendothelial system (Benacerraf et al., 1959), demonstrated a similar transient release of $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$-loaded livers (unpublished data).

Discussion

In a number of different cell types reponsive to AGEPC, calcium has been shown to be involved in the response to AGEPC stimulation. In platelets (Lee et al., 1981; Hallam et al., 1984), endothelial cells (Bussolino et al., 1985), isolated parotid gland lobules (Soling et al., 1984), and neutrophils (O'Flaherty et al., 1981a), AGEPC stimulates calcium uptake. Cultured endothelial cells stimulated with AGEPC have been shown to have altered calcium homeostasis (Bussolino et al., 1985; Brock & Gimbrone, 1986). In endothelial cells, calcium plays an important role in several critical metabolic process, such as the production of prostacyclin (Brotherton & Hoak, 1982) and the interaction with thrombin (D'Amore & Shepro, 1977).

The data presented in the present study indicate that the action of AGEPC on the isolated perfused liver leads to an enhanced mobilization of cellular calcium, though not to the degree observed with agents such as vasopressin and phenylephrine which are known to have direct effects on the parenchymal cells of the liver. That no changes in perfusate calcium levels accompany the perturbation of liver calcium by AGEPC is noteworthy. Phenylephrine and vasopressin, which effect glycogenolysis by mobilizing internal stores of hepatocyte calcium, produce a large transient efflux of calcium from the liver (Blackmore et al., 1979). The steady-state washout data presented here do not show any evidence of major perturbations of the kinetics of calcium exchange by AGEPC in the perfused liver, in contrast to phenylephrine which elicits major changes in the parameters of calcium efflux.

Since hepatocytes comprise the majority of cells in the liver, and consequently the bulk of liver calcium, it seems unlikely that AGEPC acts on the hepatocyte, directly or indirectly, to mobilize calcium, particularly in view of the smaller amount of calcium released compared to phenylephrine. In isolated heptatocytes, AGEPC does not lead to any changes in the measurable levels of phosphorylase *a* (Fisher et al., 1984); consequently, it is not likely that AGEPC mobilizes hepatocyte calcium directly. This is supported by the studies using the calcium indicator QUIN-2, which demonstrated no perturbation of the cytosolic calcium level in isolated hepatocytes following AGEPC exposure (Charest et al., 1985). In the isolated perfused liver, AGEPC does lead to an increase in the levels of phosphorylase *a*; however, since this increase is not related to increases in cAMP concentration of major changes in the relative levels of the adenine nucleotides (Buxton et al., 1986), some other mechanism must be operating. Mobilization of hepatocyte calcium stores in the perfused liver as an indirect effect of AGEPC exposure seems improbable. It is not likely that AGEPC could, directly or indirectly, trigger the release of hepatocyte calcium stores without perturbing the kinetic parameters of calcium efflux. Since there was no alteration in the efflux parameters of the washout of $^{45}Ca^{2+}$ during AGEPC exposure, then mobilization of hepatocyte calcium stores by AGEPC must be excluded.

Comparison of the release of $^{45}Ca^{2+}$ and glucose from the perfused liver following infusion of either AGEPC or latex beads indicates that calcium release generally precedes glucose release, though often the two are nearly coincident. The

calcium efflux in response to AGEPC is refractory also as has been reported for other effects following AGEPC infusion, such as glucose release (Buxton et al., 1984b). It is not clear whether depletion of calcium is involved here since subsequent release of calcium by AGEPC following a prior infusion of phenylephrine was not affected. We have confirmed this using release of labeled calcium from $^{45}Ca^{2+}$-equilibrated perfused livers.

Sinusoidal cells (endothelial and Kupffer cells) are likely targets for AGEPC in perfused liver. The similarity of response between AGEPC stimulation of calcium and glucose release and that of the infusion of latex beads on calcium and glucose release suggests this possibility. AGEPC has been shown also to stimulate $^{45}Ca^{2+}$ fluxes in cultured human endothelial cells derived from umbilical cords (Bussolino et al., 1985) and cultured bovine aortic endothelial cells (Brock & Gimbrone, 1986). In these cells, the application of AGEPC leads to losses in the amount of cell-associated calcium, in a dose-dependent manner, and increases in cytosolic Ca^{2+} concentration. In the liver, release of calcium from these cells, small in number compared with the hepatocytes, would not be expected to perturb greatly the perfusate calcium levels. The calcium release following AGEPC exposure using cultured endothelial cells shows refractory behavior similar to the results obtained the experiments reported here (Brock & Gimbrone, 1986).

In perfused livers, AGEPC induces vasoconstriction in a dose-dependent manner, which is sensitive to perfusate calcium levels, further suggesting sinusoidal cells as the site of action of AGEPC (Buxton et al., 1986). The action of β-adrenergic agonists, such as isoproterenol, to attenuate the response of the liver to AGEPC, particularly the glycogenolytic response, and the lack of effect of the α-adrenergic agonist phenylephrine on AGEPC action further implicate the nonparenchymal cells as the target for AGEPC action (Fisher et al., 1986). Also of note are the published effects of cyclooxygenase and lipooxygenase inhibitors on AGEPC action (Mendlovic et al., 1984; Garcia-Sainz & Hernandez-Sotomayor, 1985). Kupffer and endothelial cells phagocytose particulate matter passing through the liver, but only the Kupffer cells are capable of removing large particles (>0.1 μm). While the latex beads used in the experiments reported here are taken up by Kupffer cells, due to the beads diameter, it is not certain which cell type AGEPC affects, Kupffer or endothelial. Further, it has been shown that infusion of heat-aggregated IgG, which is removed by the reticuloendothelial system (Benacerraf et al., 1959), produces similar responses in the liver as does AGEPC infusion with respect to glucose production. However, the production of AGEPC as a result of IgG aggregate infusion has been demonstrated (Buxton et al., 1984a). In addition, infusion of the IgG aggregate prior to infusion of AGEPC desensitizes the response to AGEPC, while reversal of the infusion order does not desensitize the response to the IgG aggregate.

Whether hepatic calcium is released as a primary response to AGEPC or this effect is secondary to AGEPC action remains to be determined. In platelets, AGEPC leads to a rapid transient breakdown in membrane phospholipids (Shukla & Hanahan, 1982; Billah & Lapetina, 1983) and a rapid mobilization of platelet Ca^{2+} (Lee et al., 1981; Hallam et al., 1984). Breakdown of membrane phospholipids can result in the mobilization of calcium in a number of ways. Degradation of membrane lipids can lead to loss of binding sites for calcium on the cytoplasmic side of the membrane. Perturbations in membrane lipid composition can lead to alterations in the activities of integral membrane proteins responsible for ion translocation such as the Ca^{2+}-ATPase. Alternatively, inositol 1,4,5-triphosphate, formed from phosphoinositides as a result of membrane lipid breakdown, has been shown to lead to rapid mobilization of internal stores of calcium (Charest et al., 1985; Berridge, 1983). In many cell types, the need for calcium mobilization is coupled tightly to secretory and metabolic processes. It is possible that the mobilization of calcium in perfused liver as a result of stimulation by AGEPC or latex bead infusion, though smaller in magnitude than the response to α-adrenergic agents, could be crucial for the initiation of a train of metabolic and secretory processes necessary for the coupling of the sinusoidal cell events to glycogenolysis in the hepatocyte. The involvement of cell calcium in the metabolism of arachadonic acid metabolites (Brotherton & Hoak, 1982), which have been suggested to be involved in the response of the liver to AGEPC (Mendlovic et al., 1984; Garcia-Sainz & Hernadez-Sotomayor, 1985), would be a likely role. The precise nature of the coupling between cells stimulated by AGEPC and the parenchymal cells remains to be elucidated.

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Purification of Bleomycin Hydrolase with a Monoclonal Antibody and Its Characterization†

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ABSTRACT: We established a hybridoma clone that produced anti-bleomycin hydrolase antibody. The subclass of the monoclonal antibody was immunoglobulin M. The antibody significantly reacted with bleomycin hydrolase from rabbit tissues, mouse livers, sarcoma 180, and adenocarcinoma 755 but not significantly with that from MH 134 and Ehrlich carcinoma. The enzyme from L5178Y cells showed an intermediate reactivity. Bleomycin hydrolase was purified from rabbit liver by immunoaffinity with the monoclonal antibody and DEAE gel chromatography. Approximately 1300-fold-purified bleomycin hydrolase was obtained. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing on a polyacrylamide slab gel of purified bleomycin hydrolase showed a single band with an apparent M_r of 48K and an isoelectric pH of 5.2. The molecular weight of bleomycin hydrolase determined on gel filtration high-performance liquid chromatography was ca. 300K, suggesting a hexameric enzyme. The enzyme showed an optimum pH of 6.8–7.8 and gave a V_{max} value of 6.72 mg min^{-1} mg^{-1} for peplomycin and 9.24 mg min^{-1} mg^{-1} for bleomycin B_2 and a K_m value of 0.79 mM for both substrates. The enzyme was inhibited by E-64, leupeptin, *p*-tosyl-L-lysine chloromethyl ketone, *N*-ethylmaleimide, Fe^{2+}, Cu^{2+}, and Zn^{2+} but was enhanced by dithiothreitol. The results suggest that bleomycin hydrolase is a thiol enzyme.

Bleomycin (BLM)[1] is a group of glycopeptide antibiotics, which differ from one to another in the terminal amine moiety. The drug displays remarkable therapeutic activity for squamous cell carcinoma and malignant lymphoma. The molecular target of BLM is DNA. The antibiotic binds to double-stranded DNA, causing a single strand scission. Peplomycin (PEP) is an analogue of BLM.

Human and animal tissues contain BLM hydrolase (BLMase), which hydrolyzes the carboxamide bond in the

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[1] Abbreviations: BLM, bleomycin; BLMase, bleomycin hydrolase; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; NEM, *N*-ethylmaleimide; PEP, peplomycin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLCK, *p*-tosyl-L-lysine chloromethyl ketone.

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