γ-Hexachlorocyclohexane activation of alveolar macrophage phosphatidylinositol cycle, calcium mobilization and O_2^- production

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The influence of γ-hexachlorocyclohexane (HCC) on phosphatidyl inositol (PI) cycle activity was investigated in the guinea pig alveolar macrophage. Similar to stimulation by the chemotactic peptide *N*-formyl-nle-leu-phe (FNLLP), 125 μM HCC was found to stimulate PI cycle activity, calcium mobilization ($^{45}Ca^{2+}$ efflux and cytosolic $[Ca^{2+}]$ elevation) and O_2^- production, although the action of HCC was prolonged in comparison. HCC treatment did not block subsequent stimulation by FNLLP. HCC is proposed to act at an early stage in the same activation sequence as does FNLLP.

Macrophage *Hexachlorocyclohexane* *Phosphatidylinositol* *Quin2* *Formyl peptide* *Calcium*

1. INTRODUCTION

The insecticide γ-hexachlorocyclohexane (HCC) is toxic in a number of isolated cells and unicellular organisms (review [1]). The biochemical mode of action of HCC was originally proposed to be due to antagonism with inositol resulting from the related stereochemistry of the two compounds. HCC has subsequently been shown to not correspond configurationally to myoinositol and myoinositol does not protect against HCC animal toxicity. However, HCC has been shown to inhibit phytohemagglutinin-stimulated lymphocyte growth and stimulated phosphatidyl inositol (PI) turnover (2) and block both acetylcholine-stimulated PI synthesis in cerebral cortex slices and phosphatidic acid-inositol transferase in cerebral cortex microsomal preparations [3]. No effect of HCC was observed in unstimulated cerebral cortex slices [3].

Due to the current interest in investigating the relationship between PI turnover and cellular activation, we examined the influence of HCC on *N*-formyl-nle-leu-phe (FNLLP)-stimulated alveolar macrophage superoxide anion (O_2^-) production. FNLLP stimulation leads to increased PI turnover in the alveolar macrophage in parallel with Ca^{2+} mobilization and O_2^- production [4,5]. Here, we report that HCC itself stimulated PI turnover, Ca^{2+} mobilization and O_2^- production without blocking subsequent FNLLP stimulation of O_2^- production.

2. MATERIALS AND METHODS

Alveolar macrophages were isolated from guinea pigs by lung lavage as in [6]. Cells were suspended in Ca^{2+}-Mg^{2+} free Hanks' balanced salt solution (MHBSS). Macrophage purity (85–95%, PMN < 5%), viability by trypan blue (>93%) and total cell counts were assayed as previously described [4]. All assays were conducted at 21–23°C.

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Extracellular levels of superoxide dismutase-inhibitable superoxide anion were monitored continuously as previously described [7] using a Perkin-Elmer Lambda 3 recording spectrophotometer by measuring ferricytochrome *c* reduction (75 μM, type III, Sigma, St. Louis, MO) at 550 nm. An extinction coefficient of 18.5 mM (reduced-oxidized) was used for ferricytochrome *c*.

Cytosolic calcium concentrations were measured using cells preloaded with 1.5 mM quin-2 as previously described [4]. Fluorescence intensity of intracellular quin-2 was monitored continuously with a Perkin-Elmer MPF-3 fluorimeter with excitation at 339 nm and emission at 492 nm.

$^{45}Ca^{2+}$ efflux measurements were conducted using cells preloaded with $^{45}Ca^{2+}$ as previously described [8]. Cells were exposed to HCC (125 μM) immediately after suspension in fresh $^{45}Ca^{2+}$-free medium. At specified times after HCC addition, 250-μl aliquots were removed and centrifuged through 10 μl of silicone oil (S.G. 1.015) in a Beckman B microfuge. Radioactivity in the cell pellets was measured in an Intertechnique LS 30 liquid scintillation counter using ACS II (Amersham, IL).

The flux of metabolites through the phosphatidyl inositol cycle was measured as in [6]. Analysis of phosphatidic acid (PA), phosphatidyl inositol-4-phosphate (DPI) and phosphatidyl inositol-4,5-bisphosphate (TPI) was carried out by a modification of the method of [9] as previously described [10].

$^{45}Ca^{2+}$ and quin-2 ester were obtained from Amersham (Arlington Heights, IL) and $^{32}P_i$ from New England Nuclear (Boston, MA). FNLLP and HCC were from Sigma.

3. RESULTS

3.1. O_2^--measurements with HCC

The addition of 125 μM HCC alone resulted in the stimulation of O_2^- production (fig.1), which was superoxide dismutase inhibitable. Higher concentrations of HCC (such as those used in [2] and [3]) were insoluble in MHBSS, resulting in large changes in the absorbance due to solubility artifacts. Maximum initial rates of superoxide anion production with 125 μM HCC were 10.8 ± 1.5 nM $O_2^-/10^6$ cells per 5 min (mean ± SE, $n = 5$). It was observed that the time course of HCC-stimulated

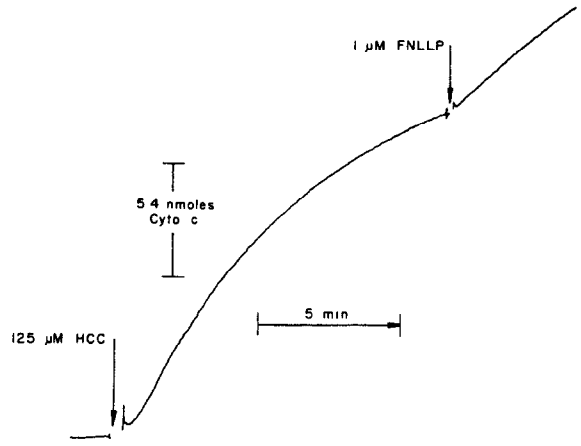

Fig.1. HCC- and FNLLP-stimulated alveolar macrophage O_2^- production in MHBSS. Superoxide anion measurements were conducted as described in section 2. HCC (125 μM) and FNLLP (1 μM) were added at the times shown. The rate of O_2^- production after FNLLP stimulation (above) was 40% of that observed after stimulation by FNLLP alone (not shown). The trace represents 6 sets of measurements.

O_2^- production was significantly more prolonged than that observed for FNLLP in MHBSS medium [4,7]. While this concentration of HCC produced high initial rates of O_2^-, it was not able to completely block stimulation by the subsequent addition of 1 μM FNLLP (fig.1) or another potent macrophage stimulant, phorbol-12,13-dibutyrate (not shown).

3.2. *Effect of HCC on $^{45}Ca^{2+}$ efflux and cytosolic $[Ca^{2+}]$*

The addition of 125 μM HCC resulted in a rapid and significant additional loss (20%) of cell-associated $^{45}Ca^{2+}$ (fig.2). Stimulated rates of $^{45}Ca^{2+}$ loss were observed within 1 min, after which the rates of $^{45}Ca^{2+}$ efflux were similar to those previously reported for formyl peptides [8].

Changes in cytosolic $[Ca^{2+}]$ upon HCC exposure were monitored via fluorescence using cells preloaded with 1.5 mM quin-2. To permit maximum possible changes of cytosolic $[Ca^{2+}]$ during stimulation [4], 1 mM Ca^{2+} was added extracellularly, followed by 125 μM HCC (fig.3). The addition of 1 mM Ca^{2+} resulted in an increase in cytosolic $[Ca^{2+}]$, from basal levels in MHBSS of approximately 40 nM, followed by a redistribution of intracellular Ca^{2+} as in [4]. The addition of 125 μM

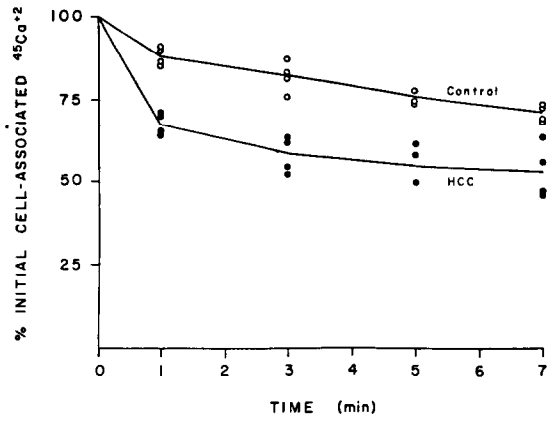

Fig.2. Control and HCC-stimulated efflux of $^{45}Ca^{2+}$ from alveolar macrophages. Efflux was measured from macrophages loaded with $^{45}Ca^{2+}$ for 30 min as described in section 2. HCC (125 μM) was added concurrently with measurement of zero time cpm ($^{45}Ca^{2+}$ cpm in untreated (control) samples taken immediately after resuspension in $^{45}Ca^{2+}$-free medium). Data are shown as the percent of the zero time cpm remaining cell-associated as function of time. The lines connect the average of duplicate measurements made in two experiments.

HCC resulted in a rapid rise of cytosolic [Ca^{2+}] to approximately 100 nM followed by a slow decrease. The redistribution of cytosolic [Ca^{2+}] following HCC stimulation was significantly prolonged compared to that observed with FNLLP [4]. Addition of 1 μM FNLLP 4 min subsequent to the addition of HCC resulted in only a slight increase in cytosolic [Ca^{2+}], suggesting that both stimulants mobilize the same pool of membrane-associated Ca^{2+}.

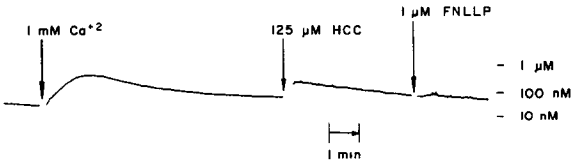

Fig.3. Cytosolic [Ca^{2+}] in alveolar macrophages treated with HCC. Quin-2 was loaded for 30 min at 21–23°C as described in section 2. Cytosolic calcium concentrations (values on right hand scale) were calculated as described in [4]. Calcium (1 mM), HCC (125 μM) and FNLLP (1 μM) were added at the times indicated. The figure represents 4 experiments.

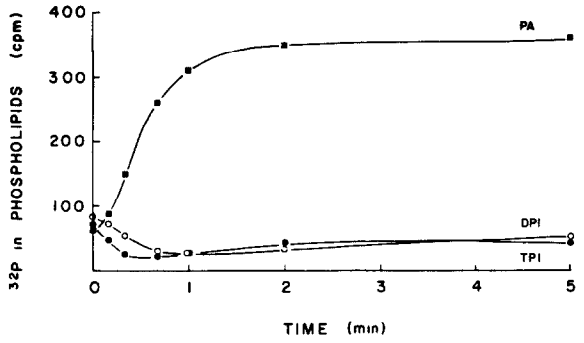

Fig.4. HCC-stimulated change in ^{32}P label in alveolar macrophage phospholipids at 21–23°C. Cells were loaded with 40 μCi/ml $^{32}P_i$ for 60 min and analyzed after stimulation as in section 2. HCC was added at zero time. Measurements were made of TPI (●), DPI (○) and PA (■) for cells in MHBSS. The points represent the average of data from two duplicate experiments.

3.3. *Effect of HCC on PI cycle activity*

The direct effect of 125 μM HCC on PI cycle activity was examined in macrophages prelabelled with $^{32}P_i$. The addition of HCC resulted in the rapid loss of label in both TPI and DPI, with that in TPI being more rapid (fig.4). In contrast to results with other reported stimulators of PI cycle activity [5], the loss of label in both TPI and DPI was much more sustained, with only partial recovery observed by 5 min. At the same time there was over a 5-fold increase in label in PA that continued to increase slightly after 2 min.

4. DISCUSSION

The addition of 125 μM HCC to guinea pig alveolar macrophages stimulated O_2^- production, PI turnover and calcium mobilization (increased $^{45}Ca^{2+}$ efflux and elevation of cytosolic [Ca^{2+}] measured with quin-2) in a manner similar to that previously observed for stimulation by FNLLP. However, HCC stimulated changes were prolonged in comparison [4,5,8], as evidenced by delayed recovery of ^{32}P-labelled TPI and DPI, delayed recovery of cytosolic [Ca^{2+}] and prolonged O_2^- production. The temporal relationship between PI turnover and Ca^{2+} mobilization cannot be determined in these experiments. However, it has been proposed that PI turnover elicits Ca^{2+} mobilization in activation by FNLLP [4,5]. The

similarities in PI turnover, $^{45}Ca^{2+}$ efflux and intracellular Ca^{2+} mobilization between exposure to both FNLLP and HCC suggest that HCC activates the cell at an early stage within the same mechanism by which FNLLP activates O_2^- production.

FNLLP stimulation of O_2^- production after the addition of HCC was not completely blocked, but the observed rate of O_2^- production in these circumstances was less than rates observed after exposure to 1 μM FNLLP alone. Since FNLLP stimulation alone in MHBSS is transient, prior HCC activation via the same mechanism may result in partial depletion of a regulatory substrate (e.g., PI and/or Ca^{2+}) which would limit subsequent FNLLP stimulation to a degree which may be a function of time after HCC exposure.

The mechanism of macrophage activation by HCC is not clear, except that it most likely acts early within the same sequence as for peptide-stimulated activation. It is likely, then, to affect either (a) phospholipase C (the activation of which commences stimulated PI cycle turnover; (b) the FNLLP receptor or a similar type of receptor which activates phospholipase C; or (c) a transducer protein (if one exists) between the FNLLP receptor and phospholipase C.

These results demonstrate that HCC is a stimulant of alveolar macrophage O_2^- production, most probably via a mechanism very similar to activation by chemotactic peptides. Furthermore, these results provide another example of the probable association between calcium mobilization, PI turnover and O_2^- production in macrophage activation.

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