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PHOTODYNAMIC ACTION OF SULPHONATED ALUMINIUM PHTHALOCYANINE (SALPC) ON ISOLATED RAT PANCREATIC ACINI

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Abstract—The photodynamic action of SALPC has been investigated on dispersed, perifused, acini isolated from the rat pancreas. Stimulation of secretion was assessed by measuring amylase release and membrane permeabilization determined by the leakage of cytoplasmic lactate dehydrogenase (LDH) and by the efflux of ^{86}Rb from preloaded acini. Light alone (> 570 nm, ≤ 18,400 lux), or SALPC (≤ 1 μM) in the absence of light, had no effect on pancreatic acini but cellularly bound SALPC when illuminated caused a dose-dependent, light intensity-dependent and temperature-dependent release of amylase. Singlet oxygen generated by photon-activation of SALPC was measured by the formation of an imidazole adduct and bleaching of the secondary substrate, RNO. Whereas illumination caused a rapid increase in photodynamically-evoked pancreatic amylase release, the efflux of ^{86}Rb and loss of cytosolic LDH were markedly delayed in onset: similar results were obtained with monochromatic laser light (633 nm). In contrast, the muscarinic agonist bethanechol evoked a rapid increase in amylase release but with an almost immediate efflux of ^{86}Rb. Finally, electron microscopy confirmed that the structural integrity of the pancreatic acinar cells was maintained after the photodynamic action of SALPC. It is concluded that the stimulation of amylase secretion and membrane permeabilization by SALPC is due to the generation of singlet oxygen. However, the consistent difference between the time course of amylase secretion and membrane permeabilization makes it likely that an initial stage in photodynamic drug action involves oxidation of plasma membrane protein and activation of secretagogue receptors or the G-proteins and their effector systems.

Photon-activated drugs may be used to sensitize selected cellular populations to light of specified wavelength for photodynamic therapy (PDT) [1]. The phthalocyanines are an important new group of compounds suitable for PDT but little is known of the biological processes involved in the action of these photosensitizers on normal or tumour cells at the cellular or molecular level except that the generation of singlet oxygen is implicated in a Type II photodynamic action [2]. In studying the basic pharmacological mechanisms of photoactivated molecules we have reported previously that membrane-located halogenated fluoroscein derivatives can cause contraction of smooth muscle cells [3], permeabilization of thymocytes [4] and secretion [5, 6]. Since the exocrine cells of the pancreas are a prime target for PDT [7], we have investigated the effects of the new photodynamic agent, sulphonated aluminium phthalocyanine (SALPC) on the acinar cells of the exocrine pancreas in terms both of membrane permeabilization and of secretion. SALPC was chosen for the present work since not only does it possess a high aqueous solubility without dimerization or aggregation over a wide concentration range, but it has an absorbance maximum of 675 nm, a good quantum yield of the triplet state, $\phi = 0.4$, and an extended triplet (^3S) life time, $\tau = 500\ \mu sec$, on photon-activation [8], all properties required for the generation of highly reactive singlet oxygen. For precise control of both drug action and photon flux we have developed a multichannel perifusion system for dispersed acini isolated from the rat pancreas. Stimulation of secretion was assessed by measuring amylase released from the acini and membrane permeabilization kinetics determined by the leakage of cytoplasmic lactate dehydrogenase (LDH), and by the efflux of ^{86}Rb (as a marker of intracellular K^+) from preloaded acini. The combination of these techniques, together with an ultracytological study by electron microscopy, has allowed us to gain greater insight into the acute pharmacological effects of photodynamic agents on the cellular secretory system of pancreatic acini.

MATERIALS AND METHODS

Preparation and perifusion of pancreatic acini

Dispersed pancreatic acini were isolated by sequential collagenase digestion of the pancreas removed from male Sprague–Dawley rats of 200–400 g body weight [9, 10]; at least 95% of the acini so prepared were viable by the eosin exclusion test. One-mL aliquots of the acini suspension were mixed with 25 mg Biogel beads (P2), loaded into tissue columns and perifused at 0.5 mL/min with oxygenated buffer (see below) for 60 min before any stimulation was applied. Tissue columns were constructed from transparent 2 mL plastic hypodermic syringes, 1 cm diameter, equipped at their base with a polystyrene filter. For multichannel perifusion up to four columns were placed in a small circulating water bath at 37° and the incubated cells illuminated

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from above when required. Two min fractions of the perifusion effluent were collected from the base of each tissue column and the amylase content of the fractions assayed spectrophotometrically. Usually three fractions were collected immediately before stimulation and the amylase content in all later fractions normalized to the mean value calculated for the three pre-stimulation fractions: the pre-stimulation fractions therefore also define the basal release. A buffered solution of the following composition (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.16, $CaCl_2$ 2.0, NaH_2PO_4 1.16, glucose 14, HEPES 25 and pH adjusted to 7.3 with NaOH 1 N was used for perifusion; it was gassed continuously with 100% O_2. In some experiments the isolated acini were first incubated for 1 hr at 37° in a solution containing ^{86}RbCl 0.925 MBq before perifusion.

Enzyme and ion measurement

Amylase. Amylase was assayed spectrophotometrically at 595 nm (Gilford 250) against standard porcine α-amylase using amylose azure as substrate which upon hydrolysis releases free azure blue [11].

Lactate dehydrogenase (LDH). LDH was assayed [12] by monitoring the initial rate of transformation of non-fluorescent NAD to the highly fluorescent NADH in a Turner Model 111 fluorometer.

Protein. Protein was measured according to Bradford [13] using the Biorad assay and bovine serum albumin as standard.

Rubidium. ^{86}Rb was detected by Cerenkov counting [14] using 7-amino-1,3-naphthalene disulphonic acid (ANDA) as a wavelength shifter and DPM measured by liquid scintillation spectrometry (TRICARB 2000 AC).

Uptake of SALPC. Acini and beads were removed from the tissue columns and pelleted by centrifugation. The pellet was resuspended in 1 mL NaOH 0.1 N, mixed to digest the acini in the alkali, centrifuged at 20,000 *g* for 10 min in a refrigerated Beckman microfuge (5°) and the fluorescence of the supernatant determined (Turner Model 111) against a standard curve derived from the addition of known concentrations of SALPC to the acini before extraction.

Light sources

The cells of the dispersed acini in the tissue columns were illuminated from above by light from one of two sources:

Halogen–quartz light source (Schott KL 1500) equipped with fibre optic probe and heat filter (KG1); emission wavelength > 400 nm and constant colour temperature of 3200° K. For all SALPC experiments a sharp-cut optical filter (23A) transmitting wavelengths > 570 nm was used unless stated otherwise.

Helium–neon laser (Uniphase: 1105P) with power output 8 mW at 632.8 nm. The laser beam was expanded with a two-lens collimating system to a parallel beam configuration (diameter 1 cm) and, by reflection from a front-surface mirror at 45°, illuminated the tissue column from above.

Output illuminance (lux) of the light source was measured, as a function of distance, by a Minolta T-1H illuminance meter.

Electron microscopy

Tissue samples for electron microscopy were fixed overnight at 4° with glutaraldehyde 1% and paraformaldehyde 1% in PIPES 0.1 M. Fixed cells were treated with osmium tetroxide 1%, dehydrated and embedded in Spurr's resin. Thin sections were cut, stained with uranyl acetate 2% and lead citrate 0.4%, and examined by transmission electron microscopy (Philips EM 300).

Chemical detection of singlet delta oxygen ($^1\Delta g\ O_2$)

The method is based on that of Kraljic and Moshni [15] using imidazole as a substrate to form the singlet oxygen adduct, imidazole peroxide, which stoichiometrically bleaches *p*-nitrosodimethylaniline (RNO); this is measured spectrophotometrically at the E_{max} 440 nm.

Statistics and data presentation

The kinetic data are expressed either by normalization to internal controls or as a percentage of the total (amylase, LDH) present in the acini at the beginning of an experiment and determined by cellular lysis with Triton X-100 0.1% of equal aliquots of an acinar suspension. For normalization the mean values of either amylase or ^{86}Rb efflux from the 52nd to the 58th min of perfusion was taken as 1.0, all other values being expressed as a ratio of this mean. For testing the significance of difference between means, Student's *t*-test (two-tailed) was used and $P < 0.05$ was taken as significant. Unless otherwise stated, all experiments were done at least three times and for each experiment the acini were exposed to SALPC from only the 34th to 44th min of perifusion.

Materials

Biogel beads (P2) and Biorad reagent for protein assay were from Biorad (Watford, U.K.); 7-amino-1,3-naphthalene disulphonic acid (ANDA) and *p*-nitrosodimethylaniline (RNO) from Aldrich (Gillingham, U.K.), ^{86}RbCl from Amersham International (Amersham, U.K.); sulphonated aluminium phthalocyanine was a gift from Ciba-Geigy (Basle, Switzerland); all other enzymes and chemicals were the best grade available from the Sigma Chemical Co. (Poole, U.K.).

RESULTS

Photodynamic effects of SALPC on amylase secretion: concentration, illumination, and temperature dependence

Initial experiments established that SALPC at concentrations < 1 μM caused no amylase secretion from freshly isolated pancreatic acini, neither did light alone at illuminances ≤ 18400 lux. In contrast, acini perifused with SALPC, 10–1000 nM for 10 min and irradiated (4500 lux: 10 min) 15 min later showed a time- and concentration-dependent increase in the

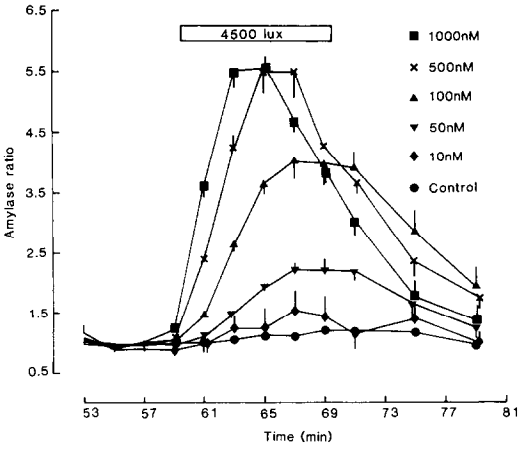

Fig. 1. Photodynamic action of SALPC on amylase release from pancreatic acini at different concentrations of SALPC. Acini were exposed to SALPC at the indicated concentrations from the 34th to the 44th min of perifusion and irradiated at 4500 lux for 10 min at the time indicated by the horizontal bar. SE bars are omitted from the mean plotted points from the 52nd to the 58th min for clarity; for all other points where the standard error bar is not seen, it lies within the symbol (N = 3–4).

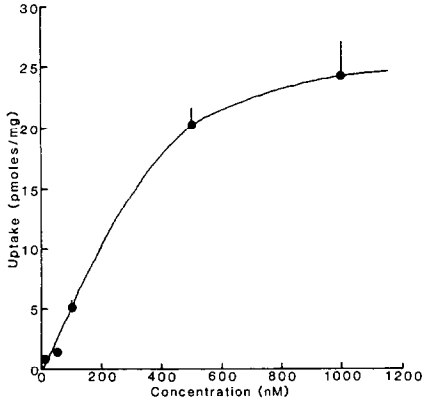

Fig. 2. Uptake of SALPC by perifused pancreatic acini. Acini were exposed to SALPC for 10 min followed by perifusion with SALPC-free medium for 15 min. The amount of SALPC remaining bound to the acini at the 58th min was determined fluorometrically and expressed as pmol/mg cell protein (N ≥ 3).

rate of amylase release (Fig. 1). At high SALPC concentrations (500–1000 nM) secretion reached a maximum before the end of illumination but at lower concentrations peak secretion continued for several minutes after illumination. In all these experiments the free SALPC in the perifusion buffer had been removed before tissue irradiation. The increased rate of amylase release must therefore be due to the photon activation of cellularly bound SALPC. To fully assess its photodynamic action the actual amount of SALPC bound to the acini at the time of light irradiation was determined after perifusion with increasing concentrations of SALPC. Figure 2 indicates that the uptake of SALPC was almost linear

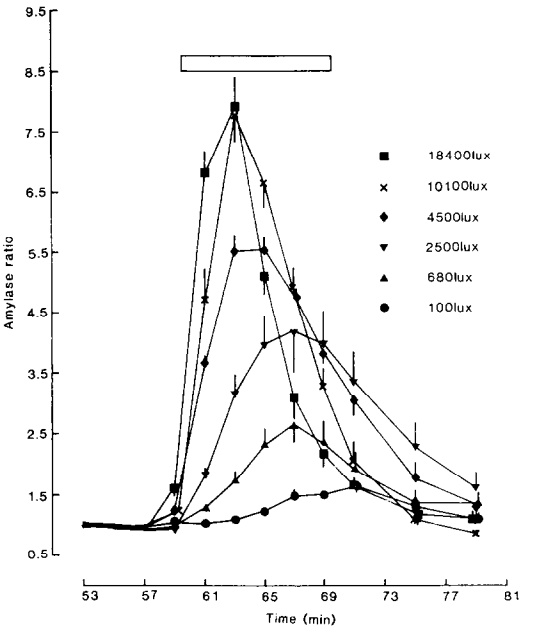

Fig. 3. Illuminance dependency of the photodynamic action of SALPC on amylase release. Acini were exposed to SALPC, 1 μM, and irradiated with light at the illuminance indicated for the time designated by the horizontal bar. Other details as for Fig. 1 (N = 4–6).

at low concentrations (10–100 nM) and tended to saturate at higher concentrations (i.e., up to 1000 nM).

The stimulation of amylase secretion by photodynamic action was dependent also on the illuminance as illustrated in Fig. 3 in which the perifusion concentration of SALPC was set at 1 μM for 10 min before extracellular washout. Amylase release was detectable at an illuminance as low as 100 lux but at higher values (680–18,400 lux) the peak increase in amylase release becomes even more marked (Fig. 3).

The effect of temperature was investigated in further experiments for two reasons: first, to examine the possible effect of membrane fluidity on photodynamic action and secondly, to establish whether hyperthermia might directly enhance the photodynamic action of SALPC, 1 μM. Photodynamic amylase release obviously was temperature-dependent (Fig. 4A) since with increasing temperature from 9° to 42° the maximum response not only increased in magnitude but became more rapid in onset. Basal release was also affected by temperature and this response together with the photodynamically-evoked amylase release is seen more clearly in Fig. 4B.

The effect of concentration of SALPC, illuminance and temperature on photodynamic action can be summarized by two parameters: (i) the net total amount of stimulated amylase release expressed as an increase above basal value and (ii) the initial rate. This is illustrated in Fig. 5. It is evident that the initial rate for photodynamic action increased steadily with increasing concentrations of SALPC

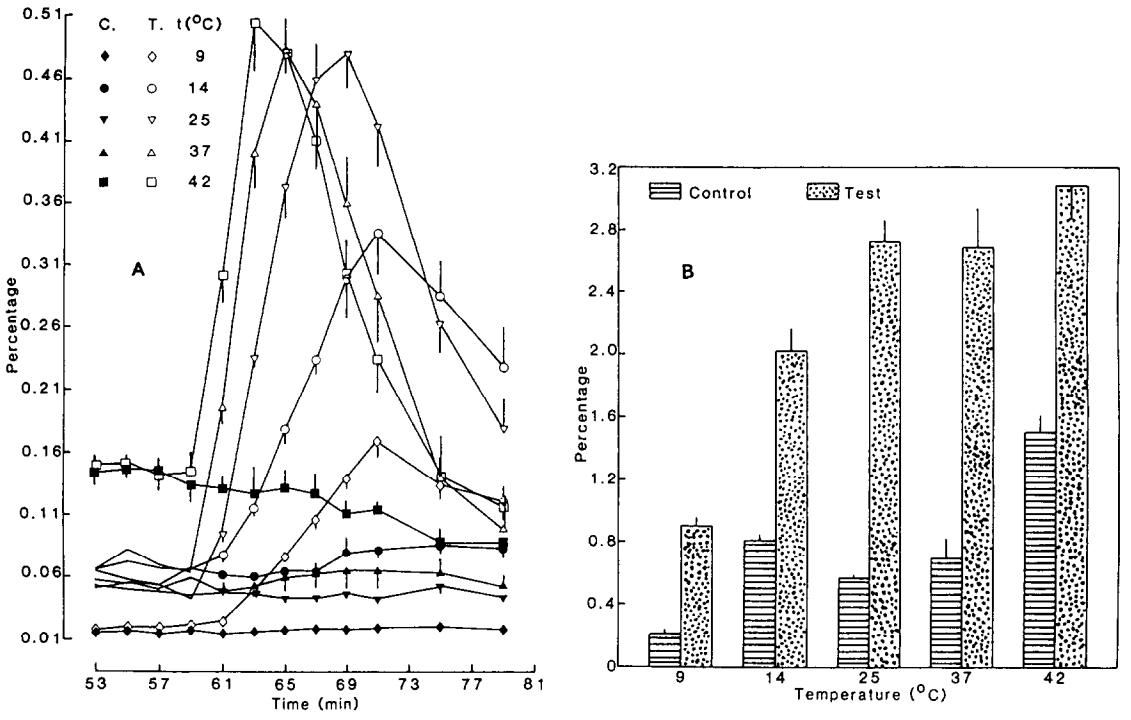

Fig. 4. Effect of temperature on the photodynamic action of SALPC, 1 μM. In (A) acini perifused at set temperatures were exposed to SALPC (open symbols). Corresponding control experiments with no exposure to SALPC are indicated by the closed symbols. Irradiation with light, 4500 lux, was from the 60th to the 70th min (N = 4–6). In (B) the amount of amylase released from the 52nd min until the end of the experiment is plotted as a percentage of the total cellular amylase.

from 10 to 1000 nM, with increasing illuminance from 100 to 18,400 lux, and with increasing temperature from 9° to 25°.

It is perhaps not surprising that the initial rate increases with increasing concentrations of SALPC or increasing illuminance, the reason being that in either situation, the rate of singlet oxygen production will also increase (see below). An explanation for the effect of temperature on the initial rate of amylase release is not so straightforward. It has been established [16] that temperature changes over the range 0° to 23° have little effect on the lifetime of singlet oxygen and even a temperature increase over a larger range from −50° to +25°, causes only a 50% decrease in the lifetime of singlet oxygen [17]. However, it has been found that temperature significantly affects photooxidation reactions involving singlet oxygen. A 6- to 7-fold increase in the photooxidation of cholesterol was observed in liposomal membranes when the temperature was increased from below, to above, the transition temperature [18]. Therefore, the reaction of singlet oxygen with the membrane substrate appears to be strongly temperature-dependent. In a cellular system such as the isolated acini, photooxidations other than that of cholesterol may also be involved, and it is likely that these too will be temperature-dependent.

The initial rate of amylase release may be determined by the rate of singlet oxygen production in Fig. 5A and B and the rate of photooxidation in Fig. 5C, but it is quite apparent that the production of singlet oxygen in 10 min (i.e., the period of illumination) is not reflected in the *total* amount of amylase released. Thus, in Fig. 5A a maximal release of amylase release was achieved at a SALPC concentration of 100 nM, whereas the initial rate can be further increased with increasing SALPC concentrations. This suggests that the total amount of singlet oxygen produced by SALPC 100 nM and an illuminance of 4500 lux for 10 min is sufficient to cause a maximal release of amylase over the experimental period. A further increase in SALPC concentration then either did not produce more singlet oxygen or more singlet oxygen was produced, but caused no further amylase release. The latter seems the more likely, since the initial rate was further increased with SALPC > 100 nM. The conclusion is therefore that although the production of singlet oxygen can be increased steadily with increasing concentrations of SALPC from 10 to 100 nM at a set illuminance of 4500 lux, the amount of singlet oxygen generated by SALPC 100 nM was sufficient to cause maximal release of amylase; this has important implications for the stoichiometry of the photodynamic process (see Discussion).

Similarly, it is evident from Fig. 5B that an illuminance of 10,100 lux was sufficient to elicit maximal release of amylase, because a greater intensity of illumination (up to 18,400 lux) though likely to generate more singlet oxygen, caused no further increase in the total release of amylase.

In Fig. 5C, a near maximal initial rate for amylase

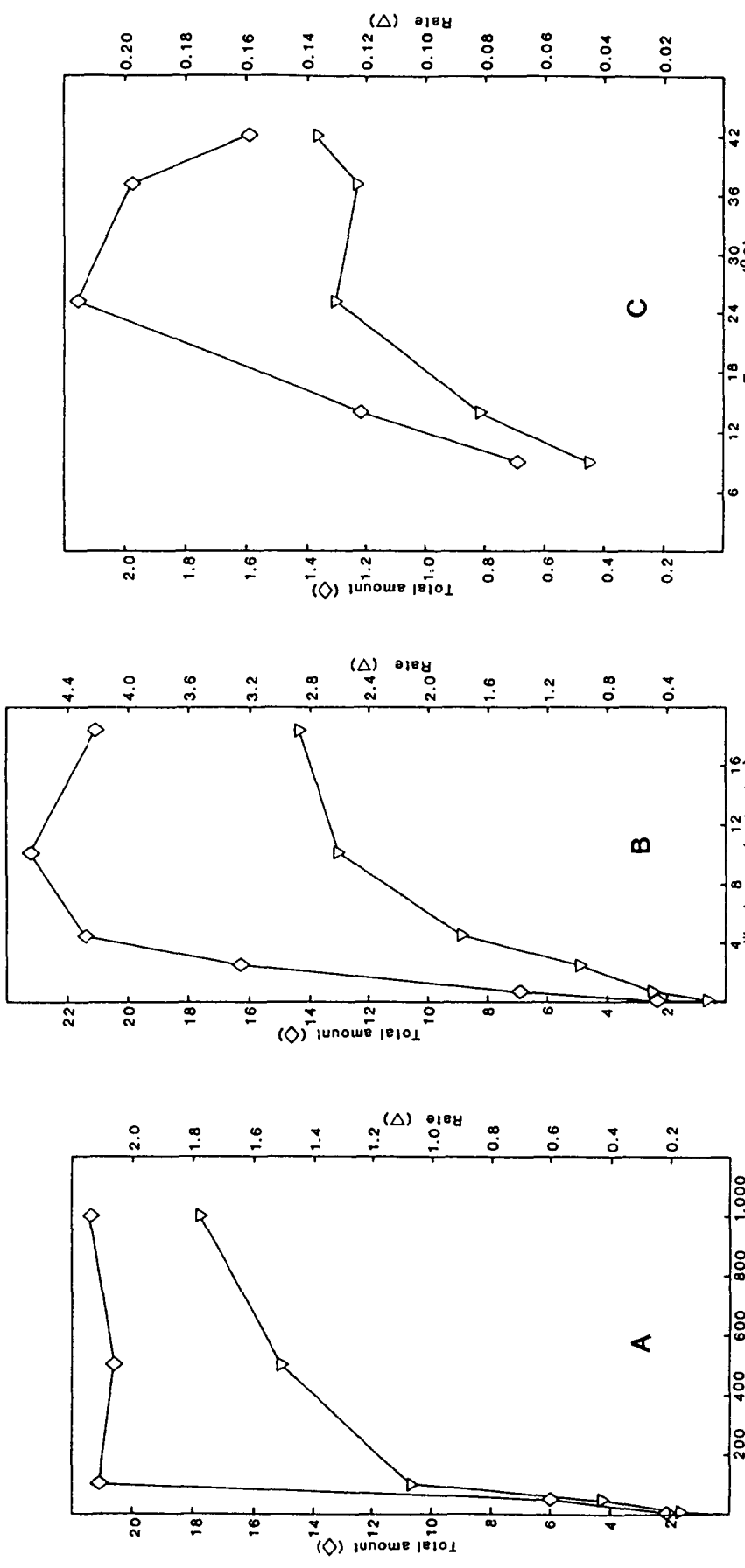

Fig. 5. Photodynamic action of SALPC on (i) the total amount, and (ii) the rate, of amylase release. (A) Effect of SALPC concentration at a set illuminance (4500 lux), (B) effect of illuminance at a set concentration of SALPC (1 μM), and (C) effect of temperature at a set concentration of SALPC (1 μM) and set illuminance (4500 lux). Rates (▽) are derived from the ascending part of each curve in Figs 1, 3 and 4A, and the total amount (◇) is the area below each curve of the same figures after subtraction of basal (control) release.

secretion was seen at 25°, raising the temperature to 42° caused no further increase. At the same time, a maximum of total amylase release was seen at 25° and higher temperatures led to an actual decrease in total amylase release. This is likely to be due to a direct inhibition of the secretory machinery at the higher temperature (i.e. 42°). A further possibility for the temperature-dependence of photodynamic action is that the uptake of SALPC by perifused acini is temperature-dependent, but the literature suggests otherwise [19]. It has been reported that the uptake of phthalocyanine was not dependent on temperature so long as the incubation with phthalocyanine was for less than 60 min. The experiments described in Fig. 5C were carried out only 15 min after incubation with SALPC (which was for 10 min), thus effectively eliminating any major action of temperature on the uptake of SALPC. The temperature-dependence of photodynamically-induced amylase secretion may therefore reflect the effect of membrane fluidity, or the temperature-dependence of the secretory process itself.

Photodynamic action of SALPC: membrane permeabilization

Evidence from previous studies [3, 4] is that permeabilization of the plasma membrane may underlie photodynamic drug action. Experiments were therefore designed to detect membrane permeabilization of acinar cells by measuring the efflux of ^{86}Rb (as a marker of intracellular K^+) from preloaded acini and the leakage of endogenous cytoplasmic lactate dehydrogenase (LDH). It was first necessary, however, to distinguish ^{86}Rb efflux associated with muscarinic receptor activation from that due to any photodynamic permeabilization of the plasma membrane. The effects of the muscarinic agonist bethanechol on amylase secretion and ^{86}Rb efflux were therefore measured in parallel (Fig. 6). Both parameters showed a similar time-course with the onset of amylase release preceding that of ^{86}Rb efflux by 30 sec, which could be explained by the activation of Ca^{2+}-dependent K^+-channels [20]. The photodynamic action of SALPC on ^{86}Rb efflux from preloaded acini is illustrated in Fig. 7. The efflux of ^{86}Rb declined slowly in control acini without exposure to SALPC but in those acini perifused with SALPC 1 μM previously, light irradiation caused a clear increase in ^{86}Rb efflux although with a considerable delay in onset of some 6 min when compared to amylase secretion (i.e., Figs 1 and 7). The net increase in ^{86}Rb efflux did not cease after illumination but remained persistently above control values until the end of the experiment. The efflux after the photodynamic action of SALPC was also greater than that seen with maximal bethanechol stimulation.

When the SALPC concentration was maintained at 1 μM and the illuminance increased to 18,400 lux, net ^{86}Rb efflux was markedly increased (Fig. 8A) reaching a maximum 4 min after the start of illumination and returning to control values 2 min after the end of illumination.

In all the experiments described so far, SALPC was removed from the extracellular environment of

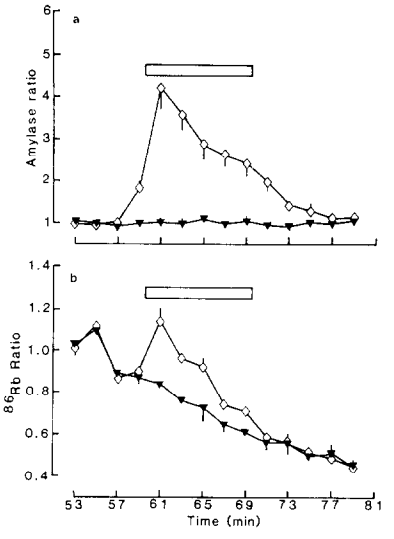

Fig. 6. Effect of bethanechol on (a) amylase release and (b) ^{86}Rb efflux from perifused acini. Normal (a) or ^{86}Rb-loaded (b) acini were exposed to bethanechol, 100 μM (◇) at the time indicated by the horizontal bar. Control acini (▼) were not exposed to bethanechol (a; N = 5–6: b; N = 3–5).

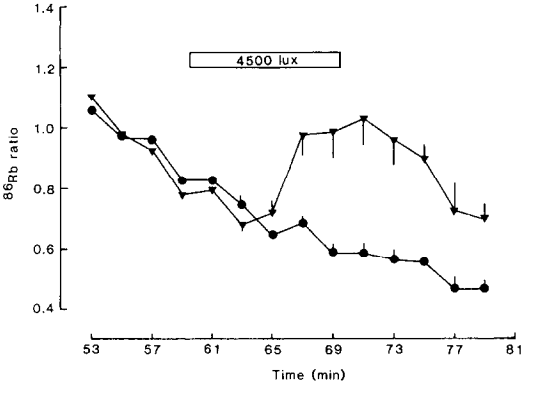

Fig. 7. Photodynamic action of SALPC on ^{86}Rb efflux from pancreatic acini. ^{86}Rb-loaded acini were exposed to SALPC, 1 μM (▼) and subsequently illuminated (4500 lux) for the time indicated by the horizontal bar. Control acini (●) were not exposed to SALPC but were illuminated (N = 6).

the acini before photon-activation of the membrane-bound SALPC. To help in assessing the localization of SALPC at the time of light irradiation, the time course and magnitude of photodynamic action by SALPC maintained free in the extracellular space was evaluated. Such experiments are not practicable when amylase release was used to assess photodynamic action, since amylase itself could be inactivated by the photodynamic process [21, 22]. Such problems do not exist when using ^{86}Rb efflux as an indicator of photodynamic action. Thus, as described in Fig. 8B perifused acini were exposed to SALPC 1 μM for 15 min, starting 2 min before light

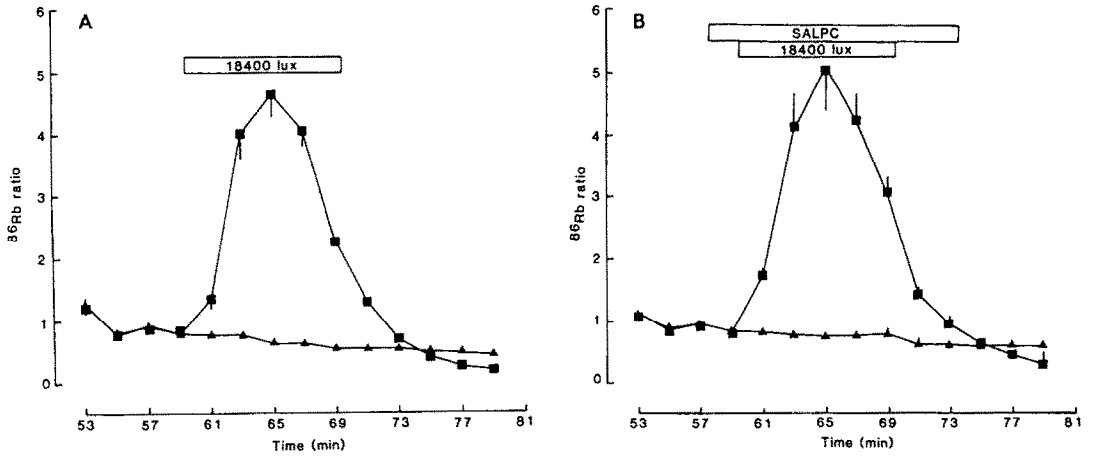

Fig. 8. Photodynamic action of SALPC on ^{86}Rb efflux at higher illuminance. (A) ^{86}Rb-loaded acini were exposed to SALPC, 1 μM (■) and subsequently illuminated, at 18,400 lux for the time indicated by the horizontal bar. (B) Effect of free extracellular SALPC on the photodynamic efflux of ^{86}Rb. ^{86}Rb-loaded acini were exposed to SALPC, 1 μM (■), before, during and after irradiation as indicated by the longer horizontal bar. Illumination at 18,400 lux is designated by the shorter horizontal bar. Control acini (▲) were not exposed to SALPC but were illuminated (N = 3–6).

irradiation at 18,400 lux. Efflux of ^{86}Rb from acini not exposed to SALPC decreased slowly (i.e., there was no effect of light alone) but illumination of the acini with free SALPC present in the perifusion buffer caused an immediate increase in ^{86}Rb efflux. Both the magnitude and the kinetics of the increase in ^{86}Rb efflux were almost identical in these experiments to those when the usual protocol was employed with an illuminance of 18,400 lux (compare Fig. 8A and B).

It has been shown above that ^{86}Rb efflux was increased by the photodynamic action of SALPC. Since ^{86}Rb is a very small ion, with strong β-emitting capacity (1.78 MeV), even a very small increase in the plasma membrane permeability to ^{86}Rb can be readily detected. To determine the extent to which the plasma membrane is permeabilized during the photodynamic action of SALPC, it is necessary to investigate what size of molecule can diffuse from the cell or gain the cell interior after permeabilization. The leakage of a large cytosolic enzyme, lactate dehydrogenase (LDH; 130 kD) was therefore examined. Figure 9 illustrates amylase release and LDH leakage measured concurrently from the same population of perifused acini during photodynamic action (SALPC 1 μM, 4500 lux). Amylase release increased immediately upon illumination and reached a maximum 4 min later, while LDH leakage did not increase until 6 min after beginning exposure to light. The stimulation of amylase release diminished with time, but was still significantly greater than control values at the end of the experiment, whilst LDH leakage increased steadily and reached a level four times greater than control values at the end of the experiment.

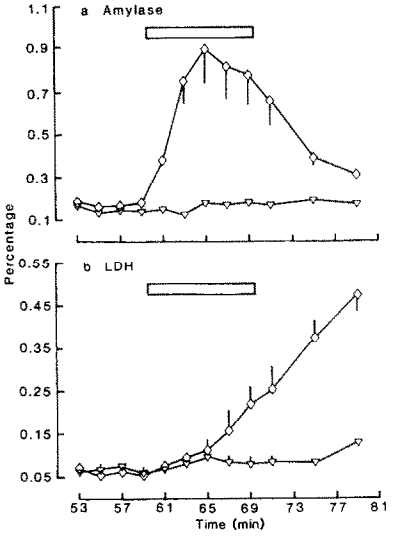

Fig. 9. Amylase release (a) and LDH leakage (b) from the same population of perifused acini during the photodynamic action of SALPC. Acini were exposed to SALPC, 1 μM (◇) and irradiated at 4500 lux at the time indicated by the horizontal bars (N = 4).

Photodynamic action of SALPC: effect of high-intensity monochromatic irradiation

Unlike ordinary light sources, lasers emit extremely high power coherent light. Moreover, the recent development of tunable dye-lasers offers the opportunity not only of selecting light at the wavelength of maximum absorption for each photosensitizer, but also of studying the action spectrum of the photodynamic agent. Here, in some initial experiments, we have examined the effects of coherent light from a helium–neon laser (632.8 nm).

As can be seen in Fig. 10A, laser light alone caused no significant change in amylase release, but laser irradiation of acini containing bound SALPC caused a very marked increase in the rate of amylase release. As with the filtered quartz–halogen source, the

response to monochromatic light was also time-dependent, a maximum amylase output being achieved about 4 min after irradiation, thereafter the rate declined steadily towards the basal level.

In addition to stimulating amylase release, SALPC activation by monochromatic light also elicits ^{86}Rb efflux as illustrated in Fig. 10B. In acini perifused in the absence of SALPC, ^{86}Rb efflux steadily declined and this decrease was not altered by light at 632.8 nm. However, with perifused acini exposed to SALPC 1 μM previously, light irradiation at 632.8 nm evoked a large net increase in ^{86}Rb efflux. The onset of the increase was slower than that for amylase and was not seen until 2 min after beginning irradiation with laser light, reaching a maximum 6 min later. The increase in ^{86}Rb efflux persisted until the end of the experiment. The magnitude of the net increase in ^{86}Rb efflux in the photodynamic action of SALPC 1 μM with laser light (8 mW) was greater than red light of illuminance 4500 lux, but smaller than that with light of illuminance 18,400 lux (see Fig. 8A and B), and consistent with a calculated laser illuminance of 16,000 lux.

Photodynamic action of SALPC on acinar cell ultrastructure

Following investigation of the effects of photodynamic action on amylase release and plasma membrane permeability any corresponding morphological changes were also studied by transmission electron microscopy (Fig. 11). It is evident that no major ultrastructural changes occurred in the acinar cells after moderate photodynamic action (SALPC 1 μM, 4500 lux: compare Fig. 11A, B and C). Only with more severe photodynamic action (SALPC 1 μM, 18,400 lux) were definite morphological changes produced in individual acinar cells (Fig. 11E). Vacuolization was apparent and the mitochondria appeared swollen but the zymogen granules remained intact. These changes are remarkably similar to those identified in electro-permeabilized chromaffin cells [23].

Production of singlet oxygen by SALPC

All the evidence in this and other studies [3, 4] implicates a Type II photodynamic action [2] involving the highly reactive singlet oxygen molecule. We have now been able to substantiate this by demonstrating directly that singlet oxygen is produced under conditions similar to those evoking photodynamic amylase release, i.e., SALPC 1 μM, light 4500 lux (23A filter), pH 7.3. In these experiments (see Materials and Methods) any singlet oxygen produced reacts with imidazole to form an endoperoxide which bleaches a secondary substrate, RNO (Fig. 12).

In additional experiments with SALPC and other sensitizers at a concentration of 1 μM, light of 14,100 lux (but without filter 23A), the order of potency for RNO bleaching was rose bengal (RB) > SALPC > erythrosine (EB). RB and EB also both cause photodynamic amylase release [6, 22]. In contrast merocyanine 540, which does not evoke amylase release (data not shown) was found not to bleach RNO (Fig. 12), consistent with its very low triplet yield [24]. The bleaching of RNO is dependent on imidazole concentration and the concentration–response curves of Fig. 12 are of the typical bell shape found previously [15, 25]; this is believed to be due to the direct interaction between imidazole and its peroxide at high imidazole concentration. The imidazole concentration for maximal RNO bleaching (3 mM) is also of a similar order to that of 8 mM previously reported [15, 25].

DISCUSSION

Our ultracytological studies establish that, with SALPC 1 μM and an illuminance of 4500 lux, no major changes in acinar cell structure are observed. Under these conditions, therefore, amylase released from perifused rat pancreatic acini cannot be attributed to photodynamic action causing widespread lysis or membrane fragmentation of acinar cells nor of the zymogen granules themselves. However, the data with ^{86}Rb efflux and LDH leakage confirm unequivocally that under these conditions (i.e., SALPC 1 μM, and light 4500 lux), the cell membrane *is* permeabilized during photodynamic action. Amylase release could therefore occur as a direct consequence of calcium influx following permeabilization because calcium has a pivotal role in exocytotic secretion in these cells [26, 27]. It was, however, a consistent observation in the present experiments that amylase secretion preceded both ^{86}Rb efflux and LDH leakage by about 6 min and maximal amylase release was often achieved before either ^{86}Rb efflux or LDH loss became detectable. With other photosensitizers there is evidence also of a calcium-independent component of amylase secretion in pancreatic cells [6]. It is reasonable to state, therefore, that although plasma membrane permeabilization occurs it is not a prerequisite for amylase release in the first few minutes of photodynamic action. Membrane permeabilization would certainly contribute to the later stages of amylase secretion but even then cytoplasmic LDH leakage was still continuing to increase at a time when amylase release was actually decreasing (Fig. 9). Laser light was effective, with SALPC, in eliciting ^{86}Rb efflux and amylase release. An interesting point is that here too ^{86}Rb efflux occurred several minutes after amylase release; furthermore, ^{86}Rb efflux was at only a fraction of its maximum when near maximal amylase release was observed. Thus, as with the quartz-halogen light source, membrane permeabilization is not required for maximal amylase release induced by laser light.

If there is no evidence that the plasma membrane is permeabilized extensively during the initial phase of amylase release (i.e., in the first 6 min) then the photodynamic release of amylase must be due to factors other than a direct influx of calcium. Since, in common with several other photosensitizers [4], SALPC is highly negatively-charged, with a sulphonate/phthalocyanine ratio of approximately 3 [28, 29], it cannot easily cross the plasma membrane and enter the cell. SALPC taken up by perifused acinar cells will therefore be confined largely to the cell membrane. The ability of photon-activated SALPC, 1 μM, when free in the extracellular space to cause a permeabilization of the plasma membrane

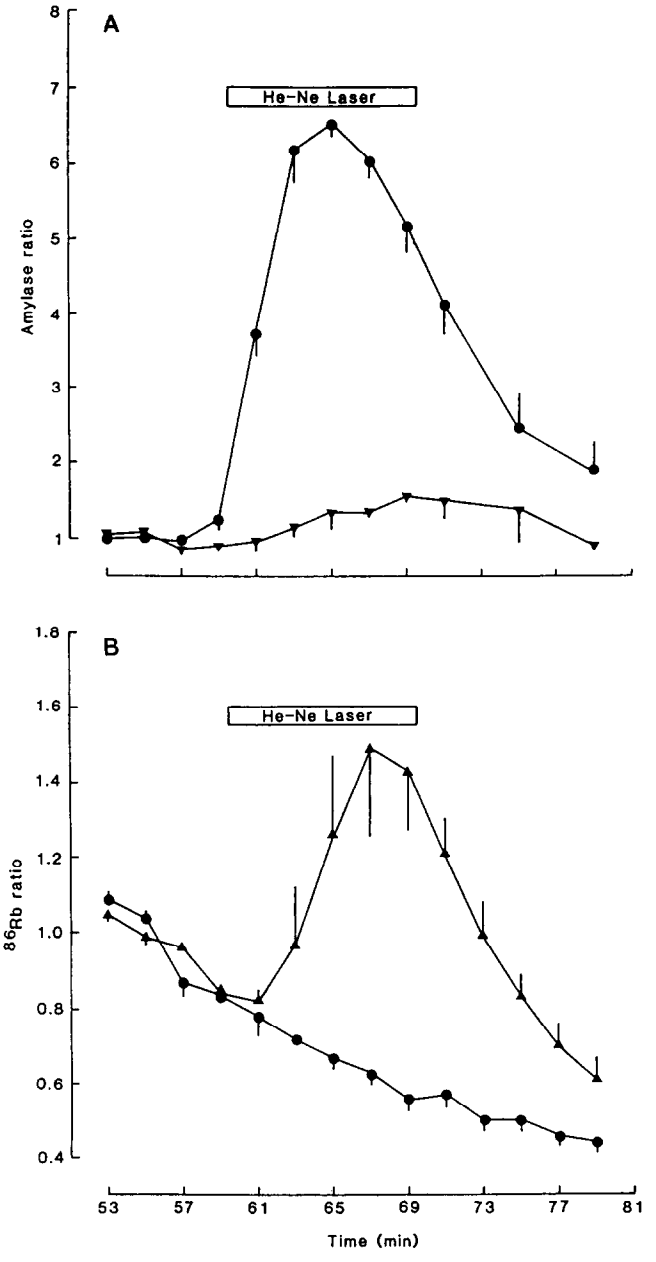

Fig. 10. Photodynamic action of SALPC on (A) amylase release and (B) ^{86}Rb efflux; helium–neon laser light source (632.8 nm). Acini were exposed to SALPC, 1 μM (A, ●; B, ▲) and irradiated with laser light (632.8 nm) for the time indicated by the horizontal bars. Control acini (A, ▼; B, ●) were exposed to laser light but not to SALPC (N = 3–4).

no greater than that occurring in response to cellularly-bound SALPC confirms the plasma membrane as the primary locus of SALPC action, a conclusion strengthened recently by fluorescence microscopy [30]. Any action that SALPC may have leading ultimately to the release of amylase must therefore also be located primarily, if not exclusively, at the plasma membrane. The most obvious explanation would be a direct stimulation by singlet oxygen of secretagogue receptors, or their coupled G proteins or effectors, resulting in the formation of second messengers and hence secretion of amylase by exocytosis.

SALPC is chemically very stable and even after very intense photodynamic action appreciable amounts of membrane-bound SALPC will remain fully effective. Therefore, during illumination for 10 min, SALPC molecules will be activated repetitively with a high probability that each SALPC molecule could be excited to the triplet state more than once. The stoichiometry is such that for every membrane-located SALPC molecule, more than one molecule of singlet oxygen can be produced and react with a target molecule. Singlet oxygen has a lifetime of 5 μsec in aqueous solutions; it therefore has a

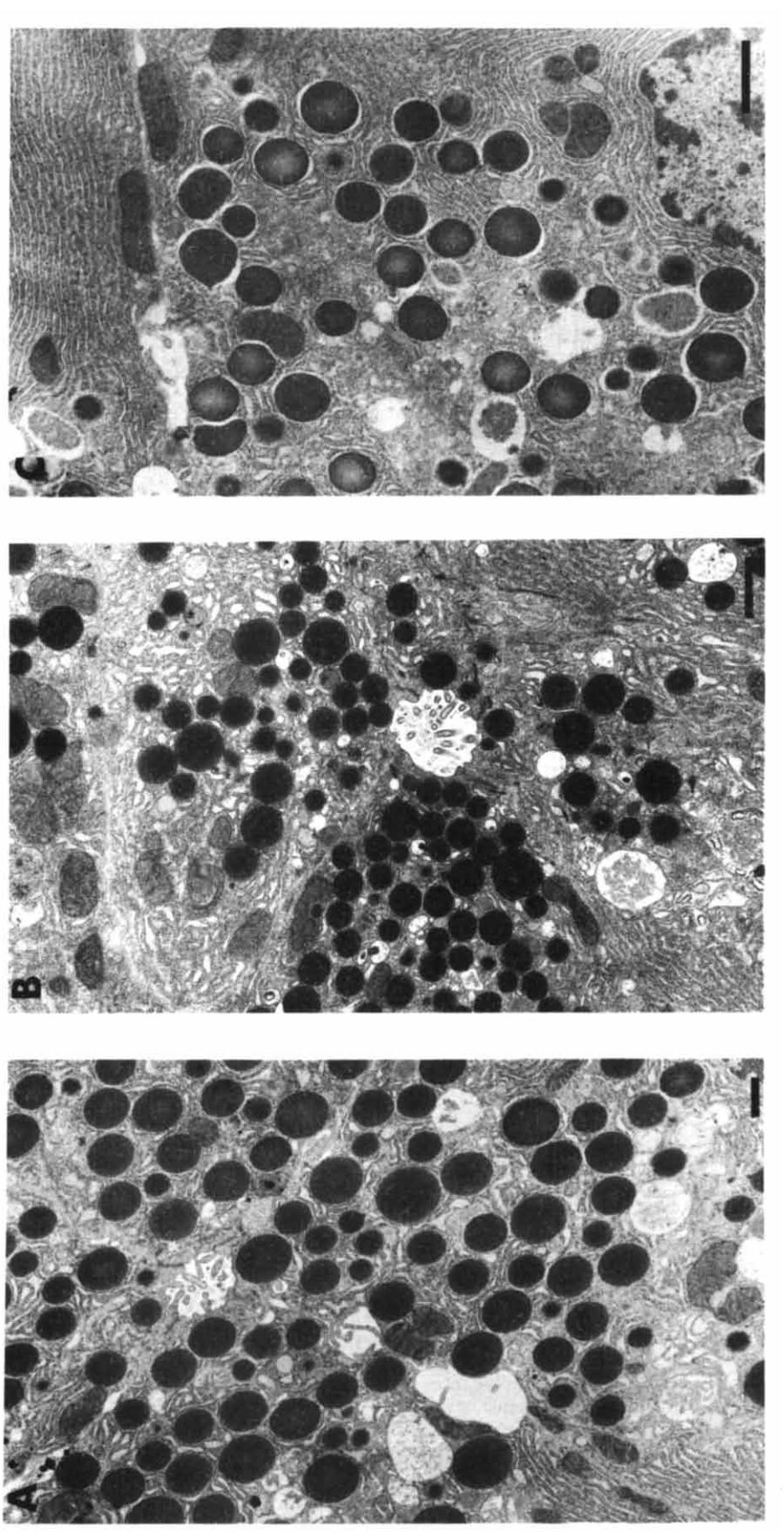

Fig. 11. Acinar cell ultrastructure. (A) Isolated pancreatic acini in the absence of SALPC or light. (B) Acini after light irradiation (4500 lux, 10 min) in the absence of SALPC. (C) Acini after photodynamic action (SALPC, 1 μM; 4500 lux, 10 min). (D) Acini after light irradiation (18,400 lux, 10 min) in the absence of SALPC. (E) Acini after photodynamic action at the higher illuminance (SALPC, 1 μM; 18400 lux, 10 min). Calibration bar: 1 μm.

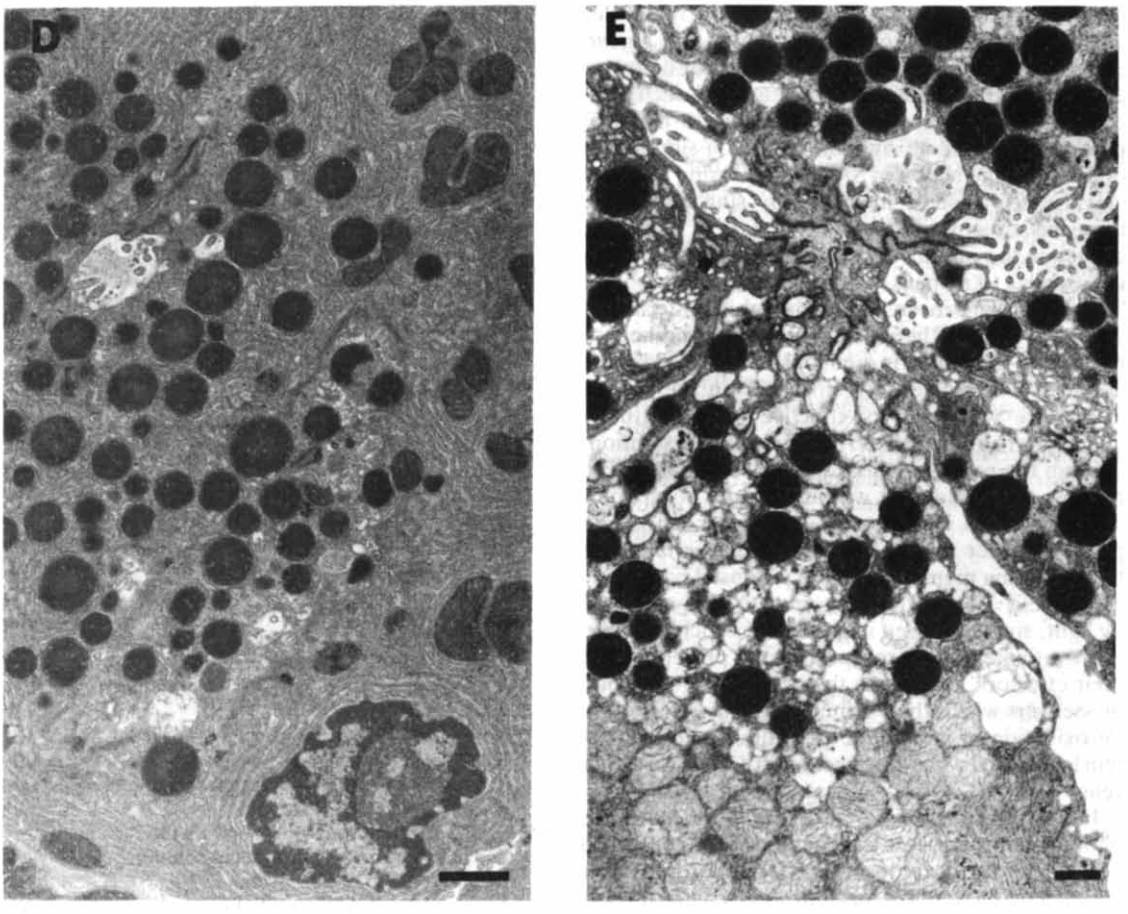

Fig. 11. Continued.

mean free diffusion pathway of 33 nm [31] and if the potential target site of action of singlet oxygen is separated from the site of production by a distance greater than this, it will not reach the target. Singlet oxygen produced in the plasma membrane for example, is unlikely to reach the nucleus or other subcellular structures laying deeper within the cell. At present the factors which govern the uptake of negatively-charged photosensitizers into the plasma membrane are not completely understood but it has been claimed that the uptake conforms to the Freundlich adsorption isotherm rather than to the linear relationship of simple solvent partitioning [32]. In the present experiments SALPC uptake appears to saturate at higher concentrations. There may therefore be a limited number of membrane adsorption or insertion sites even though specific SALPC receptors are unlikely to exist. Alternatively, progressive accumulation of SALPC may alter the membrane surface charge and so limit the uptake of further molecules.

It is noteworthy that the maximal concentration of SALPC causing amylase secretion is 100 nM (Fig. 5). The amount of SALPC taken up at this concentration is 5.2 pmol/mg cell protein which, since the protein content of a single acinar cell approximates to 0.285 mg [33], is equivalent to 8.9×10^5

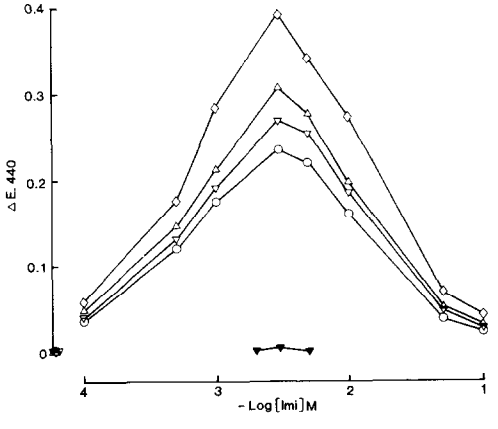

Fig. 12. Measurement of singlet oxygen production. Dependence on imidazole concentration of RNO bleaching by rose bengal, 1 μM (◇); SALPC, 1 μM (△); erythrosine B, 1 μM (○); merocyanine 540, 1 μM (▼), all at 14,100 lux (unfiltered) and SALPC, 1 μM (▽) at 4500 lux (23A filter). The reduction of extinction (E_{440}) after light irradiation for 10 min is plotted against the concentration of imidazole used for each determination.

molecules per cell. Assuming that these molecules are mainly confined to the membrane, are spread evenly over the surface of an acinar cell of diameter 20 μm, and that the active target area of each molecule can be represented by a hexagon for maximal packing, then the hexagon must have a side length of 23 nm. If the action of SALPC in causing maximal amylase secretion is mediated by singlet oxygen, then the effective diffusion path length of singlet oxygen in the plasma membrane must be 23 nm also i.e., similar to its path length in aqueous solution of 33 nm [31]. This means that although the lifetime of singlet oxygen in the plasma membrane is likely to be longer than that in aqueous solution [34], the abundance of singlet oxygen targets in the plasma membrane will limit its effective path length by quenching to approximately that in aqueous solution.

From the results discussed so far, it is possible to propose the following working model for photodynamic action in rat pancreatic acinar cells. The photosensitizer after a brief incubation is taken up by the plasma membrane of the perifused pancreatic acinar cells. When the photosensitizer is activated by light, singlet oxygen is produced, which can then react with membrane receptors (or G proteins), and their effectors, leading to the production of second messengers which then stimulate amylase secretion. Peroxidation of membrane structural lipid and protein leads also to photopermeabilization of the acinar cells.

In conclusion, we have shown that the photodynamic process can be precisely controlled not only by the local concentration of photosensitizer but also by the photon flux and wavelength or spectral content of the incident light. Photodynamic action has the major advantage that it can be used both *in vivo* and *in vitro*, in single cell preparations, or in cell clusters, as here in pancreatic acini; it is hence a powerful technique for the control of cellular function. Finally, our results have important implications not only for further investigations of the molecular mechanisms of secretion and of photodynamic drug action but also for defining the way in which secretory cell function can be affected by photodynamic agents in both normal and in pancreatic tumour cells [35, 36].

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