J. Membrane Biol. 96, 243–249 (1987)

The Journal of
Membrane Biology
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Calcium Transport Affinity, Ion Competition and Cholera Toxin Effects on Cytosolic Ca Concentration

D.D. Maenz*, S.E. Gabriel, and G.W. Forsyth
Department of Veterinary Physiological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N OWO, Canada

Summary. The physiological relevance of an apparent ionophore activity of cholera toxin towards Ca^{2+} has been examined in several different systems designed to measure affinity, specificity, rates of ion transfer, and effects on intracellular ion concentrations. Half-maximal transfer rates across porcine jejunal brush-border vesicles were obtained at a concentration of 0.20 μM Ca^{2+}. When examined in the presence of competing ions the transfer process was blocked by very low concentrations of La^{3+} or Cd^{2+}. Sr^{2+}, Ba^{2+} and Mg^{2+} were relatively inefficient competitors for Ca^{2+} transport mediated by cholera toxin. The relative affinities observed would be compatible with a selectivity for Ca^{2+} transfer at physiological ion concentrations, as well as an inhibition of this ionophore activity by recognized antagonists of cholera toxin such as lanthanum ions. Entry rates of Ca^{2+} into brush-border vesicles exposed to cholera toxin were large enough to accelerate the collapse of a Ca^{2+} gradient generated by endogenous Ca, Mg-ATPase activity. The treatment of isolated jejunal enterocytes with cholera toxin caused a significant elevation in cytosolic Ca^{2+} concentrations as measured by Quin-2 fluorescence. This effect was specifically prevented by prior exposure of the cholera toxin to excess ganglioside G_{M1}. We conclude that cholera toxin has many of the properties required for promoting transmembranes Ca^{2+} movement in membrane vesicles and appears to be an effective Ca^{2+} ionophore in isolated mammalian cells.

Key Words cholera toxin · ionophore · calcium · brush-border membrane vesicles

Introduction

Cholera enterotoxin is a versatile agent which can affect physiological processes in many different cell types. The actions of the toxin in its normal location in the intestinal lumen, and on a variety of other tissues and isolated cell lines is usually considered to be mediated via activation of adenylate cyclase [4, 5, 10, 18]. The mechanisms of adenylate cyclase activation have been studied intensively, placing a major emphasis on the role of cyclic AMP as the intracellular mediator which causes physiological responses such as fluid secretion when the toxin is introduced into the lumen of the small intestine. Alternative mechanisms for the intestinal secretory response to cholera toxin have not received serious consideration in spite of some strong supporting evidence. We have reported that cAMP concentrations did not increase over basal levels in mucosal samples from pig jejenum exposed *in situ* to strongly secretory doses of cholera toxin [6, 9]. The sensitivity of the methodology used in these assays has been verified by showing cAMP concentration increases after tissue treatment with isobutylmethylxanthine, and a doubling of cGMP concentrations in this issue on exposure to secretory concentrations of heat stable enterotoxin [6, 7]. Further evidence against cAMP as a sole mediator of the secretory response to cholera toxin was obtained by adding adenylate cyclase inhibitors to the luminal fluid of intestinal loops pretreated with cholera toxin. Reduction of cAMP concentration to basal levels in rabbit ileal mucosa or to 50% of basal levels in pig jejunal mucosa failed to reduce the net fluid secretion induced by prior exposure to cholera toxin [7]. Intestinal fluid secretion is also known to be altered substantially by several agents which have no effect on adenylate cyclase activity or on cytosolic cyclic AMP concentrations [2, 3, 8, 12, 13].

Cholera enterotoxin is particularly interesting because at least parts of the protein are capable of post-translational transmembranous movement [23]. This properly implies hydrophobicity which has been subsequently confirmed for the A subunit of the toxin [24]. Along with this hydrophobicity there is evidence for effects of cholera toxin on ion permeability of black lipid membranes [19]. The possible significance of these observations was suggested by Knoop and Thomas who showed that

* Department of Physiology, University of Alberta, Edmonton, Alberta T6G 4N3, Canada.

cholera toxin stimulated the rate of Ca uptake by rat basophilic leukemia cells [11]. As would be expected for intact viable cells the efflux rate of Ca was not altered by treatment with cholera toxin. We have studied this system further using pig jejunal brush-border vesicles to measure ionophore activity of several agents, including cholera toxin [14–16]. In this vesicle system it was possible to show both increased rates of Ca uptake into and efflux from an osmotically active space in the presence of cholera toxin. The observed rates of Ca transport facilitated by physiological levels of cholera toxin were somewhat less than corresponding Ca transfer rates caused by A23187. Thus there are questions remaining about the rate and the specificity of the ionophore activity of cholera toxin, relative to a role for this activity in mediating physiological effects of the toxin via increases in cytoplasmic Ca concentration.

In the present study we have investigated the specificity of cholera toxin for promoting the transmembranous movement of Ca in the presence of competing cations. The ability of the ionophore to affect intravesicular and intracellular Ca concentrations in the presence of functional Ca, Mg-ATPase has also been studied to determine the potential relevance of Ca as a cytosolic messenger compound for cholera toxin.

Materials and Methods

Brush-border vesicles (BBV) were prepared from samples of frozen mucosal scrapings. The scrapings were taken from 50-cm segments of proximal jejunum which had been removed surgically from 6- to 8-week-old pigs of mixed Yorkshire-Landrace breeding. On the day of use a sample of scrapings was thawed and vesicles prepared in a buffer containing 250 mM sorbitol, 5 mM Tris-HCl, pH 7.4, 240 units/ml penicillin and 125 units/ml streptomycin by homogenizing, followed by differential centrifugation and Mg^{2+} precipitation of nonbrush-border material [14]. This procedure gives right-side-out vesicles as indicated by electron microscopy.

The Ca indicator dye, arsenazo III (AIII), was incorporated into selected batches of vesicles by adding 3.0 mM AIII to the sorbitol Tris-HCl buffer prior to homogenization. Extravesicular dye was removed from these vesicles in the three subsequent washing steps of the standard preparation protocol. The rate of uptake of nonradiolabeled Ca by brush-border vesicles was determined by measuring rates of change in absorbance at 652 nm in suspensions of vesicles containing the AIII indicator dye [22]. Endogenous vesicle Ca was first depleted by exposing the vesicle suspension to 5.0 mM ethyleneglycol-bis(β-aminoethyl ether)N,N′-tetraacetic acid (EGTA) for 30 min at 37°C. The following sequential additions were made to the depleted vesicles in a spectrophotometer cuvette; 10 mM dithiothreitol, followed 2 min later by 10 μg of cholera toxin per mg of vesicle protein. Then after a further 5 min $CaCl_2$ was added to give the desired free Ca concentrations for uptake rate determinations. For competition experiments the competing metal ion was added after the cholera toxin, but 5 min prior to addition of 1.0 mM Ca ($[Ca]_f$ of 0.14 μM). Rates of absorbance change at 652 nm were measured with the Kinetics II Compuset of a Beckman DU8 spectrophotometer. Absorbance values were converted to nanomoles of Ca using an extinction coefficient of 19,600 liters · $moles^{-1}$ for the AIII-Ca complex (λ = 652 nm) at pH 7.4 [17]. Spectral studies were carried out in the absence of vesicles to determine the effect of the competing ions used in this study on the absorbance changes at 652 nm caused by Ca addition to the AIII dye.

Concentrations of free EGTA [$(EGTA]_f$) in the presence of two binding metal ions were determined according to Bartfai [1] using an IMSL library subroutine for an iterative solution to the third degree polynomial. $[EGTA]_f$ was used to determine $[Me]_f$ also according to Bartfai, using equilibrium constants which were corrected to the operating pH of 7.40.

The active efflux of Ca from BBV was studied by loading vesicles with ATP and $MgCl_2$ during the preparation procedure and equilibrating the resulting vesicles with 0.30 mM $CaCl_2$ plus 1.0 μCi $^{45}CaCl_2$ per ml by incubation at 0°C. Efflux was initiated by warming vesicles to 37°C, and monitored by removal of samples of vesicles from the suspension for filtration on 0.45 μm pore size cellulose acetate membranes, followed by liquid scintillation counting to determine ^{45}Ca retained on membrane filters. After 5 min at 37°C the vesicle suspensions were divided to allow addition of test solutions containing A23187 or cholera toxin. The effect of these additions on intravesicular Ca content was monitored as before by removing samples of the vesicle suspension to measure intravesicular ^{45}Ca.

Whole enterocytes were prepared by a modification of Weiser's method [21]. Jejunal segments were flushed with isotonic saline and filled with an oxygenated buffer containing (mM): KCl 1.5, NaCl 96, KH_2PO_4 8, Na_2HPO_4 5.6, Na citrate 27, and maintained at 37°C for 5 min with gentle agitation. The segments were drained, and the lumen filled with a second oxygenated buffer solution containing the following ingredients (mM): NaCl 120, KCl 5.5, K_2HPO_4 0.25, HEPES 20, dextrose 10, β-OH butyrate 2.5, L-glutamine 1, dithiothreitol 0.5, and EDTA 1.5. After a second 5-min period with agitation at 37°C the segment was drained, and the cells collected by centrifugation at 3000× g for 5 min. Cells were washed twice with the same buffer, and counted. Viability was determined by exclusion of trypan blue, and only preparations with a viability greater than 75% were used for further studies.

Isolated jejunal enterocytes were loaded with the acetoxymethyl ester of Quin-2 (Quin-2 AM) by mixing 5.0 ml of cell suspension (2 × 10^7 cells/ml) with 5 μl of a 75-μM solution of Quin-2 AM in dimethyl sulfoxide. Loading was carried out at 37°C with frequent agitation for 20 min, when the solution was made 1.0 mM in free Ca and Mg for a further 20-min period. At this time the cells were diluted fivefold in a modification of the second isolation buffer described above, with 1.5 mM EDTA replaced by 1.0 mM $CaCl_2$ and 1.0 mM $MgCl_2$. After washing twice in this buffer the cells were resuspended to give a working concentration of 1 × 10^6 cells/ml. The extent of Quin-2 AM hydrolysis in the loaded cells was assessed by examining the excitation spectra for a maxima at 339 nm (emission λ_{max} 490 nm). The Ca content of cells containing the Quin-2 was determined from fluorescence intensities as described by Tsien et al. [20] except that lysis was carried out with 0.5% sodium dodecyl sulfate. Effects of ionomycin and cholera toxin on the cytoplasmic Ca content were determined.

The Student's t-test was used to determine the probability that observed differences in means were statistically significant.

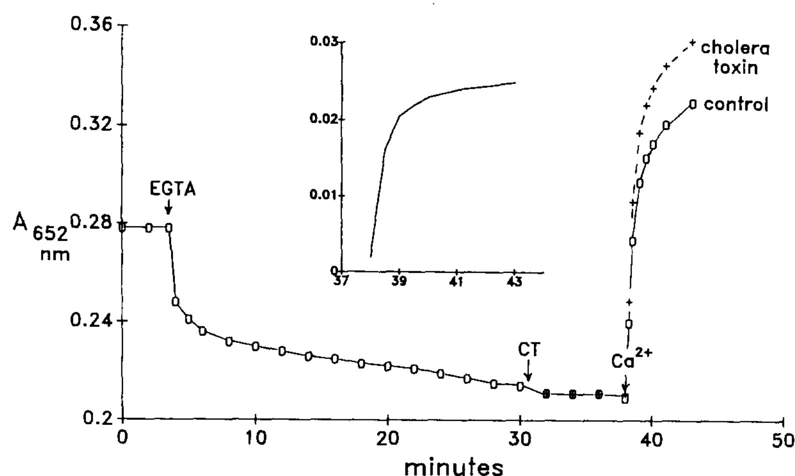

Fig. 1. Effect of cholera toxin on the time course of Ca binding to arsenazo III contained within jejunal brush-border membrane vesicles. The data were generated from two cuvettes zeroed against a water blank, each containing 2.5 mg of vesicle protein in 675 μl of 250 mM sorbitol, 5 mM Tris HCl, pH 7.4. The two suspensions were treated identically through addition of 5.0 mM EGTA at 4 min, and 10 mM dithiothreitol at 28 min. Then 25 μg of cholera toxin was added to only one of the suspensions at 30 min. Ca was added to both suspensions at 38 min. The difference in absorbance after Ca addition is shown in the inset

Results

A Ca ionophore activity for cholera toxin has been demonstrated previously in a system of pig jejunal brush-border vesicles. Increased rates of ^{45}Ca uptake into an osmotically active space, and increased rates of Ca binding to the indicator dye arsenazo III were seen in vesicles treated with cholera toxin [16]. An experimental protocol which was developed to examine the effect of cholera toxin (CT) on the rate of added extravesicular Ca binding to intravesicular AIII dye is shown in Fig. 1. Vesicles prepared in buffer lacking added Ca (divalent cation precipitation employing $MgCl_2$) had significant levels of Ca (approximately 1.0 nmol/mg vesicle protein) which could be depleted by exposure to EGTA at 37°C. Depletion of this endogenous Ca content required approximately 30 min to reach a uniform minimum absorbance value.

The Ca permeability of vesicles loaded with AIII was similar to vesicles which did not contain the indicator dye (*see* reference [16] for direct comparison). The basal Ca entry rate shown for Fig. 1 was 1.8 nmol/min/mg vesicle protein. This basal rate was not affected by the addition of dithiothreitol to the vesicles loaded with AIII. We have shown previously that the rate of initial Ca entry varies as a function of CT concentration, and is approaching saturation at CT concentrations of 10 μg CT per mg vesicle protein [16]. With 10 μg CT per mg vesicle protein, and a free Ca concentration of 1.0 μM, the initial Ca entry rate of 2.18 nmol/min/mg vesicle protein was obtained as shown in Fig. 1. The difference in Ca entry rates between the control condition and the vesicles with added CT is shown in the inset to the Figure. This difference in initial rate was 0.028 absorbance units per min or 0.38 nmol Ca/min/mg vesicle protein.

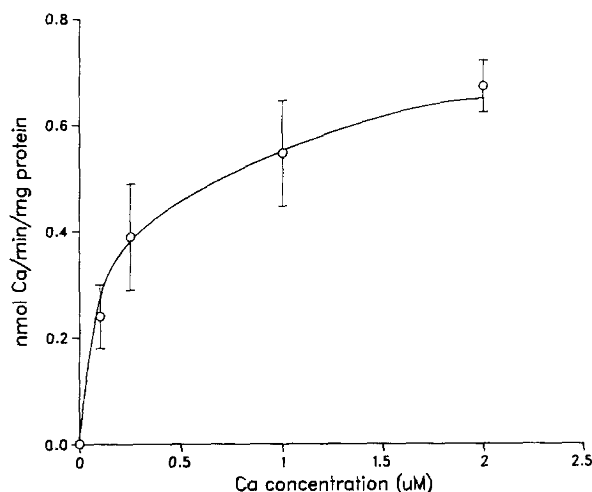

Fig. 2. Effect of Ca concentration on the cholera toxin-dependent rate of Ca uptake by jejunal brush-border membrane vesicles. Increments above the control rate of Ca uptake which were caused by adding 10 μg of cholera toxin per mg of vesicle protein are shown. $n = 6 \pm$ SEM

Using this difference in initial rates of absorbance change after Ca addition we have examined a range of free Ca concentrations to determine the K_m of CT for Ca transport (Fig. 2). Values of 0.19, 0.25, 0.197 and 0.196 μM were obtained from Lineweaver-Burk, Hanes-Woolf, Eadie-Hofstee, and direct linear plots, respectively, using Enzpack software (Elsevier-Biosoft). The data used to generate the direct linear plot are shown in Fig. 2. The calculated V_{max} for Ca transport by CT varied from 0.692 (Lineweaver-Burk) to 0.748 (direct linear) nmol Ca/min/mg vesicle protein.

The ability of several cations to interfere with the cholera toxin-dependent component of Ca uptake by vesicles loaded with AIII was examined to

Table 1. Competing ion concentrations which cause a 50% reduction in the rate of Ca transport by cholera toxin[a]

Ion	$K_{pH\ 7.4}^{Me \cdot EGTA}$	$[Me]_f$ (mol · liter^{-1})	$[Ca]_f$ (mol · liter^{-1})	Increment in Ca uptake (pmol · min^{-1} · mg protein^{-1})	I_{50}^{Me} (mol · liter^{-1})
La	1.2×10^{11}	1.1×10^{-13}	1.4×10^{-7}	140 ± 50	1.0×10^{-13}
Cd	2.5×10^{11}	3.8×10^{-10}	1.3×10^{-7}	120 ± 30	1.0×10^{-10}
Ca	4.4×10^{6}	—	1.4×10^{-7}	320 ± 90	2.0×10^{-7}
Mg	3.0×10^{0}	1.0×10^{-5}	1.4×10^{-7}	170 ± 60	1.1×10^{-5}
Sr	2.2×10^{3}	5.7×10^{-5}	1.5×10^{-7}	140 ± 40	3.2×10^{-5}
Ba	1.9×10^{3}	6.5×10^{-5}	1.5×10^{-7}	140 ± 50	5.0×10^{-5}
Zn	5.9×10^{7}	4.3×10^{-10}	1.4×10^{-7}	790 ± 240	?

[a] $[Me]_f$ concentrations given are the experimental values used which were closest to the determined I_{50} values. $[Ca]_f$ values given are the concentration of free calcium in solution in the presence of the amount of the corresponding competing ion listed in column 3. The values for Ca increments are averages of eight observations ± SEM.

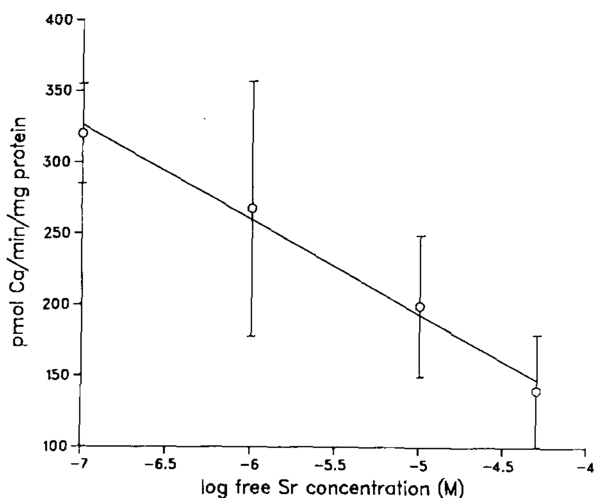

Fig. 3. Determining the concentration of Sr^{2+} which gives a 50% reduction in the cholera toxin-dependent rate of Ca^{2+} uptake by brush-border vesicles. The I_{50} concentration was determined from the point where the regression line intercepts the value of 160 nmol Ca/min/mg vesicle protein, corresponding to one-half of the maximal rate of Ca uptake observed in the presence of 10 μg of cholera toxin per mg of vesicle protein

determine the specificity of the ionophore effect which has been observed. Except for Zn, the competing ions used in the study had no influence on the changes in absorbance at 652 nm caused by Ca addition to the AIII indicator dye. Zn formed a chromophore with the AIII dye which absorbed at the wavelength used for detecting Ca binding. The general procedure involved the empirical choice of a range of cation concentrations which spanned the concentration required to cause a 50% inhibition of the control or basal rate of Ca uptake. Plots of the initial rate of CT-dependent Ca uptake versus the log of competing ion were constructed to determine I_{50} concentration values. The plot for Sr is shown in Fig. 3. Sample values of free metal and Ca concentrations for several cations are reported in Table 1, along with the I_{50} concentrations determined by graphical extrapolation. The order of affinity of the ions in this competition assay was La > Cd > Mg ≥ Sr ≥ Ba. La and Cd ions appeared to have very high affinities for the putative divalent cation binding site on cholera toxin, while Mg, Sr and Ba competed relatively poorly with Ca. Zn appeared to be transported and to form a chromophore with the AIII dye.

The observed velocities of Ca transport by CT are relatively low in comparison to passive Ca leakage rates in brush-border vesicle membranes. The important question relates to the significance of these transport rates in comparison to Ca entry which would be required to produce second messenger effects by elevating cytosolic Ca concentrations. We have shown previously that Ca transport rates across brush-border vesicle membranes are increased approximately twice as much by 20 μM A23187 as by 7.5 μg of cholera toxin [16]. The significance of these influx rates is not clear until they are compared with physiological, energy-dependent extrusion processes. The experiment shown in Fig. 4 was performed to compare the magnitude of transmembranous Ca flux rates induced by the activity of the brush-border Ca, Mg-ATPase with the back fluxes caused by A23187 and by cholera toxin acting as Ca ionophores. Activation of Ca, Mg-ATPase by warming vesicle suspensions to 37°C caused a rapid Ca extrusion from preloaded vesicles. The addition of either A23187 or cholera toxin after 5 min of Ca pumping by the ATPase accelerated the collapse of the Ca gradient generated by the physiological activity of the Ca, Mg-ATPase.

If CT does allow Ca entry into vesicles at a rate

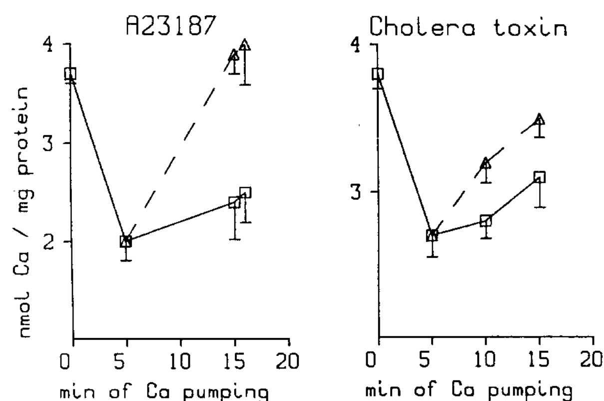

Fig. 4. Ionophore effects on a Ca gradient generated across jejunal brush-border membrane vesicles by endogenous Ca, Mg-ATPase. Vesicles were prepared in the presence of 2.0 mM ATP and equilibrated with 0.30 mM $CaCl_2$ plus ^{45}Ca at 0°C to inhibit Ca, Mg-ATPase activity. Equilibrated vesicle Ca was measured at 0°C before warming vesicles to 37°C. Effects of warming on vesicle Ca content are shown after 5 min at 37°C. A23187 (20 μM) or cholera toxin (7.5 μg per mg vesicle protein) was added at 5 min to determine ionophore effects on the Ca gradient generated by Ca, Mg-ATPase. $n = 6 \pm$ SEM. The Ca content of vesicles was elevated significantly from the control condition (open squares) at both of the time points shown with open triangles after addition of A23187 or cholera toxin ($P < 0.05$)

comparable to *in vitro* Ca, Mg-ATPase activity then it is pertinent to ask what effect it will have on the cytosolic Ca levels in intact cells where ATP supplies are maintained by metabolic activity, and cytosolic membrane permeability has not been increased drastically by mechanical procedures used in preparing isolated membrane fractions. Ca levels in intact enterocytes were estimated by the fluorescence intensity of Quin-2 [20]. Control $[Ca]_i$ measurements reported in Table 2 gave an average value of 119 nM. This value of cytosolic Ca concentration was increased significantly upon addition of 2.0 μM ionomycin, and also after addition of 5.0 μg of cholera toxin per 1×10^6 cells. The full ionophore effect of the toxin was evident after 1 min of exposure to cells suspended in the fluorometer cuvette. The addition of excess ganglioside G_{MI} to the cell suspension had no effect on basal $[Ca]_i$, but caused a 20% decrease in the $[Ca]_i$ level attained with 2.0 μM ionomycin ($P = 0.08$). Exposure to the ganglioside totally prevented the increase in $[Ca]_i$ caused by addition of cholera toxin to the cell suspension.

Discussion

Activation of adenylate cyclase may be sufficient to produce many of the physiological responses which have been observed following cholera toxin addition in a wide range of species, tissues and cell types. The emphasis on cholera toxin catalyzing the transfer of ADP ribose to the G_s regulatory subunit of adenylate cyclase [18] has overshadowed questions about systems which have shown a response to the addition of cholera toxin without detectable increases in cAMP concentrations. Suggestions of alternate modes of action of the toxin have not been widely accepted.

Table 2. Effects of ionomycin and cholera toxin on enterocyte cytosol Ca concentrations as determined by Quine-2 fluorescence

Treatment	n	$[Ca]_i$ (nM ± SEM)	Comparison and P value
a. Control	10	119 ± 9	—
b. Ionomycin 2.0 μM	5	318 ± 27	*vs.* a <0.001
c. C toxin 0.5 μg	5	144 ± 12	*vs.* a 0.14
d. C toxin 5.0 μg (1 min)	5	225 ± 19	*vs.* a <0.001
e. C toxin 5.0 μg (3 min)	5	190 ± 50	*vs.* d 0.23
f. C toxin 5.0 μg (5 min)	5	255 ± 43	*vs.* d 0.22
g. G_{MI} 5.0 μg	5	117 ± 0	*vs.* a 0.46
h. Ionomycin + G_{MI}	5	250 ± 0	*vs.* b 0.08
i. C toxin 5.0 μg + G_{MI}	5	109 ± 6	*vs.* a 0.25 *vs.* d 0.002

There are at least three reports of increased rates of ion transport in membrane systems exposed to cholera toxin. These reports range from artificial lipid bilayers [19] through membrane vesicles [16] to intact cells [11]. The present experiments were conducted to try to characterize possible ionophore activity of cholera toxin beyond the level of initial reports. Ionophore activity toward Ca was of particular interest since Ca is well recognized as a second messenger agent which can have several intracellular actions, including the activation of C kinase. Most of these actions require significant elevations of cytosolic Ca, which in turn would require that the rate of Ca influx exceed the capacity of intracellular Ca sequestration and extrusion.

Direct physical measurements of the affinity of cholera toxin for Ca indicated that half-saturation occurred at 1.0 μM Ca [16]. The K_m for Ca transport measured under more physiological conditions, i.e., with the A subunit inserted into the lipid bilayer of pig jejunal brush-border vesicle membranes, was measured at approximately 0.20 μM in this present study. These affinity values are in relatively good agreement, and they shift in the predicted direction if the ionic cation binding site were exposed to a more hydrophobic environment during insertion into the lipid bilayer. The measured affinities for binding and transport are of the proper magnitude to ensure maximal Ca transport across plasma

membranes faced with external Ca concentrations in the range of 1 to 2 mM.

Ion competition for Ca transport provides some information about the nature of the cation binding site of the ionophore, and of the degree of specificity of the transport process. The very high affinity of the system for La ions may indicate the presence of several anionic groups at the ligand binding site. It may also have some bearing on the reported inhibitory effects of La toward the secretory effects of cholera toxin [12]. These competition experiments indicated that the Ca transport affinity of the toxin exceeds that of Mg by two orders of magnitude, implying a relatively strong specificity or preference for Ca relative to Mg. The possibility also arises that the ionophore effect could occur in the form of a Ca/Mg exchange *in vivo*, with electoneutrality maintained by export of one Mg ion for each Ca entering the cytosol.

Rates of Ca transport by cholera toxin have been reported previously in vesicles, and compared to transport rates induced by A23187 [16]. Since saturating levels of cholera toxin gave transport rates which were somewhat lower than the rate measured with the peptide ionophore it is relevant to compare the ion leak rate induced by the ionophores to the rate of active Ca extrusion from vesicles via the Ca, Mg-ATPase activity. In these experiments cholera toxin acted like A23187 to accelerate the collapse of the transmembranous Ca gradient generated by supplying ATP to the vesicles. While these results suggest that the ionophore activity may have a rate somewhat similar to the brush-border Ca, Mg-ATPase the interpretation may be questioned due to the constraints of the artificial system. Limited ATP supply for Ca pumping, a high basal Ca leak rate common to vesicle preparations, and the very small transmembranous Ca gradient could all cause departures from *in vivo* rates of ion movement.

The dispersed whole cell preparations are not ideal for measuring rates of Ca uptake, but they do provide evidence that the ionophore rates must be substantial, as cytosolic Ca levels almost doubled after addition of cholera toxin. Based on these results it seems likely that cholera toxin may be an effective ionophore *in vivo*, and that changes in intracellular Ca concentration should be considered when assessing the effects of cholera toxin on any system containing intact cells.

This research was supported by grants from Medical Research Council and the Natural Sciences and Engineering Research Council of Canada.

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Received 22 September 1986; revised 15 December 1986