Chloroquine inhibition of cholera toxin

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Cholera toxin (CT) stimulated adenylate cyclase and a phospholipase which elevated cellular levels of 3',5'-cyclic adenosine monophosphate (cAMP) and arachidonic acid (AA). The AA was quickly converted to prostaglandins (PGs) via the cyclo-oxygenase pathway. Chloroquine exerted minimal inhibition of cAMP levels in CT-treated cells, although CT-induced release of [^{3}H]AA and PGs was blocked completely when the drug was added in concentrations as low as 0.1 mM (50 μg/ml). Inhibition of [^{3}H]AA release was complete when chloroquine was added before or within 30 min after CT. The capacity of chloroquine to inhibit either phospholipase C (PLC) or phospholipase A_2 (PLA_2) could explain the antisecretory activity of this drug.

1. INTRODUCTION

Cholera toxin (CT) exerted a stimulatory effect on arachidonic acid (AA) metabolism as well as on adenylate cyclase [1-4]. The precise mechanism of CT activation of phospholipase activity was not clear, although an initial study by Burch et al. [1] indicated that PLA_2, rather than PLC, may be involved. Their studies showed that CT and pertussis toxin caused the accumulation of lysophosphatidylinositol and glycerophosphoinositol in a murine macrophage/monocyte cell line. Further, minimal appearance of inositol phosphates was observed. Only phosphatidyl inositol 4,5-bisphosphate (PIP_2) and not other phospholipids decreased in concentration in response to these toxins. Indirect evidence favoring PLC activation by CT indicated that CT-induced [^{3}H]AA release from murine macrophages was blocked by aspirin but not by dexamethasone [5]. Whether hydrolysis of PIP_2 occurred by a one step (PLA_2) or a two step reaction (PLC and diglyceride lipase), the end result was AA accumulation in the cells. Since AA was a limited substrate, it was quickly converted to a variety of PGs by the cyclo-oxygenase pathway [6]. Therefore, we searched for points in the metabolic pathway where the effects of CT might be interrupted by administration of a metabolic inhibitor. This report indicated that chloroquine, a potent inhibitor of PLA_2 and PLC [7,8], effectively inhibited CT-induced AA release and PG synthesis in murine macrophages.

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Abbreviations: CT, cholera toxin; CQ, chloroquine; AA, arachidonic acid, cAMP, 3',5'-cyclic adenosine monophosphate; PGs, prostaglandins

2. MATERIALS AND METHODS

2.1. *Macrophage cultures and labeling*

Monolayers of a macrophage/monocyte cell line (Raw 264.7) (ATCC, Rockville, MD) with [^{3}H]AA-labeled phospholipids were established as described in detail elsewhere [1,5]. Briefly, 1 ml of a labeled macrophage cell suspension (4×10^5 cells) was added to each well of 24-well tissue culture plates (Falcon). All plates were incubated overnight at 37°C with 5% CO_2. Before CT or chloroquine was added, the monolayers were washed 3× with PBS and 1 ml aliquots of fresh culture medium were added.

2.2. *Administration of drugs and CT to macrophage cell cultures*

Labeled macrophages were treated with chloroquine phosphate (Sigma Chemical Co., St. Louis, MO) at designated concentrations for 1 h before CT (100 ng/ml) was added to the culture medium, then, they were incubated for another 2 h with or without CT. In the kinetic study, chloroquine (500 μg/ml) was added at various time intervals relative to the time of CT addition. Assay for [^{3}H]AA metabolite release was performed by sampling the culture supernatants as described by Reitmeyer and Peterson [2].

2.3. *HPLC analysis of prostaglandins*

Labeled monolayers (2×10^8 cells), growing in 75 cm^2 flasks, were washed (3×) with PBS. Fresh Dulbecco's medium (10 ml) was added to all flasks, which were randomly selected to serve as cell controls, CT controls (2 μg/ml), or drug + CT treatment groups. After addition of drug and/or CT, flasks were incubated for 4 h at 37°C with 5% CO_2, at which point the media were removed and analyzed for PGs. Extraction and HPLC separation of prostaglandins were performed as described by Peterson *et al.* [6].

2.4. *Cyclic AMP extraction and assay*

Two ml aliquots of a macrophage cell suspension (4×10^5 cells/ml) were incubated (37°C and 5% CO_2) overnight in tissue culture dishes (Falcon). Culture supernatants were aspirated and replaced with 2 ml of fresh medium. CT (100 ng/ml) with or without chloroquine (10 or 100 μg/ml) was added, and the cultures were incubated for 4 h. Then, cAMP was extracted from the macrophage cell monolayers and assayed by a radioimmunoassay as described by the manufacturer (Advanced Magnetics Inc., Cambridge, MA).

2.5. *Statistics*

Data were analyzed by a one-way ANOVA followed by the Tukey test. The dose response and kinetic experiments reflect the means of quadruplicate determinations ± 1 SD. The cAMP data depict pooled values of two experiments each performed in quadruplicate. All experiments were performed at least twice with comparable results.

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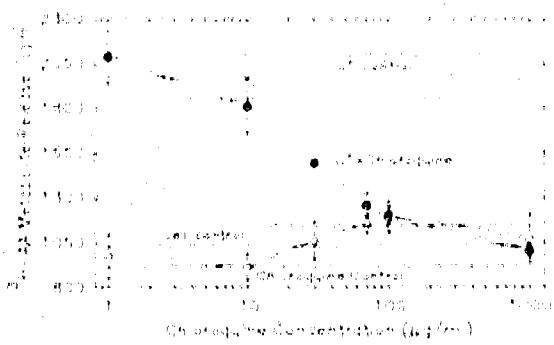

Fig. 1. Chloroquine dose-response curve depicting progressive inhibition of CT-induced [^{3}H]AA release from macrophages/monocytes. Exposure to CT (100 ng/ml) was 2 h for all samples, while chloroquine was added in various concentrations 1 h before the toxin. The vertical bars and dashed horizontal lines depict 1 SD. The CT Control indicates the mean ± 1 SD deviation and shows the maximum [^{3}H]AA release response in the absence of chloroquine. Similarly, the Cell Control shows the amount of [^{3}H]AA release in normal cells.

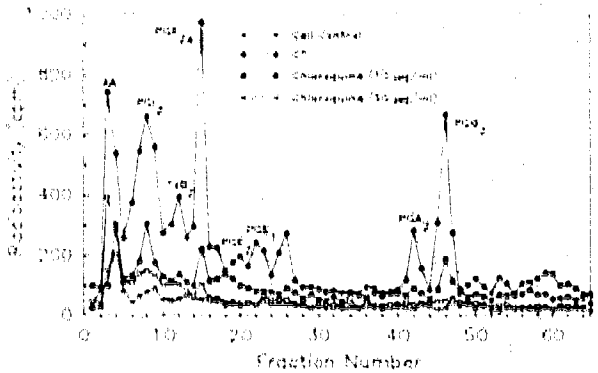

Fig. 3. Inhibitory effect of chloroquine on CT-induced PG synthesis. The HPLC system was fitted with a C18 column (Serva) and eluted with an isocratic gradient of 30% acetonitrile in 0.1% trifluoracetic acid. Comparisons were made with control cells, cells treated with CT (2 μg/ml), and cells exposed to both CT (2 μg/ml) and the drugs (10 μg/ml and 50 μg/ml). The identity of the prostaglandins was determined by chromatography of purified prostaglandin standards under identical conditions.

3. RESULTS

3.1. *Effect of chloroquine on [^{3}H]AA release*

Fig. 1 illustrates the inhibitory effects of chloroquine on CT-induced [^{3}H]AA release in murine macrophages/monocytes. Addition of chloroquine to the culture medium 1 h before CT (100 ng/ml) in concentrations of 50 μg/ml and above blocked [^{3}H]AA release completely ($P \leq 0.05$). Addition of chloroquine in concentrations of 10 μg/ml and below was not effective in altering the stimulatory effects of CT on AA metabolism ($P \geq 0.05$).

3.2. *Kinetics of chloroquine administration*

Fig. 2 indicated that the stimulatory effects of CT on AA metabolism occurred very early. Addition of chloroquine (500 μg/ml) before, or as late as 30 min after CT (100 ng/ml) application, resulted in total inhibition of CT-induced [^{3}H]AA release ($P \leq 0.05$). Partial inhibition was noted when the drug was added 1–2 h after the toxin. By 3 h, no inhibitory effects of the drug were detected ($P > 0.05$).

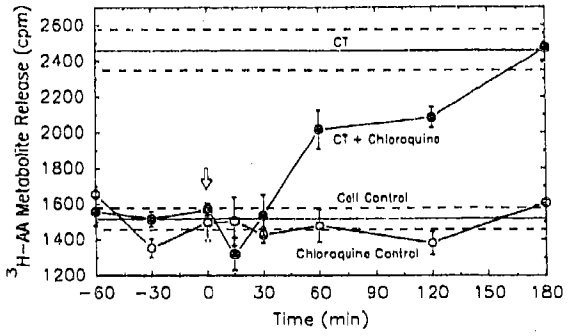

Fig. 2. Effect of time of chloroquine addition on CT-induced [^{3}H]AA release. Chloroquine (500 μg/ml) was added at various time intervals relative to the time of CT (100 ng/ml) application. The arrow indicates the time at which CT was added. Incubation time with CT was 3 h for every sample. All data points are means of quadruplicate determinations on replicate monolayers, and the vertical bars and dashed horizontal lines denote one standard deviation. The CT and Cell Controls were derived as indicated in Fig. 1.

3.3. *Chloroquine inhibition of PG synthesis*

Inhibition of [^{3}H]AA release from CT-treated cells constituted a metabolic blockade for subsequent synthesis of a variety of PGs. Fig. 3 illustrated the relative effectiveness of chloroquine in preventing CT-induced synthesis of PGs. The uppermost curve (CT treatment), when compared to the lowermost curve (cell control), depicted increased synthesis of PGI_2, TxB_2, $PGF_{2\alpha}$, PGE_2, and PGE_1. PGA_2 and PGB_2 are nonenzymatic degradation products of the relatively labile PGE_2. PG synthesis in cells exposed to CT in the presence of chloroquine (50 μg/ml) appeared similar to that of the cell control. When only 10 μg/ml of chloroquine was

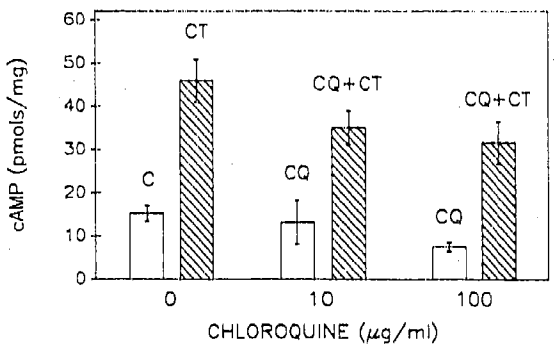

Fig. 4. Effect of chloroquine (CQ) on cellular cAMP levels. Chloroquine (10 μg/ml and 100 μg/ml) decreased basal levels of cAMP in the macrophages/monocytes; it also had a minimal inhibitory effect on cholera toxin (CT)-induced increased cAMP levels ($P \leq 0.05$).

added, limited synthesis of PGI_2, $PGF_{2\alpha}$, and PGB_2 was observed.

3.4. *Effect of chloroquine on cAMP levels*

Fig. 4 summarized the effects of CT on intracellular levels of cAMP in murine macrophages in the presence and absence of chloroquine. Chloroquine lowered basal levels of cAMP in the macrophages/monocytes, although these changes were not considered significant ($P > 0.05$). Chloroquine had minimal inhibitory effects on CT-induced cAMP levels, although the decrease was considered statistically significant by the Tukey test ($P \leq 0.05$) at either dose. The CT-induced cAMP accumulation in chloroquine-treated cells was significantly elevated above the respective controls ($P \leq 0.01$).

4. DISCUSSION

Chloroquine proved to be a very effective inhibitor of CT-induced [^{3}H]AA release and subsequent PG synthesis in murine macrophages/monocytes. The dose of chloroquine effective in inhibiting [^{3}H]AA release was 50 μg/ml (Fig. 1), and it was important that the drug be added to the culture medium within 30 min of the toxin (Fig. 2). Such kinetic characteristics indicated that CT evoked an early stimulatory effect on arachidonic acid metabolism suppporting the earlier observation of Reitmeyer and Peterson [2] that [^{3}H]AA release began within 5 min of exposure to CT. This inhibition of CT-induced AA release also was reflected in the PG synthesis profile (Fig. 3). Normally, CT caused the synthesis of numerous PGs including PGI_2, TxB_2, $PGF_{2\alpha}$, PGE_2, PGE_1, PGA_2, and PGB_2. Intracellular levels of cAMP were increased in response to CT, while chloroquine reduced cAMP minimally in both CT and control groups (Fig. 4). These observations suggested that CT-induced synthesis of cAMP occurred independent of the toxin's effect on AA metabolism leading to PG synthesis. Chloroquine blocked CT action by uncoupling cAMP-mediated events required for AA release [6]. Without AA release occurring in CT-treated cells, PG synthesis also was not possible.

Chloroquine previously has been shown to inhibit secretion of water and electrolytes in rat intestinal perfusion loops in vivo and Cl^- transport across isolated rabbit mucosa in vitro [10]. These observations clearly documented the protective effect of chloroquine against CT-induced alterations in vascular permeability; however, the molecular basis for the drug's activity was unknown. We attributed the antisecretory effect of chloroquine to its capacity to inhibit phospholipase activity, which in turn, prevented CT-induced AA release. A related drug, chlorpromazine, that blocked PLC and PLA_2 activity [8,9] also has been demonstrated to possess antisecretory activity; however, we observed less inhibition of CT-induced AA release by the latter drug (data not shown). Interestingly, drugs that blocked cyclo-oxygenase activity (e.g. indomethacin and aspirin) also possessed antisecretory activities [11–14]. We also have observed that aspirin inhibited [^{3}H]AA release from macrophages/monocytes [5]; however, high doses (1 mg/ml) of indomethacin failed to block [^{3}H]AA release (data not shown) even though lower doses (10 μg/ml) blocked CT-induced PG synthesis [5]. This information, combined with that of other studies correlating PG synthesis with CT-induced secretion [15], implicated AA metabolites in the mechanism of action of CT.

Chloroquine has been tested in cholera patients without diminishing the severity or duration of the disease [16]. CT effects on AA metabolism occurred early, and as shown in this study, chloroquine had to be added within 30 min of the toxin to elicit any inhibitory effect. Thus, it is not surprising that past clinical trials with this drug were less than encouraging. Based upon the kinetic data presented in this report, it might be anticipated that cholera patients would not respond to drug treatment once symptoms of fluid loss began. If this drug were to have clinical application, it would have to be administered very early. It is unknown whether chloroquine could be used prophylactically to prevent symptoms of cholera in the same manner that the drug is used to prevent malaria. Nevertheless, the drug appears useful in defining the molecular mechanism of cholera toxin.

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