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Short communication

'In vivo' studies on the pathophysiological mechanism of *Vibrio parahaemolyticus* TDH$^+$—induced secretion

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Abstract

The thermostable direct haemolysin (TDH) is considered to be the major virulence factors of *Vibrio parahaemolyticus*; however, poor information is available about its mechanism of action. In our study we examined the capacity of two *V. parahaemolyticus* TDH-producers (strains 2067 and 3305) to induce fluid secretion in rat ileal loop and to reveal the role of calcium ions (Ca^{2+}), calmodulin (CaM), and protein kinase C (PKC) in *V. parahaemolyticus* TDH$^+$-induced fluid secretion. The results show that *V. parahaemolyticus* TDH$^+$ strains were able to induce secretion in small intestine; on the contrary, this ability was not evidenced in the *V. parahaemolyticus* TDH$^-$ strain used as negative control. The data suggest an enterotoxic activity of haemolysin. Calcium ionophore A23187 and 1-verapamil (calcium channel blocker), when injected alone, induced fluid accumulation in the control loops. A further increase in fluid accumulation ($P<0.001$) was noted when calcium ionophore was injected along with bacterial suspension of both TDH$^+$ strains and a significant decrease ($P<0.001$) in experimental loops when 1-verapamil was inoculated along with bacterial suspension. The other modulating agents increased fluid accumulation in both control and experimental loops, without significant differences with respect to the positive control.

Our findings suggest that Ca^{2+} appears to be an important messenger involved in the stimulation of intestinal secretion, contrary to PKC and calmodulin which do not appear to have any role.

Keywords: *Vibrio parahaemolyticus*; TDH; Pathophysiological mechanism

1. Introduction

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium that naturally inhabits marine and estuarine environments. *V. parahaemolyticus* infections in humans, are often associated with the consumption of raw or undercooked shellfish and exposure of wounds to contaminated seawater. In the last decade, it has been recognised as one of the most common causes of gastroenteritis due to the ingestion of contaminated seafood worldwide [1,2]. Acute enteritis caused by *V. parahaemolyticus* is characterized by a self-limiting watery diarrhoea, often accompanied by nausea, vomiting and/or abdominal cramps; occasionally bloody diarrhoea can also occur [3,4].

Although, the mechanisms underlying the diarrhoeal action are not fully understood, the intestinal disease is linked to some virulence properties of this pathogen including adhesiveness and other virulence factors such as exoenzymes and toxins [5]. Almost all *V. parahaemolyticus* strains isolated from clinical specimens exhibit a beta-type hemolysis on Wagatsuma agar, called the Kanagawa phenomenon (KP) [6]. Originally, this haemolysin has been considered an important putative virulence factor and the KP reaction was extensively used as a marker for the detection of pathogenic strains of *V. parahaemolyticus*. Subsequently, on the basis of its characteristics, this protein has been called thermostable direct haemolysin (TDH) and presently it is conventionally considered to be the main virulence factor for this species. TDH shows a variety of biological activities, such as haemolytic activity, enterotoxicity, miocardiotoxicity as well as cytotoxicity on cultured cells [7]. In fact, some studies suggest that

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the cytotoxic effect of TDH on enterocytes, is due to a number of TDH-generated channels underlining its role as a pore-forming toxin which causes membrane permeabilization and colloid osmotic lysis [8,9]. On the other hand, TDH is also reported to act as an enterotoxin, the action of which is mediated by intracellular calcium [10]. The enterotoxins give, as a final common pathway, fluid secretion in ligated rat ileal loops [11], that seems to be elicited by different intracellular mediators, such as cAMP, cGMP and Ca^{2+} [12]. Several authors, in fact, have reported a role for cyclic nucleotides, Ca^{2+}, calmodulin (CaM) and protein kinase C (PKC, an intracellular Ca^{2+}- and phospholipid-dependent enzyme) in the intestinal electrolyte transport [13,14]. However, complete data regarding the biochemical/pathophysiological mechanism(s) involved in the pathogenesis of *V. parahaemolyticus* TDH^+-induced diarrhoea, are still not available. In particular, for those concerning Ca^{2+} dependency of TDH action, the investigations conducted so far lead to conflicting conclusions [10,15].

In this study, we examined the ability of *V. parahaemolyticus* TDH^+ strains to induce fluid secretion in the rat small intestine, and we investigated the role of calcium ions (Ca^{2+}), calmodulin (CaM), and protein kinase C (PKC) in *V. parahaemolyticus* TDH^+-induced fluid secretion in rat ileum.

2. Results

To evaluate the pathogenic mechanism of *V. parahaemolyticus*-induced fluid accumulation, we used the rat ileal loop assay. Experiments proved that bacterial suspensions of the haemolysin producers (TDH^+) *V. parahaemolyticus* 3305 and *V. parahaemolyticus* 2067 strains caused secretion in the small intestine. In fact, TDH^+ strains inocula induced fluid accumulation when injected in positive control loops; no fluid accumulation was observed in negative control loops containing Mueller Hinton (MH) broth alone (Tables 1 and 2). As expected, no secretion was induced by the TDH^- *V. parahaemolyticus* 14335 strain (Table 3).

As the role of different modulating agents is concerned, a set of experiments was carried out. As shown in Tables 1 and 2, referred to both TDH^+ strains, the calcium ionophore A23187 induced secretion both in control (containing modulating agent only) and experimental control (containing MH broth along with modulating agent) loops, showing values similar to those of the correspondent positive control loops, containing bacterial inocula. On the other hand, a significant increase in the fluid accumulation was observed in experimental loops when this modulating agent was injected along with live cells of both TDH^+ *V. parahaemolyticus* strains. When the calcium channel blocker 1-verapamil was used, fluid accumulation was revealed in the control and experimental control loops, with values equivalent to those of positive control loops, while a significant decrease in fluid secretion was observed in both experimental loops injected with TDH^+ strains. The values of fluid accumulation obtained with the CaM inhibitor W-7 did not significantly decreased when this modulating agent was injected along with inocula of TDH^+ strains.

Experiments performed using the PKC activator PMA, showed that fluid accumulation was induced both in control and experimental control loops injected with each of the two TDH^+ strains tested; on the other hand, the values observed in the experimental loops did not show significant differences. Analogous results were also obtained when the potent, specific inhibitor of PKC, H-7, was used alone or together with bacterial inocula.

As regards the TDH^- *V. parahaemolyticus* 14335 strain (Table 3), all the tested modulating agents increased fluid accumulation either when injected alone or in association with bacterial suspensions. When compared to those obtained from positive control loops (bacterial cells only), these results were statistically significant, whereas no differences were observed between values obtained in control loops and experimental loops for each modulating agent.

Table 1
Effect of modulating agents on stimulation of intestinal secretion by the *V. parahaemolyticus* 3305, TDH^+, cytotoxic and enterotoxic strain

Modulating agents	Fluid accumulation in rat ileal loops (ml/cm)[a]				
	1° loop (negative control)	2° loop (positive control)	3° loop (control loop)	4° loop (experimental loop)	5° loop (experimental control)
	Mueller Hinton Broth	Bacterial inoculum	Modulating agent	Bacterial inoculum + modulating agent	Mueller Hinton Broth + modulating agent
Ca^{2+} A23187	0.023 ± 0.009	0.174 ± 0.010	0.175 ± 0.005	0.295 ± 0.005[b]	0.173 ± 0.009
1-Verapamil	0.023 ± 0.010	0.172 ± 0.012	0.170 ± 0.007	0.080 ± 0.010[b]	0.172 ± 0.010
W-7	0.021 ± 0.009	0.172 ± 0.010	0.166 ± 0.007	0.160 ± 0.003	0.019 ± 0.009
PMA	0.020 ± 0.009	0.173 ± 0.012	0.170 ± 0.010	0.175 ± 0.015	0.172 ± 0.009
H-7	0.024 ± 0.011	0.174 ± 0.011	0.163 ± 0.008	0.160 ± 0.017	0.171 ± 0.010

[a] Average of six values ± standard deviation espressed as ml/cm of fluid produced after 18 h of gut exposure to bacterial suspension.
[b] $P < 0.001$ as compared to both positive control loops (inoculated with bacterial suspension) and control loops (modulator alone).