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Role of the W07-toxin on *Vibrio cholerae*-induced diarrhoea

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Abstract

Vibrio cholerae W07 strain isolated from a cholera epidemic in South India, lacked the *ctx* gene but could still secrete a novel toxin, the W07-toxin that could cause fluid accumulation in ligated rabbit ileal loop. The important intracellular messengers implicated in this study were Ca^{2+}, cyclic AMP, inositol triphosphate and protein kinase C (PKC). A number of inhibitors/channel blockers have further shown the major role of $[Ca^{2+}]_i$ in modulation of the toxin-induced cellular response. An increase in the level of reactive oxygen species (ROS) in the W07-toxin-stimulated enterocytes correlated with the decrease in the levels of antioxidant enzymes, catalase and superoxide dismutase (SOD). The reactive nitrogen intermediates (RNI) detected by measuring the levels of nitrite and citrulline, were found to be high in the enterocytes triggered with the W07-toxin, thereby indicating their role in toxin-mediated change in mucosal permeability. The precise role of the toxin has also been authenticated by conducting the experiments with W07-toxin preincubated in the presence of IgG_{WT} (IgG isolated from antitoxin sera) or GM_1. Thus, a significant increase in the levels of second messengers and a decrease in antioxidant defenses appear to be important in mediating the fluid secretion caused by this novel toxin from *V. cholerae* W07.
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Keywords: *Vibrio cholerae*; W07-toxin; Intracellular messenger

1. Introduction

Toxigenic *Vibrio cholerae* cause cholera, a severe diarrhoeal disease responsible for significant morbidity and mortality worldwide. Two determinants, cholera enterotoxin (CT) and toxin coregulated pilus (TCP) are critical factors responsible for this organism's virulence. There have been reports of the genesis of newer strains of *V. cholerae* with varied toxigenic potentials [1]. The strains can acquire toxin-producing capacity by horizontal gene transfer or recombination events. Toxigenic *V. cholerae* strains are lysogens of a filamentous phage (CTXΦ) that encodes CT and recently another filamentous phage RS1Φ has been identified that plays a role in the dissemination of the toxin genes [2]. Walia et al. [3] identified a novel toxin (W07-toxin) in the strain, *V. cholerae* W07, isolated from a cholera epidemic in South India. The enterotoxic activity of the purified W07-toxin was observed in the rabbit ileal loop assay. It is possible that binding and internalization of this potent toxin may cause the subversion of a number of cellular processes resulting in the activation of intestinal secretion.

The intracellular messengers proposed to directly regulate the small intestinal electrolyte transport include cyclic nucleotides and calcium [4]. A direct evidence of synergism between cyclic adenosine 3′,5′-monophosphate (cAMP) and Ca^{2+}-mediated secretory mechanism in the intestine has also been reported [5]. The G-proteins have been shown to activate phospholipase Cγ (PLCγ), which catalyses the hydrolysis of phosphatidylinositol 4-5,bisphophate (PIP_2) to inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) [6]. The IP_3 mobilizes Ca^{2+} from intracellular stores and DAG can activate members of protein kinase C (PKC) family, which in turn, can phosphorylate the membrane proteins, thereby affecting the transport carriers or the conductance channels and ultimately leading to an electrolyte imbalance in the intestine [7]. The release of $[Ca^{2+}]_i$ can also be mediated through cAMP, the level of which has been found to be increased

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by the stimulation of calcium-activated adenylate cyclase [8]. Reactive oxygen species (ROS) have been implicated in diseases of the gastrointestinal tract [9]. The extent of oxidative damage due to ROS depends on the balance between the pro-oxidants and the antioxidants. In addition to ROS, nitric oxide produced by inducible nitric oxide synthase (iNOS) have also been shown to play a role in the pathophysiology of gastrointestinal disorders [10]. The ROS or the reactive nitrogen intermediates (RNI) can cause lipid peroxidation in enterocytes, which in turn may be responsible for altered membrane transport process as seen during diarrhoeal diseases [11].

Wright et al. [12] have reported the ROS-induced activation of PLCγ in an epithelial cell line. These authors have also shown that the RNI could activate PLC via a mechanism dependent (at least in part) on intracellular oxidant-mediated process(es). A significant decrease in the level of antioxidants in enterocytes during *V. cholerae* 0139 infection could lead to lipid peroxidation of the membrane components and thus might contribute to the changes in membrane permeability and ultimately fluid secretion [8]. Thus, in the present study, an attempt has been made to evaluate the level of different intracellular messengers and free radicals in mice enterocytes triggered with the novel, non-CT W07-toxin.

2. Material and methods

2.1. Chemicals

In the present study, chemicals used were of analytical grade. Radiolabeled chemicals were obtained from Radiochemical Centre, Amersham (Sweden, UK).

2.2. Laboratory animals

Inbred Balb/c mice (15–20 g) and male New Zealand white rabbits (2.0–2.5 kg) were obtained from the Central Animal House of Postgraduate Institute of Medical Education and Research, Chandigarh. The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (1996, published by the National Academy Press, 2101 Constitution Ave., NW, Washington, DC 20055, USA); and the use of animals was reviewed and approved by the Institute Ethics Committee.

2.3. Bacterial strains

Clinical isolate of *V. cholerae* W07 was a kind gift from Dr. G.B. Nair, NICED, (Kolkata) India. The strain was maintained in trypticase soy broth (TSB).

2.4. Purification of toxin

The novel toxin from *V. cholerae* W07 was purified by the method of Walia et al. [3].

2.5. Isolation of enterocytes

Enterocytes were isolated from mice small intestine as described by Pinkus [13] with modifications given by Toyoda et al. [14]. Briefly, the animals were sacrificed and the small intestine of each animal was quickly excised. The intestine was opened longitudinally, cut into small pieces and enterocytes were isolated by chelation–elution.

3. Study on the mode of action of the purified W07-toxin in mice enterocytes

The mode of action of the purified W07-toxin was studied in mice enterocytes (10^6 cells ml^{-1}) and compared with the commercially available CT (2 μg). Enterocytes without the W07-toxin served as control in all the assays. Most of the assays were done in the presence and absence of GM_1 (1 μg) or IgG_{WT} (IgG against the purified W07-toxin; diluted to 1:2500 in TBS) to assess the effect of the W07-toxin on different parameters of signal transduction. Further, specific inhibitors of different intracellular mediators were used in each assay to reveal the authenticity of the result. All the assays were performed in triplicate.

3.1. Estimation of intracellular free calcium concentration $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was estimated in enterocytes by the method of Pace and Galan [15]. Briefly, the isolated enterocytes were taken in 20 mM HEPES buffer (pH 7.4) containing 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$ and 5 mM glucose. The cells were triggered with different doses of the purified W07-toxin for 1 min and incubated with 2 μM Fura-2/AM (Sigma, Chemicals, dissolved in DMSO) at 37 °C for 45 min unabsorbed dye was removed by washing the cells in HEPES buffer (thrice). Finally, the enterocytes were suspended in the HEPES buffer and fluorescence measurements of the cell suspension were performed at an excitation wavelength of 340 nm. The emission spectrum was recorded at 510 nm. After measuring the basal fluorescence (F), 2 mM EGTA (prepared in 1 M Tris buffer, pH 8.8) was added to the suspension to make the pH of the solution as 8.3. Digitonin (10 nM) was added to permeabilize the cells and release the trapped dye, which resulted in maximum fall in fluorescence (F_{min}). Then 5 mM $CaCl_2$ was added to saturate Fura-2/AM and EGTA. The resultant fluorescence signal was designated as F_{max}. The intracellular free Ca^{2+} concentration was calculated by the equation:

$$[Ca^{2+}]_i = 224 \times (F - F_{min})/(F_{max} - F),$$

where 224 is the K_d value of Fura-2/AM. The optimum dose of the toxin was then used to trigger the enterocytes for different time period and the $[Ca^{2+}]_i$ was estimated.

To study the role of intracellular calcium stores, cells were preincubated separately with dantrolene, a drug known to trap Ca^{2+} in intracellular stores (20 μM, 15 min, 37 °C) or verapamil, an L-type Ca^{2+} channel blocker (50 μM, 15 min, 37 °C) or calciseptin, an endoplasmic reticulum L-type Ca^{2+} channel blocker (1 μM, 15 min, 37 °C) and treated with the W07-toxin. The levels of Ca^{2+} were then measured as described above.

3.2. Measurement of cAMP levels

The cAMP levels were measured in the enterocytes by RIA kit (Amersham International, Radiochemical Centre, Amersham, UK code TRK 432) according to the manufacturers' instructions. The assay is based on the competition between unlabeled cAMP and a fixed quantity of ^{3}H-labeled cAMP for binding to a protein, which has high specificity and affinity for cAMP. Measurement of the protein-bound radioactivity enables to calculate the amount of unlabeled cAMP in the sample. In this method, the enterocytes were suspended in TBS (pH 7.2) and triggered with the purified W07-toxin. The cells were centrifuged and cold 0.1 N HCl (0.1 ml) was added to the cell pellet followed by incubation for 15 min at 37 °C. The cell debris were removed by centrifugation ($500 \times g$, 10 min) and the supernatant was collected. Subsequently, the supernatant was titrated to pH 7.2 with 0.1 N NaOH and used for estimation. Results were expressed as pmol of cAMP mg^{-1} $protein^{-1}$ from a standard curve plotted with Co/Cx (where Co is the cpm bound in the absence of unlabeled cAMP and Cx is the cpm bound in presence of standard or unlabeled cAMP) versus pmol standard.

A separate set of experiment was conducted with the enterocytes preincubated with 2′,5′-dideoxyadenosine (DDA), a specific inhibitor of adenylate cyclase and the level of cAMP was measured as described above.

3.3. Assessment of PLCγ$_1$ level

The level of PLCγ$_1$ in the mice enterocytes was assessed in Western immunoblot [16]. Briefly, the enterocytes were triggered with the W07-toxin for different time period. These were then sonicated and debris were removed by centrifugation ($1000 \times g$, 10 min). The supernatant was analyzed for the expression of PLCγ$_1$ using affinity-purified rabbit polyclonal antibody raised against PLCγ$_1$ [PLCγ$_1$(1249): SC 81; Santa Cruz Biotechnology, Inc.] as the primary antibody (diluted to 1:1000 in TBS containing 1% BSA) and HRP-conjugated swine immunoglobulins to rabbit immunoglobulins (DAKOPATTS, Denmark) as the secondary antibody (diluted to 1:1000 in TBS-BSA). Flow cytometric analysis of whole cells triggered with the W07-toxin preincubated in presence and absence of IgG_{WT}/GM_1 were performed with the same primary antibody (diluted to 1:1000 in TBS) as before and the FITC-conjugated swine immunoglobulins to rabbit immunoglobulins (DAKOPATTS) as secondary antibody (diluted to 1:1000) using the Cell Quest program on a FACScan (Becton and Dickinson, USA). Results were expressed as the percentage of labeled cells, which could be directly correlated to the level of PLCγ$_1$ in the enterocytes.

3.4. Inositol triphosphate (IP_3) turnover

The IP_3 turnover in the enterocytes was measured using labeled [^{3}H]-myoinositol (0.5 μCi ml^{-1}, Amersham Life Sciences, Arlington Heights) according to the method of Oldham [17]. Briefly, enterocytes were incubated with the purified W07-toxin for different time period at 37 °C. Enterocytes without W07-toxin served as control. The cells were then centrifuged ($1000 \times g$, 10 min, 4 °C) to remove the excess toxin. This was followed by treatment with lithium chloride (10 mM) for 20 min. Subsequently, the cells were incubated with [^{3}H]-myoinositol for 45 min at 37 °C. The cell suspension was centrifuged ($500 \times g$, 10 min) to wash off excess-labeled inositol. The cell pellet was suspended in TBS, treated with perchloric acid (20%) and incubated on ice for 20 min. Proteins were removed by centrifugation ($2000 \times g$, 20 min, 4 °C) and the supernatant was collected. Siliconized glassware was used in the subsequent steps to minimize the loss of inositol phosphates. Supernatant was titrated to pH 7.5 with ice-cold KOH (10 N) and incubated on ice for 15 min. Precipitated $KClO_4$ was removed by centrifugation ($2000 \times g$, 20 min, 4 °C). The supernatant was applied on Amprep™ mini columns (SAX, 100 mg, Amersham), preconditioned with 1 M $KHCO_3$ and distilled water. Elution was done in a stepwise manner with 5 ml of each eluent [distilled water, 50 mM $KHCO_3$ (to elute IP_1), 100 mM $KHCO_3$ (to elute IP_2), 170 mM $KHCO_3$ (to elute IP_3) and 250 mM $KHCO_3$ (to elute IP_4), respectively]. Each elutate (1 ml 5 ml^{-1}) was transferred to a scintillation vial containing aqueous scintillation fluid (7 ml) and counted in liquid scintillation counter (Rack beta 1214).

3.5. Measurement of PKC levels

The PKC activity in the enterocytes was measured by using a PepTag® Non-radioactive PKC Assay kit (Promega, USA, Cat. No. V5330) according to the manufacturers' instructions. The PepTag® Assay utilizes bright coloured fluorescent peptide substrate, i.e. PepTag® C1 peptide, PLSRTLSVAAK that is highly specific for the kinase. Phosphorylation by PKC of this specific substrate alters the peptide's net charge from +1 to −1. This change in the net charge of the substrate allows the phosphorylated and the non-phosphorylated forms of the substrate to be rapidly separated on an agarose (1%) gel at neutral pH. The phosphorylated species migrates toward the positive electrode while the non-phosphorylated

substrates migrate toward the negative electrode. The phosphorylated substrate was extracted from the gel, heated at 95 °C, solubilized, acidified with glacial acetic acid and finally evaluated by measuring the optical density at 550 nm. For this assay, the enterocytes were suspended in TBS (pH 7.2) and incubated with the W07-toxin for different time period. Each tube containing the cells was centrifuged ($1000 \times g$, 10 min at 4 °C) to remove the excess toxin. The cells were resuspended in TBS (pH 7.2) and sonicated. The debris were removed by centrifugation ($500 \times g$, 10 min) and the supernatant of each tube was used for the estimation of PKC. The activity of PKC was expressed as units 10^6 cells^{-1}.

In a separate set of experiment, the cells were pretreated with H7 (6 μM), a serine-threonine kinase inhibitor and incubated with the W07-toxin. The activity of PKC was measured as above.

3.6. Estimation of total free oxygen radicals

Total free oxygen radical released from the enterocytes was measured by estimating the luminol-dependent chemiluminescence response of the enterocytes [18]. This method is based on the principle that mammalian cells undergoing respiratory burst, while coming in contact with the stimulating agent, release various ROS which emit light when electrons come down from the excited state to the ground level. The biological reaction produces chemiluminescence (CL), which is less than 100 photons s^{-1} cell^{-1}. Thus, luminol (5-amino-2,3-dihydro-1,4-phthalazinedine) is used as chemiluminogenic probe for the amplification of the luminescence to 10^3 to 10^4 times by its conversion to phthalate anions. Latex acts as a nonspecific stimulant of CL. In this assay, the isolated enterocytes suspended in HBSS were taken in the wells of chemiluminescence plate. The enterocytes were incubated with the W07-toxin for different time period. Background counts (A) were recorded for 1 min in Berthold luminometer (BioLumat, LKB 9500C) set at integration mode at 37 °C. Luminol (20 μl) was added to it and the counts (B) were recorded. Latex (20 μl, 0.81 μm diameter, Difco, USA) was added to the suspension and mixed properly. Counts were taken till a peak was attained which was recorded as 'C'. Results were calculated as chemiluminescence index: $(C-A)/(B-A)$.

3.7. Superoxide dismutase (SOD) assay

SOD activity was measured by the method of Kono [19]. This assay is based on the rate of nitrobluetetrazolium (NBT) dye reduction by superoxide anion radical (generated by photooxidation of hydroxylamine hydrochloride) in the presence of the enzyme, SOD. The absorbance of the reduced NBT is recorded at 560 nm. In this assay, enterocytes were incubated with the W07-toxin for different time periods and washed in PBS. The cells were disrupted by sonication and debris were removed by centrifugation. SOD activity was estimated in the supernatant fractions. Small aliquots of the supernatant were added to the reaction mixture containing 50 mM Na_2CO_3/100 mM EDTA (pH 10.0) containing 96 μM NBT, 0.6% Triton X-100 and 20 μM NH_2OH/HCl. The absorbance of the reduced NBT was recorded at 560 nm. The decline in the rate of NBT reduction in presence of SOD in the supernatant was assessed. One unit of SOD was taken as the inverse of the amount of protein (mg) required to inhibit the reduction of NBT by 50%. Finally, the activity was expressed as IU 10^6 cells^{-1}.

3.8. Catalase assay

Catalase was assayed in the enterocytes by the method of Beers and Sizer [20]. The assay is based on the principle that catalase causes time-dependent decomposition of H_2O_2 which can be monitored spectrophotometrically at 240 nm. The change in absorbance was read at 240 nm for 60 s at 10-s intervals. The specific activity was calculated using a molar absorbance index (ε) 43.6 for H_2O_2 and expressed as nmol mg protein^{-1} min^{-1}. In this assay, enterocytes were incubated with the W07-toxin for different time periods and washed in PBS. The cells were disrupted by sonication and debris were removed by centrifugation. Catalase was estimated in the supernatant fractions. The assay system comprised of 3 ml of 150 mM phosphate buffer (pH 7.0) containing 0.16 ml H_2O_2 (30% v/v). Twenty microliters of the supernatant was added to the reaction mixture. The change in absorbance was read for 60 s at 10-s intervals. The rate of decomposition of H_2O_2 by catalase was measured by recording the time required for decrease in OD by 0.05 at 240 nm.

3.9. Measurement of nitric oxide (NO)

NO released from enterocytes incubated with the W07-toxin was measured by the determination of nitrite and citrulline levels in culture supernatant of the cells.

Nitrite levels were measured by the method of Green et al. [21]. In this assay, nitrites in the sample react with a Griess reagent to form a purple azo dye. The colour of the product (dye) is developed by incubation in a 60 °C water bath followed by cooling in a 0 °C water bath. Finally, the absorbance is monitored at 546 nm. Briefly, the enterocytes were incubated in the presence of purified W07-toxin for different time periods and the excess toxin was removed by washing the cells. The cells in each tube were then incubated in RPMI containing L-arginine (1 mM) at 37 °C for 2 h. The cell suspensions were centrifuged ($500 \times g$, 10 min) and supernatant of each tube was taken for nitrite estimation. The supernatant was added to an equal volume of reagent C [prepared by mixing *N*-1-napthylethylene diamine dihydrochloride (0.1%) along with sulfanilamide (1%)and phosphoric acid

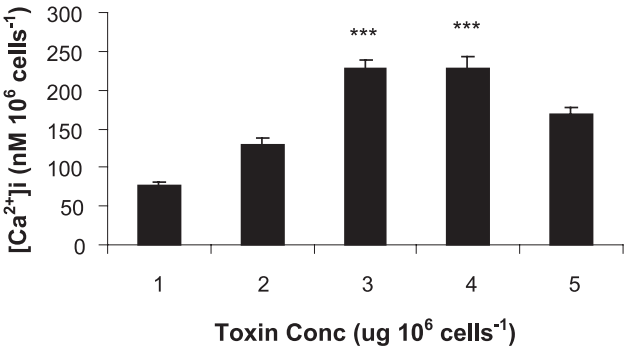

Fig. 1. $[Ca^{2+}]_i$ levels in enterocytes triggered with various concentrations of W07-toxin.

(5%) at a ratio of 1:1 (v/v)] followed by incubation for 10 min at room temperature and the OD was recorded at 546 nm. Blank and standards (1–10 nmol) were also run in parallel. The results were expressed as μmol 10^6 $cells^{-1}$.

3.10. Measurement of citrulline

Citrulline levels were measured by the method of Boyde and Rahmattulah [22]. In this method, citrulline, a carbamido compound produced from L-arginine (obtained from the acid hydrolysis of proteins/peptides) forms purple coloured complex with diacetyl monoxime in acid solution that can be monitored spectrophotometrically at 530 nm. Briefly, enterocytes were incubated in the presence of W07-toxin for different time periods and the excess toxin was removed by washing the cells. The cells in each tube were then incubated in RPMI containing L-arginine (1 mM) at 37 °C for 2 h. The cell suspensions were centrifuged and supernatant of each tube was taken for citrulline estimation. The supernatant of each tube (50 μl) was treated with 0.1 N HCl (450 μl) and 1.5 ml of reagent C [prepared by mixing two parts of reagent A (550 ml of DW, 250 ml of H_2SO_4 (95–98%), 200 ml of *o*-phosphoric acid (85%) and 250 mg of ferric chloride) and one part of reagent B (0.5% diacetyl monoxime and 0.01% thiosemicarbazide)]. Tubes containing the reaction mixture were immersed in water bath at 100 °C for 5 min followed by cooling to room temperature. Blank and standard (25–300 nmol) were run simultaneously. Results were expressed as μmol 10^6 $cells^{-1}$.

3.10.1. Statistical analysis

The data was analyzed by standard statistical methods (mean, S.D., analysis of variance and unpaired Student's *t*-tests wherever applicable). In comparing groups, 'P' < 0.05 was taken as significant.

4. Results

To understand the mechanism of action of the W07-toxin in mice enterocytes, different parameters of signal transduction were studied. The $[Ca^{2+}]_i$ (nM 10^6 $cells^{-1}$) in mice enterocytes incubated with different doses of the purified W07-toxin (1, 2, 5, 10 μg) for 1 min revealed that 2 μg of the W07-toxin was the optimum dose to cause effective increase in the $[Ca^{2+}]_i$ level (Fig. 1). Thus, 2 μg purified W07-toxin 10^6 $enterocytes^{-1}$ was used in the study of all the parameters. The enterocytes were triggered with 2 μg of the purified W07-toxin for various times (1, 5, 10, 15, 20 min) showed a five-fold increase in $[Ca^{2+}]_i$ at 5 min in W07-toxin-triggered mice enterocytes (280.5 ± 3.3) as compared to that in control enterocytes (50.7.7 ± 3.6). However, in the presence of dantrolene or verapamil or calciseptine, the W07-toxin-induced $[Ca^{2+}]_i$ in the enterocytes was significantly reduced to 67.15 ± 4.3, 190 ± 5.4, and 199.4 ± 18.9 nM, respectively, compared to that in W07-toxin-triggered cells (239.7 ± 8.9). Control values in each set were subtracted from the respective test values. Further, in enterocytes triggered with the W07-toxin preincubated with IgG_{WT} or GM_1, the $[Ca^{2+}]_i$ was found to be 12.3 ± 2.9 or 23.7 ± 4.0 nM, respectively. The commercially available CT (positive control) could induce an eight-fold increase in $[Ca^{2+}]_i$ (484 ± 6.0 nM) in the enterocytes as compared to that of control cells (50.7 ± 3.6 nM) (Fig. 2).

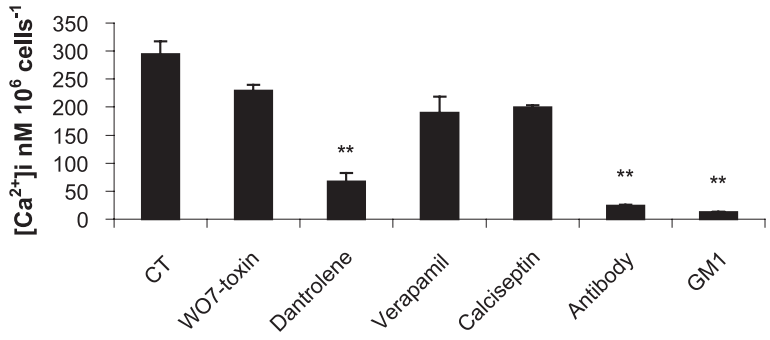

Fig. 2. $[Ca^{2+}]_i$ levels in enterocytes triggered with W07-toxin in the presence of inhibitors/channel blockers.

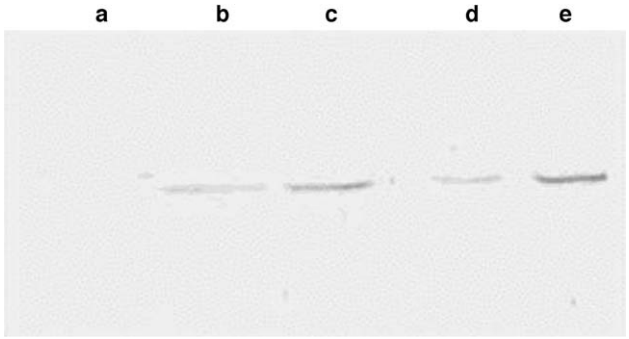

Fig. 3. Western immunoblot showing the PLCγ_1 activity in mice enterocytes membrane triggered with the W07-toxin at different time periods. Lane a: control enterocyte membrane. Lanes b, c and d: enterocytes triggered with W07-toxin for 15 s, 30 s and 1 min, respectively. Lane e: enterocyte membrane triggered with cholera toxin.

The level of cAMP (pmol mg protein^{-1}) in enterocytes triggered with the W07-toxin or W07-toxin preincubated with IgG_{WT}/GM_1 showed a statistically significant ($P<0.01$) increase in cAMP level in enterocytes triggered with the W07-toxin for 1 min (1.65 ± 0.40) as compared to that of control enterocytes (0.32 ± 0.03). However, the level was found to be decreased in the presence of IgG_{WT} (0.51 ± 0.03) and GM_1 (0.17 ± 0.05). In the presence of DDA, the cAMP level was reduced to non-detectable levels in the W07-toxin-triggered enterocytes. The CT-triggered enterocytes revealed a significant ($P<0.01$) increase in the level of cAMP (0.95 ± 0.07) as compared to that of control cells.

The time-profile study of PLCγ_1 in the Western immunoblot (Fig. 3) revealed an increase in the level of this enzyme within 30 s of the W07-toxin-triggering of the enterocytes. However, the expression of PLCγ_1 could be reduced by $43.75 \pm 0.91\%$ and $48.61 \pm 4.5\%$, respectively, when the cells were triggered with the W07-toxin preincubated separately with IgG_{WT} and GM_1 (the level of PLCγ_1 in enterocytes stimulated with the W07-toxin taken as 100%) as assessed by flow cytometric analysis.

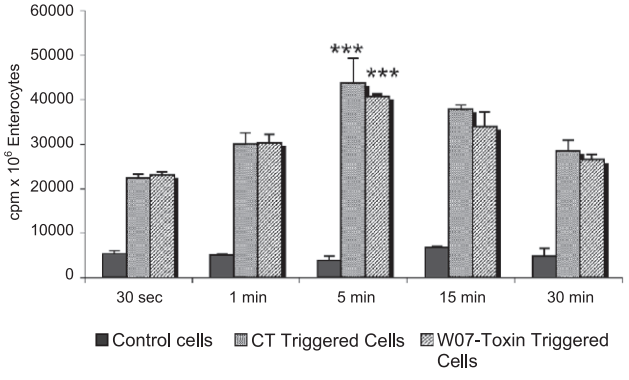

Fig. 4. Estimation of IP_3 in enterocytes triggered with cholera toxin and W07-toxin separately at different times.

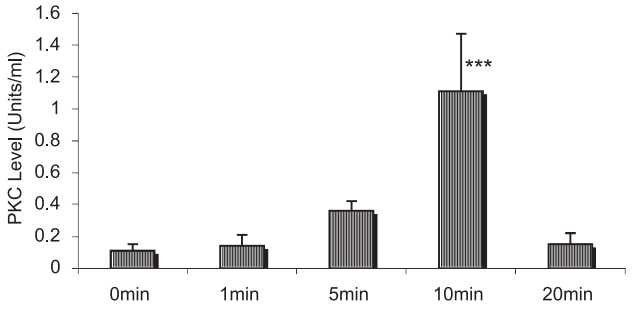

Fig. 5. PKC activity in enterocytes triggered with W07-toxin at different times.

The IP_3 turnover (cpm 10^6 cells^{-1}) was measured in control enterocytes as well as in cells triggered separately with the purified W07-toxin and CT (positive control) (Fig. 4). A gradual increase in the IP_3 level was observed on triggering the cells with the purified W07-toxin for 0.5, 1 and 5 min (23115 ± 578, 30255.0 ± 1946 and 40749 ± 494, respectively). Then a gradual decline in the value of IP_3 was noticed in the enterocytes stimulated with the purified W07-toxin for 15 and 30 min (33837 ± 3379 and 26409 ± 1098, respectively). Maximum IP_3 formed in the W07-toxin induced enterocyte at 5 min was significantly higher ($P<0.001$) as compared to that of control enterocytes (3771 ± 1024). The IP_3 level in CT-triggered (positive control) cells was 43736 ± 5535 at 5 min.

The PKC activity (units 10^6 cells^{-1}) was found to be maximum in the W07-toxin-triggered mice enterocytes at 10 min (Fig. 5) as compared to that in the control cells. The PKC activity was reduced to a value below detectable range when the cells were triggered with the W07-toxin preincubated in presence of IgG_{WT} and GM_1 separately. Further, complete reduction in the PKC activity was observed in the W07-toxin-triggered enterocytes, preincubated with H-7, a serine-threonine kinase inhibitor. The CT (positive control) could increase the PKC activity (1.04 ± 0.3) significantly ($P<0.001$) as compared to that of control enterocytes.

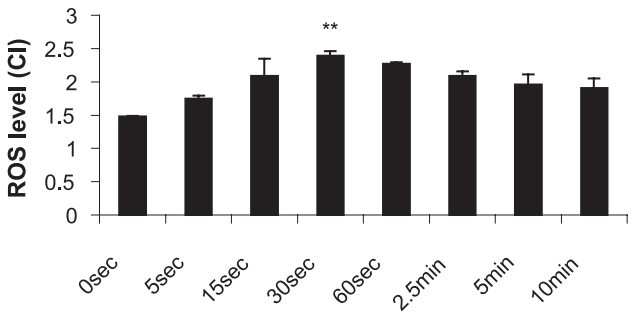

Fig. 6. Release of ROS in enterocytes triggered with W07-toxin at different times.

Chemiluminescence response [chemiluminescence index (CI)] was measured in the W07-toxin-triggered enterocytes at various times (Fig. 6). Release of ROS was highest at 30 s (2.39 ± 0.07) as compared to that of control (1.48 ± 0.01). The level was reduced significantly when the enterocytes were triggered with the W07-toxin preincubated with IgG_{WT} or GM_1 separately. The values were 1.40 ± 0.06 and 1.85 ± 0.07, respectively. The CT (positive control) could also release ROS (3.00 ± 0.20) of statistically significant levels ($P < 0.05$).

The catalase activity (nmol mg protein^{-1} min^{-1}) and the SOD activity (IU 10^6 cells^{-1}) in the W07-toxin-triggered mice enterocytes at different time periods are shown in Table 1.

A two-fold decrease in the catalase activity and a statistically significant ($P < 0.05$) reduction in the SOD activity was observed in the enterocytes at 30 s as compared to that of the control cells. However, when enterocytes were triggered with the W07-toxin pretreated with IgG_{WT}, the activities of both the enzymes were increased. The CT (positive control) could decrease the activity of catalase and SOD in the enterocytes significantly as compared to that in the control cells.

The levels of nitrite and citrulline (μmol 10^6 cells^{-1}) in the supernatant of the ESP-triggered enterocytes at different time periods are shown in Table 2. A significant increase in the levels of both nitrite and citrulline was found in the enterocytes at 5 min as compared to that of control cells. However, the levels were found to be partially decreased in the enterocytes stimulated with the W07-toxin preincubated separately with GM_1 and IgG_{WT}. The CT revealed a significant increase in the levels of both nitrite and citrulline in the enterocytes as compared to that in the control cells.

Table 1
Antioxidant enzyme activity in enterocytes triggered with the W07-toxin at different times

Groups	Catalase levels (nmol mg protein^{-1} min^{-1})	SOD levels (IU10^6 cells^{-1})
Enterocytes (control)	31.85 ± 2.29	4.63 ± 0.70
Enterocytes + toxin (5 s)	42.47 ± 1.87	4.13 ± 0.33
Enterocytes + toxin (15 s)	33.18 ± 3.75	3.65 ± 0.55
Enterocytes + toxin (30 s)	15.92 ± 1.8**	3.40 ± 0.47*
Enterocytes + toxin (60 s)	26.54 ± 3.75	3.76 ± 0.73
Enterocytes + toxin (2.5 min)	47.78 ± 1.87	4.28 ± 0.33
Enterocytes + toxin (5 min)	33.84 ± 2.82	3.46 ± 0.06
Enterocytes + toxin (10 min)	41.81 ± 4.69	4.11 ± 0.57
Enterocytes + toxin + IgG_{WT} (30 s)	26.54 ± 2.12	4.18 ± 0.23
Enterocytes + CT (30 s)	11.28 ± 2.80	2.84 ± 0.16

Values are expressed as mean ± S.D. of triplicate experiments.
* $P < 0.05$ as compared to control.
** $P < 0.05$ as compared to control.

Table 2
Nitrite and citrulline levels in enterocytes triggered with ESP at different times

Groups	Nitrite levels (μmol 10^6 cells^{-1})	Citrulline levels (μmol 10^6 cells^{-1})
Enterocytes (control)	0.29 ± 0.0	48.75 ± 4.61
Enterocytes + toxin (5 s)	1.29 ± 0.10	68.65 ± 5.90
Enterocytes + toxin (15 s)	1.72 ± 0.25	101.50 ± 1.49
Enterocytes + toxin (30 s)	2.30 ± 0.29	114.18 ± 8.21
Enterocytes + toxin (60 s)	2.55 ± 0.25	159.70 ± 7.40
Enterocytes + toxin (5 min)	2.90 ± 0.63	317.10 ± 3.70
Enterocytes + toxin (10 min)	3.80 ± 0.98***	328.30 ± 13.40***
Enterocytes + toxin (15 min)	3.16 ± 0.76	292.50 ± 4.40
Enterocytes + toxin + IgG_{WT} (10 min)	1.24 ± 0.43	284.07 ± 32.19
Enterocytes + CT (10 min)	5.32 ± 0.20	305.46 ± 21.39

Values are expressed as mean ± S.D. of triplicate experiments.
*** $P < 0.001$ as compared to control.

5. Discussion

V. cholerae as a species includes both pathogenic and non-pathogenic strains, which vary in their virulent gene content. The *V. cholerae* 01 is generally associated with the production of cholera toxin which is regarded as the cause of epidemics. A number of unknown secretogenic factor(s) have been implicated to play a role in inducing cholera-like symptoms observed in patients. A study by Dalsgaard et al. [1] has stressed the importance of monitoring *V. cholerae* serogroups (including non-01 and non-0139) for their virulent gene content as a means of assessing their epidemic potential. In this context, the ability of *V. cholerae* W07 to elaborate a novel toxin in absence of the known toxin genes is a matter of concern and needs better understanding. In the intestinal epithelium, alteration in the level of cAMP, Ca^{2+}, PKC and phospholipid metabolites have been implicated in the action of enteric pathogens or their toxins [23]. Thus, in the present study, an attempt was made to explore the mechanism by which this novel toxin from *V. cholerae* W07 could exert its effect on the secretory pathway in the intestine.

It has been observed that after being released into the jejunum, CT could recognize the oligosaccharide unit of the GM_1 present on the surface of the epithelial cells through their B-subunits. Angstrom et al. [24] did binding

studies of various glycolipids to CT and observed that minimally required sequence for optimal binding was Galβ3GalNAcβ4 (NeuAcα3) Galβ. Competitive binding experiments with GM_1-derived gangliosides have demonstrated the importance of both galactose and *N*-acetylneuraminic acid residues of GM_1 for binding of CT. The heat labile toxin (LT) from *Escherichia coli* has been shown to have the highest affinity for GM_1 [25]. In fact, LT-1 has been shown to bind mainly to GM_1 while GD_{1b} and GD_{1a} were found to be the best inhibitors of LTIIa and LTIIb, respectively [26]. These observations have suggested that the ganglioside binding domains of these toxins may be structurally and evolutionarily related despite their diverse origins. The W07-toxin too has been reported to have a good specificity for GM_1 [3]. So, in all our further studies, GM_1 has been used as an inhibitor of the W07-toxin along with the antitoxin IgG (IgG_{WT}).

Calcium is known to regulate numerous functions of all types of cells as it acts as a universal second messenger in a variety of cells. Ca^{2+} has been established as an intracellular regulator of small intestinal as well as colonic transmembrane electrolyte transport [27]. Intracellular calcium can be regulated both by alterations in Ca^{2+} permeability as well as intracellular Ca^{2+} binding activity (i.e. redistribution and sequestration). An increase in $[Ca^{2+}]_i$ has been reported to decrease active Na^+ and Cl^- absorption and/or stimulate active Cl^- secretion, whereas a decrease in $[Ca^{2+}]_i$ has been found to stimulate Na^+ and Cl^- absorption [28].

Fasano et al. [29] have reported that the zot toxin, elaborated by *V. cholerae* could modulate intestinal tight junctions and the permeabilizing effect of the toxin could lead to intestinal secretion. The mechanism of action of zot represented the paracellular pathway as an alternate route for intestinal secretion. Khurana et al. [30] have observed an enhancement in the level of $[Ca^{2+}]_i$ in *Salmonella typhimurium* enterotoxin-treated enterocytes. Further, the $[Ca^{2+}]_i$ was found to be increased in enterocytes isolated from *V. cholerae* 0139-treated rabbit ileum [11]. Recently, *E. coli* heat stable enterotoxin (STa) has been reported to bind to isolated rat epithelial cells and trigger a chain of events including increase in $[Ca^{2+}]_i$ levels [31].

In the present study, an increase in $[Ca^{2+}]_i$ levels was observed in W07-toxin-stimulated enterocytes. The increase in the $[Ca^{2+}]_i$ may have its source either from increased Ca^{2+} entry from the extracellular milieu to the cell or from the release of Ca^{2+} from intracellular calcium stores [32].

Dantrolene is a drug which is used clinically as a skeletal muscle relaxant and is known to trap calcium in intracellular calcium stores. It acts specifically on calcium channels by reducing the steady and phased leakage of Ca^{2+} into cytosol without changing the calcium flux at plasma membranes [11]. In this study, $[Ca^{2+}]_i$ seems to have a major involvement since maximum reduction in $[Ca^{2+}]_i$ was observed in W07-toxin-triggered enterocytes pretreated with this drug. Thus, our result is in good agreement with the observation of Hoque et al. [33] in which an increase in $[Ca^{2+}]_i$ was noticed in the rat enterocytes in response to heat stable enterotoxin of *V. cholerae* non-01 and decrease in $[Ca^{2+}]_i$ response was obtained in cells pretreated with dantrolene. Further, the observed Ca^{2+} concentration in presence of dantrolene in the W07-toxin-triggered enterocytes seems to come from an extracellular source. Further, with calciseptin, an appreciable level of $[Ca^{2+}]_i$ was observed, which might be due to the involvement of other Ca^{2+} channels. L-verapamil (a phenylalkylamine) is known to block all L-type Ca^{2+} channels in intracellular stores and plasma membrane. In the present study, the $[Ca^{2+}]_i$ was found to be reduced in the W07-toxin-triggered enterocytes preincubated with verapamil. However, the decrease was not significant as compared to that due to calciseptin. Thus, the probable involvement of other voltage-gated Ca^{2+} channels along with L-type Ca^{2+} channels also cannot be ruled out. All these observations have clearly indicated that the release of Ca^{2+} from the intracellular store plays a very significant role in W07-toxin-induced alteration in $[Ca^{2+}]_i$. Quattrini et al. [34] have reported that CT could modulate calcium concentration in murine neuroblastoma cells via the activation of L-type voltage-dependent calcium channels. Ikeda [35] have suggested that the voltage-gated calcium channels could sense the membrane potential and thus cause depolarization by opening the gate, which could allow Ca^{2+} to enter the cell, which in turn could trigger a number of cellular events.

The elevation of cAMP due to CT in the intestinal epithelial cells could stimulate active Cl^- secretion and/or inhibit electroneural NaCl absorption. Further, binding of CT to GM_1 on HeLa cell surface has been reported to be associated with an increase in cAMP accumulation in these cells [36]. cAMP levels are known to be elevated in *Salmonella* enterotoxin-treated cells [37]. It has been reported that cAMP could not increase the influx of external calcium into epithelial cells but it could cause the release of calcium from intracellular sites [23]. Further, Chang and Semrad [38] have shown that cAMP could stimulate the release of endogenous Ca^{2+} in isolated enterocytes. Thus, cAMP is thought to utilize the intracellular stores of calcium as also observed in the ileum and colon of both rabbit and rat. Shapiro et al. [39] have shown that elevation in the levels of intracellular cyclic nucleotides could alter the structure of F-actin in intestinal epithelial cells, thus potentially contributing to intestinal secretion. It has been reported by Sachinidis et al. [40] that stimulation of smooth muscle cells in the presence of CT could lead to an increase in cAMP levels, which could be correlated with the change in cellular morphology, including complete disruption of actin filaments and loss of focal adhesion. In the present study, the cAMP levels were found to be upregulated in W07-toxin-treated mice enterocytes. The authenticity of our finding has been further

substantiated by a reduction in the level of cAMP in the presence of DDA, a specific inhibitor of adenylate cyclase. Ruschkowski et al. [41] have suggested that *S. typhimurium* could stimulate host phospholipase C (PLC) activity, resulting in the cleavage of PIP_2 into IP_3 and DAG. The IP_3 produced as a result of phospholipid hydrolysis in turn could mobilize Ca^{2+} release from the intracellular stores, which might be responsible for several changes including disruption of the microvillus structure, loss of transepithelial electrical resistance as well as the localized rearrangement in actin filaments and related proteins like villin in the host cell. Bhatnagar et al. [42] have shown that the cytotoxic activity of anthrax lethal toxin in J774A.1 cells could be inhibited in the presence of neomycin, a PLC inhibitor. Our results have also indicated the involvement of PLCγ_1 in the W07-toxin-triggered enterocytes.

Measurement of $[Ca^{2+}]_i$ in different cell types triggered with various stimuli have revealed that increase in IP_3 either preceded or coincided with the onset of the Ca^{2+} signal [43]. This pathway has also been implicated in the CT-induced diarrhoea [33]. In an earlier study with *Shigella* toxin (STx), Kaur et al. [44] have found a significant increase in IP_3 levels in enterocytes isolated from STx-treated rabbit ileum. Mehta et al. [45] showed that in case of *E. coli* heat-stable enterotoxin-treated rat enterocytes, the rise in IP_3 preceded the rise in $[Ca^{2+}]_i$. All these observations are in good agreement with our present finding where a significant increase in IP_3 level was observed at 5 min in enterocytes triggered with the W07-toxin. $[Ca^{2+}]_i$ was also found to be upregulated after 5 min of the toxin stimulation, thereby indicating the release of IP_3 might coincide with the onset of the calcium signal. Thus, IP_3 might play a major role in the W07-toxin-induced signal transduction in mice enterocytes.

Several authors have reported the role of activated PKC in stimulation of intestinal secretion both in vivo and in vitro. In view of the importance of Ca^{2+} as a mediator of intestinal secretion and the observation that many phospholipid-interacting drugs like chlorpromazine and trifluoperazine are potent antisecretory agents, it is possible that PKC might be involved in the secretory response. Henderson et al. [46] have suggested that PKC might directly modify the microvillus membrane and thereby regulate electrolyte transport in the intestinal epithelial cells. It was shown by Kaur et al. [44] that the appreciable level of PKC in the membrane fraction of enterocytes isolated from *Shigella* toxin-treated rabbit ileum could be reduced in the presence of H-7.The role of PKC in mediating *V. cholerae* 0139-induced lipid peroxidation was also studied by Gorowara et al. [11]. In the present investigation, it was found that H-7 could decrease the activity of PKC in W07-toxin-triggered enterocytes. Thus, the two possibilities regarding PKC activation may be (a) mobilization of Ca^{2+} from intracellular stores by cAMP, which may activate PKC and (b) phospholipid hydrolysis by PLCγ, which may induce PKC activation through DAG along with Ca^{2+} mobilization by IP_3. A synergism between PKC activation and alterations in cAMP level has been well documented. Ganguly et al. [31] have provided evidence regarding the role of Ca^{2+} in the translocation of PKC from the cytosol to membrane of enterocytes treated with *E. coli* heat-stable enterotoxin. In the W07-toxin-treated mice enterocytes, it would be difficult to comment on what comes first; whether cAMP could cause the release of Ca^{2+} from intracellular stores, which then activated PKC, or phospholipid hydrolysis by PLCγ could induce PKC activation through DAG and Ca^{2+} mobilization by IP_3, which may ultimately act synergistically to cause direct phosphorylation of the catalytic subunit of adenylate cyclase.

The evidence of a complex interrelationship between PKC and cAMP-mediated pathways already exists in the literature in the regulation of cellular responses in the etiology of diarrhoea.

Thus, at this stage, it can be suggested that PKC may be located at the crossover point of various signaling pathways in the W07-toxin-mediated electrolyte imbalance/fluid secretion involving Ca^{2+}, inositol phospholipids, and cyclic nucleotides.

The gastrointestinal tract is a rich source of antioxidant enzymes which are known to detoxify the effect of ROS. However, when the rate of production of ROS exceeds the capacity of the antioxidant defenses, substantial tissue damage occurs. Mehta et al. [45] have investigated the role of ROS in mediating the enterocyte damage during in vitro exposure to *S. typhimurium* enterotoxin (S-LT). PKC-dependent generation of ROS has been implicated in vascular smooth muscle cells from rat aorta. In fact, ROS has been shown to modulate signal transduction via a protein phosphorylation mechanism in endothelial cells [46]. A specific enzyme, NADPH-oxidase has been reported to be involved in the generation of ROS [47]. Our study indicates that the change in the ion permeability of the membrane may be through reaction of the ROS with membrane lipids and/or ion transporting proteins. The decrease in the antioxidant enzyme levels (SOD, catalase) corresponding to the increase in ROS in the present study further substantiates the role of ROS. Gorowara et al. [11] have observed a marked decrease in the activity of the antioxidant enzymes in enterocytes isolated from the *V. cholerae* (0139) infected group as compared to that from the control group. They have suggested that decrease in the level of antioxidant enzymes could sensitize the enterocytes to an increased flux of ROS, which led to membrane damage and cell death.

Nitrites and citrulline are known to be the end products of oxidative metabolism of the labile NO and their quantitation is regarded as an indicator of NO generation [48]. Forsythe et al. [49], on rat intestinal epithelial cells, have shown that enterocytes could be the target as well as the producer of NO. In the present investigation, the concomitant increase in the levels of nitrite and citrulline in the supernatants of the W07-toxin-treated enterocytes at

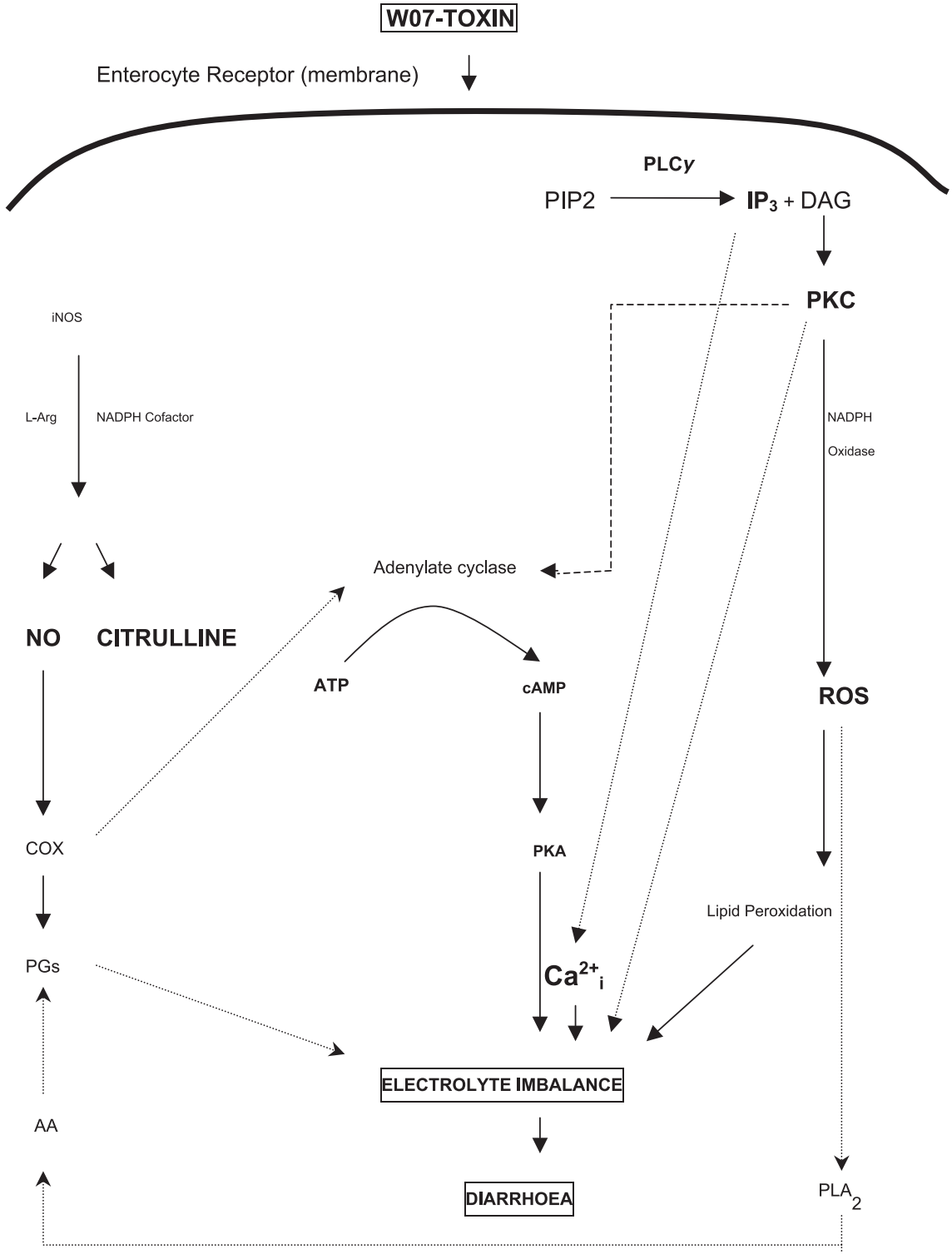

Fig. 7. Proposed model for the mode of action of the W07-toxin. PLCγ catalyses the hydrolysis of PIP_2 to IP_3 and DAG. IP_3 mobilizes Ca^{2+} from intracellular stores while DAG can activate members of the PKC family that can lead to electrolyte imbalance in the intestine by phosphorylation of transport carriers and conductance channels. PKC can also cause generation of ROS, which can lead to lipid peroxidation causing electrolyte imbalance. PKC can also induce adenylate cyclase thus increasing cAMP levels. The iNOS may be activated under the stress response generated by the W07-toxin and the NO produced can activate cyclo-oxygenase enzyme (COX) and mobilize arachidonic acid (AA) mediated by Ca^{2+}-independent phospholipase A_2 (PLA_2). The NO can induce prostaglandins (PGs), which in turn could either directly cause ion imbalance or activate adenylate cyclase. Therefore, cross-talk between different pathways of the signal transduction occurs to bring about a common secretory response due to the W07-toxin.

5 min confirmed their co-expression within the cells and thus indicating that the generation of free radicals like peroxynitrite anion may be possible due to the reactions between ROS and NO, which can induce tissue injury through lipid peroxidation and the oxidation of sulfhydryl groups in the membrane component [50]. The level of citrulline and nitrite in the enterocytes triggered separately with W07-toxin as well as with CT showed a significant increase indicating a role of NO in the W07-toxin-mediated change in mucosal permeability. It has been reported previously that cells can produce either NO or superoxide radical or both depending on the stimulus, although it is unlikely that both are generated in the same cell at the same time [51]. In our study, the peak activity of NO and ROS also occurred at different times. The alteration in the levels of all intracellular mediators could be inhibited in the presence of GM_1 (1 μg) as well as IgG_{WT} (diluted to 1:2500 in TBS). In most of the cases, preincubation of the toxin with IgG_{WT} or GM_1 could show inhibition to the same extent. This indicates that both antibody-binding epitope as well as GM_1-binding epitope of the W07-toxin might have a precise role in cell signaling. The GM_1-binding epitope might be involved in the recognition of the cell surface receptor as suggested by Minke et al. [52]. Further, complete reversal in the level of intracellular mediators was not observed in enterocytes stimulated with W07-toxin preincubated in presence of either IgG_{WT} or GM_1, thereby indicating that somehow both the IgG_{WT} and GM_1 specific epitopes of the toxin are involved in transducing signals within the cells.

The present investigation has indicated that the W07-toxin could induce the activation of $PLC\gamma_1$ in the mice enterocytes, which could lead to the production of IP_3 and DAG. The IP_3 could then mobilize Ca^{2+} release from intracellular stores. The activation of the PKC could be due to the presence of DAG formed along with IP_3 from PIP_2. Involvement of intracellular Ca^{2+} stores in the W07-toxin-stimulated enterocytes has been confirmed from the experiments conducted separately in the presence of dantrolene, verapamil and calciseptin. However, the role of extracellular calcium could not be ruled out. The alteration in the $[Ca^{2+}]_i$ could be directly involved in electrolyte imbalance. Thus, it can be suggested that in cholera-like symptoms associated with *V. cholerae* W07, the W07-toxin probably plays a crucial role in alteration of the level of the key mediators of different signal transduction pathways as shown in Fig. 7.

The studies on the mode of action of enterotoxins may yield rational and specific methods of treatment that could stop the fluid and electrolyte loss. This may be achieved through the use of molecular inhibitors of the signal transduction parameters altered as a result of the microbial toxins. Further, new vaccine candidates, based on a molecular understanding of pathogenicity, provide a scope for improved strategies for disease prevention. In this context, a detailed study of this novel toxin from a structural as well as functional point of view may provide additional input for the development of modulators which may have better therapeutic potential to control the disease caused by newly emerging epidemic strains of *V. cholerae*.

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