Bordetella heat-labile toxin causes release of radioactivity from smooth muscle cells labeled with [14C]arachidonic acid

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1. SUMMARY

The effect of Bordetella heat-labile toxin (HLT) on cultured vascular or tracheal smooth cells labeled with [14C]arachidonic acid for 3 h was examined. At 37°C, in the presence of Ca2+, HLT induced the release of radioactive substances from the cells in a dose-dependent manner but HLT had no effect on release of radioactivity from cells at 0°C or in the absence of Ca2+. After cells were exposed to HLT, a 2-h lag period occurred before release of radioactivity was detected. The substances released from cells by HLT were identified as arachidonic acid and phosphatidylcholine.

2. INTRODUCTION

Virulent strains of all four Bordetella species produce a heat-labile protein toxin (HLT) [1–3] which induces ischemic or dermonecrotic lesions in the skin of most animals [3–7]. However, adult mice do not respond to HLT in this manner, because skin layers of these animals contain oleic and linoleic acids, substances which inhibit HLT [5]. Since the inhibitory substances were free fatty acids, we investigated whether HLT might affect lipid metabolism in target cells. In this paper we report that HLT induced release of radioactive substances from cultured smooth muscle cells which were labeled with [14C]arachidonic acid.

3. MATERIALS AND METHODS

3.1. HLT preparations

HLT was purified from phase 1 organisms of B. parapertussis strain 23054 by the method described previously [8]. The purified HLT preparation contained less than 0.033 µg of endotoxin per mg of protein [4] and 10 µg did not contain detectable adenylate cyclase toxin (ACT) or hemolysin activity.

3.2. Ischemia-inducing activity of HLT

Samples (0.1 ml) of an HLT preparation which had been diluted logarithmically in phosphate...
buffered saline were injected intracutaneously into the shaved back of guinea pigs (Hartley, female, 300–350 g). At 6 h after injection, the diameters of the ischemic lesions were measured. The minimum quantity of protein required to induce an ischemic lesion 10 mm in diameter is defined as the minimum ischemia-inducing dose (MID) and one MID of the purified HLT used in this experiment was 0.36 ng protein.

3.3. Preparations of cultured muscle cells

The cultured vascular smooth muscle cells were prepared from pig aortas by the method described previously [9]. Other types of smooth muscle cells from pig trachea, intestine, uterus and vas deferens were prepared as described previously [10] using an enzyme solution containing collagenase (type I) and elastase (type IV) purchased from Sigma (St. Louis, MO). The cells were used after 5 days of culture in Dulbecco’s modified Eagle’s (DME) medium supplemented with 5% fetal calf serum (FCS). Cultured cardiac- and skeletal-muscle cells were obtained from pig ventricle and skeletal muscles, respectively.

3.4. Measurement of radioactive substances from cells labeled with [14C]arachidonic acid

Arachidonic acid release was measured by a method previously described [11]. Briefly, the cultured muscle cells were labeled for 3 h at 37°C with [14C]arachidonic acid (0.05 μCi/ml) in 6-well tissue culture plates with 1 ml of DME medium supplemented with 1% FCS. The cells were washed three times with pre-warmed DME medium containing 1% FCS. Pre-warmed DME medium (supplemented with 5% FCS) containing HLT or control buffer (total volume of 1 ml) was then added. After incubation for up to 4 h, radioactivity in the medium was measured. Cells were collected and the radioactivity remaining in the cells was also measured. Released radioactivity is reported as percentage of total radioactivity taken up by the cells (total cpm = cpm released + cpm remaining in cells).

3.5. Preparations of endotoxin, adenylate cyclase toxin and pertussis toxin

A standard Escherichia coli endotoxin available in the Pregel kit supplied by Seikagaku-Kogyo (Tokyo, Japan) was used. Adenylate cyclase toxin (ACT) was purified from phase 1 organisms of B. pertussis strain 3779B by the method described previously [12] except for using calmodulin (CaM)-Cellulofine prepared by ourselves from CaM (Sigma) and FMP activated-Cellulofine (Seikagaku-Kogyo) instead of CaM-agarose. The ACT preparation at a concentration of 3 μg protein/ml induced marked haemolysis in rabbit erythrocytes. Pertussis toxin (PT) was obtained from culture fluid of B. pertussis strain 3779B by the method described previously [13]. At a dose of 1 ng/ml, the PT preparation induced significant release of glycerol from rat adipocytes.

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Fig. 1. Effect of HLT on smooth muscle cells labeled with [14C]arachidonic acid. [14C]arachidonic acid-labeled tracheal smooth muscle cells were exposed to HLT (300 MID/ml, filled symbols) or phosphate-buffered saline (PBS, open symbols) for 0, 1, 2, 3 or 4 h at 37°C (circles and triangles) or 0°C (squares). The cells were exposed to HLT (300 MID/ml) in the presence (circles and squares) or absence (triangles) of Ca2+ in the incubation medium. After incubation, the radioactivity in the medium was measured using a liquid scintillation counter. The remaining cells were collected and radioactivity was measured. Released radioactivity is expressed as percentage of total radioactivity originally taken up by the cells (total cpm = cpm released + cpm remaining in cells). Each symbol with a vertical line represents the mean ± SD of triplicate determinations.
4. RESULTS AND DISCUSSION

When [14C]arachidonic-acid-labeled tracheal smooth muscle cells were exposed to 30–300 MID/ml of HLT, the HLT induced release of radioactive substances from the cells (Fig. 1). The toxin also induced release of radioactivity from other types of cultured smooth muscle cells such as those from aorta, intestine uterus and vas deferens (data not shown). The effect of HLT on release of radioactivity from cultured cardiac and skeletal muscle cells (Fig. 2) was less striking than that observed from smooth muscle cells. The time required for induction of release of radioactivity by HLT (300 MID/ml) was 2 h (Fig. 1) and addition of higher doses of HLT did not shorten this lag period (data not shown). HLT-induced release of radioactivity was dose-dependent (Fig. 3) and was concomitant with cell contraction. HLT did not induce release of radioactivity at 0°C or in the absence of Ca2+ in the incubation medium (Fig. 1). The ability of HLT to induce the release of radioactive substances from cultured smooth muscle cells was not affected by endotoxin, ACT or PT (Table 1). If the substance released from the cells by HLT under the conditions used in this experiment were arachidonic.

![Fig. 2. Effect of HLT on the release of radioactivity from cultured skeletal or cardiac muscle cells. Cultured skeletal (A) or cardiac (B) muscle cells labelled with [14C]arachidonic acid were exposed to HLT (300 MID/ml, filled circles) or PBS (open circles), and incubated for 0, 2 or 3 h at 37°C. After incubation, radioactivity in the medium was measured as described in Fig. 1. Each symbol with a vertical line represents the mean ± SD of triplicate determinations.](https://academic.oup.com/femsle/article/93/2/133/555885)

![Fig. 3. Effect of concentration of HLT on release of radioactivity from cells. Cultured tracheal smooth muscle cells labeled with [14C]arachidonic acid were exposed to HLT (30, 100 or 300 MID/ml) and incubated for 3 h at 37°C. After incubation, radioactivity in the medium was measured as described in Fig. 1. Each symbol with a vertical line represents the mean ± SD of triplicate determinations.](https://academic.oup.com/femsle/article/93/2/133/555885)

<table>
<thead>
<tr>
<th>Added</th>
<th>Radioactivity released (Ct)</th>
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<tbody>
<tr>
<td></td>
<td>HLT ±</td>
</tr>
<tr>
<td>Control buffer</td>
<td>14.3 ± 1.3</td>
</tr>
<tr>
<td>Endotoxin (1 µg/ml)</td>
<td>13.5 ± 1.2</td>
</tr>
<tr>
<td>ACT (3 µg/ml)</td>
<td>14.0 ± 1.4</td>
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<tr>
<td>PT (0.1 µg/ml)</td>
<td>13.8 ± 1.6</td>
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* The tracheal smooth muscle cells were exposed to HLT (300 MID/ml) or control buffer, and incubated for 4 h at 37°C.

b Mean ± SD of triplicate determinations.
Fig. 4. Effect of HLT on phosphatidylinositol. [3H]phosphatidylinositol (t-o-[2-3H]-myo-inositol), 4.4 nmol/ml) (A, B and C) or [14C]-phosphatidylinositol (f-a-cholesteryl-l-14C), 660 nmol/ml) (D, E and F) was incubated with PBS (A and D), HLT (312 MID/ml) (B and E) or bee venom (10 μg/ml) which has phospholipase A2 activity (C and F) for 18 h at 37°C. After incubation, lipid fraction was obtained by extraction in chloroform. The chloroform extracts were dried, resolved in an organic solvent (CHCl₃:CH₃OH:H₂O = 20:20:5), and spotted on Silica gel 60 thin layer chromatography (TLC) plates. A solvent (CHCl₃:CH₃OH:CH₃COOH:H₂O = 25:15:4:2) was used for TLC. Radioactivity in the silica gel collected by scratching was measured by a liquid scintillation counter. Standard lipids are shown by the arrows in this Figure (PI, phosphatidylinositol; Lyso-PI, lysophosphatidylinositol: Ins, inositol; and AA, arachidonic acid).
aaDG

ps PE PC

Fig. 5. High performance liquid chromatography (HPLC) of radioactive substances released from tracheal smooth muscle cells. Tracheal smooth muscle cells labeled with $[^{14}C] $arachidonic acid were incubated for 6 h with 300 nM/ml of HLT (A) or PBS (B) in 5 ml of DME medium containing 5% FCS in tissue culture flasks (75 cm$^2$). The incubation medium was collected from each flask and lipids were extracted with chloroform. The percentage of radioactivity released from cells exposed to HLT or PBS was 21% of 3.4% of the total counts, respectively. Each extract was dried under a nitrogen gas stream, and dissolved in 250 μl of an organic solvent (CH$_3$CN:CH$_3$OH:H$_2$PO$_4$ = 900:95:5). The samples (200 μl) were then analysed with a TSK gel Silica 60 HPLC column (0.46 × 25 cm) using the organic solvent as an eluent. Standard lipids are shown by the arrows in this Figure (AA, arachidonic acid; DG, diacylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; and PC, phosphatidylcholine).

...acid [11], then HLT might have a phospholipase A$_2$ activity. However, we reported previously that the induction of contraction of vascular smooth muscle cells by HLT was not inhibited by phospholipase A$_2$ inhibitors [14]. We also found that HLT did not have intrinsic phospholipase A$_2$ or C activity (Fig. 4). Thus, we attempted to determine whether the radioactive substance was arachidonic acid. As shown in Fig. 5, the released radioactive substances were identified as arachidonic acid and phosphatidylcholine by high-performance liquid chromatography (HPLC). These data suggest that HLT may induce release of components of the membrane of the smooth muscle cells, possibly resulting in disturbance of membrane permeability of the cells. As demonstrated previously, HLT induced an increase in permeability of vascular smooth muscle cell membranes [3,14]. Further experiments are aimed at determining whether release of phosphatidylcholine might be the first step in HLT-induced contraction of smooth muscle cells.

REFERENCES