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# Inositol 1,4,5-trisphosphate directly opens diphtheria toxin channels

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**Inositol 1,4,5-trisphosphate ($IP_3$) is a soluble second messenger, which acts by cooperatively releasing $Ca^{2+}$ into the cytosol from non-mitochondrial stores, probably by activating $Ca^{2+}$-permeable channels. We demonstrate that submicromolar concentrations of $IP_3$ can directly open the $Ca^{2+}$-permeable diphtheria toxin channel, and that this occurs by $IP_3$ binding to a specific site on the toxin protein. This provides a model for $IP_3$-induced $Ca^{2+}$ release and suggests that $IP_3$-induced channel opening may play a role in diphtheria intoxication and in protein translocation across membranes.**

## Introduction

Inositol 1,4,5-trisphosphate ($IP_3$) is produced in many cells in response to an external stimulus and is a critical element in the phosphatidyl inositol cycle in eukaryotic cells (see Ref. 1 for review). $IP_3$ is thought to act intracellularly by causing an increase in cytosolic $[Ca^{2+}]$ primarily from non-mitochondrial intracellular stores such as endoplasmic reticulum [2]. Although the precise mechanism of $IP_3$-induced $Ca^{2+}$ release remains obscure, it has often been suggested that $IP_3$ might directly open $Ca^{2+}$-permeable channels located in a membrane sequestering $Ca^{2+}$. A purified $IP_3$ receptor has been shown to mediate $Ca^{2+}$ flux in lipid vesicles [3], and an $IP_3$ receptor has been cloned and sequenced [4]. Kuno and Gardner [5], using patch clamp techniques, have demonstrated that $IP_3$ can activate $Ca^{2+}$-permeable channels in the lymphocyte plasma membrane, and they proposed that this might account for $IP_3$-induced $Ca^{2+}$ flux across the plasma membrane. $IP_3$-activated $Ca^{2+}$ channels from muscle sarcoplasmic reticulum and transverse tubules reconstituted into lipid bilayer membranes have also been described recently [6–8]. These reports are consistent with the hypothesis that $IP_3$ directly gates a $Ca^{2+}$-permeable channel, but none excludes the possibility that $IP_3$ gates the channel through some intermediary (e.g., a G-protein or another inositol phosphate compound). Since previous work had shown that the diphtheria toxin (DT) channel interacts with some molecules containing an inositol phosphate moiety [9], we decided to examine the effects of $IP_3$ and related inositol phosphates on DT channel opening.

Diphtheria toxin (DT) is a dichain protein ($M_r$ = 58342) produced by *Corynebacterium diphtheria* strains lysogenic for bacteriophage beta carrying the *tox*$^+$ gene (see Refs. 10–12 for reviews). DT kills most eukaryotic cells by enzymatically ADP-ribosylating elongation factor-2, thus inhibiting protein synthesis. ADP-ribosylating activity resides on the A chain, while the B chain of DT is responsible for cell surface binding and membrane translocation activity. After binding to a cell surface receptor, DT undergoes receptor mediated endocytosis into an acidic endosomal compartment [13,14]. The role of the DT receptor in DT translocation across membranes remains unclear [15–17].

Using lipid bilayer membranes, Kagan et al. [18] showed that the N-terminal 23000 dalton $B_{45}$ fragment of DT was able to form channels large enough ($d$ = 18 Å) to allow passage of the A chain in its unfolded state across a lipid membrane, and that channel formation

Abbreviations: DT, diphtheria toxin; $IP_3$, inositol 1,4,5-trisphosphate; ADP, adenosine diphosphate; NAD, nicotinamide adenine dinucleotide; DMG, 3,3′-dimethylglutaric acid; CRM, cross-reacting material; ApUp, adenosyl(3′-5′)-uridine 3′-monophosphate; ATP, adenosine triphosphate.

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required a low pH (< 6.0), and was maximal under conditions mimicking the pH gradient across the endosomal membrane (4.7/7.4) which DT encounters during intoxication. Exposure of DT to low pH causes denaturation and a conformational change essential for entry of DT into the cytosol. It has been proposed that the $B_{45}$ channel might play a role in translocation of the A chain across the endosomal membrane and that the pH gradient might provide the driving force for translocation [18]. Subsequent work with DT in cells and liposomes has confirmed that conditions supporting channel formation are required to obtain A chain translocation across membranes [19–24], although the precise role of the channel in A chain translocation remains uncertain [17,25–27]. Donovan et al. [10,28] showed that native DT could form channels under similar conditions and that phosphoinositide lipids in the membrane increased channel formation. They also showed that inositol hexaphosphate ($IP_6$) could stimulate channel formation (or opening) when added in millimolar concentrations to the *trans* side of the membrane (i.e., the side opposite that to which DT had been added). Kagan et al. [19] showed that $IP_6$ could stimulate channel opening at concentrations as low as 20 $\mu$M, and that it apparently acted on the phosphate binding or 'P' site of DT. Competitive binding studies by Lory et al. [26] found that the 'P' site which is located on the B chain, and the nicotinamide adenine dinucleotide (NAD) or enzymatic site which is located on the A chain, were in very close proximity.

## Experimental procedures

*Membrane formation*

Voltage-clamp conditions were employed using solvent-free planar lipid bilayer membranes formed from mixtures of purified soybean phospholipids (Avanti, Inc., Birmingham, AL) containing 20% negatively charged lipids but not containing any inositol phospholipids. The usual mixture included phosphatidyl ethanolamine (40%), phosphatidyl choline (40%), and phosphatidyl serine (20%). Membranes were formed from a union of two lipid monolayers across a 100–200 $\mu$m hole in a Teflon partition separating two aqueous phases [30]. The hole was treated with 20 $\mu$l of a 2% solution of squalene (Sigma, Aldrich) or squalane (Fluka) in pentane. Voltages ($V$) were applied using a battery powered stimulator or a signal generator. Current ($I$) was measured using a Keithly 427 amplifier, and the output was fed to an oscilloscope and a chart recorder. Silver/silver chloride electrodes were used to apply voltages and record currents. In the absence of toxin, the membrane conductance $g = I/V$ was about 10 pS. The front chamber (or *cis* side to which DT was added) contained 3 ml of 100 mM NaCl, 10 mM 3,3'-dimethylglutaric acid, 2 mM $MgCl_2$ (for membrane stability), 0.2 mM EDTA (to improve the channel forming activity of DT), all adjusted to pH 4.7. The rear chamber (or *trans* side which was taken as ground) contained 300 $\mu$l of 100 mM NaCl, 10 mM 3,3'-dimethylglutaric acid, 2 mM $MgCl_2$, 0.2 mM EDTA, all adjusted to pH 7.2.

*Toxin*

Diphtheria toxin was a gift of Dr. R.J. Collier of Harvard Medical School. The toxin preparations used were free of the dinucleotide ApUp which is often found bound tightly to DT. The toxin used was nicked and was in monomeric form.

*$IP_3$ stimulation*

$IP_3$ stimulation experiments were performed as follows: After membrane formation, 40 ng of DT was added to the *cis* side with constant stirring. The membrane was held at a constant voltage (+30 mV) which favors DT channel opening. Within a few minutes the current (and therefore conductance) began to increase, slowly at first, but soon reaching a steady rate of increase ($dg/dt$) whose value is a function of DT concentration, *cis* pH, transmembrane pH gradient, and voltage [18,28]. In the absence of inositol phosphates, the rise continued at a steady rate for 20–30 min before reaching a final plateau dependent on the DT concentration. After a steady rate of conductance increase had been established, $IP_3$ or other inositol phosphates were added to the *trans* side of the membrane to a final concentration of 0.05–100 $\mu$M. At concentrations above 200 nM $IP_3$, a sharp acceleration in the rate of conductance increase ($dg/dt$) followed, and a new steady-state rate of increase was achieved. The rate three minutes after $IP_3$ addition was then compared to the rate immediately prior to $IP_3$ addition.

## Results and Discussion

*$IP_3$ stimulation of DT-induced conductance*

Fig. 1a shows the effect of adding $IP_3$ to the *trans* side (final concentration 10 $\mu$M) on the macroscopic membrane current induced by DT. The experimental conditions (*cis* pH 4.7, *trans* pH 7.4) mimicked those that DT would encounter *in vivo* in an acidic endosome (see Fig. 1 legend). Under these conditions DT channels rapidly and spontaneously bind, insert, and open, but rarely close. Therefore in order to quantitate the effect of $IP_3$ under these physiologic conditions, we had to observe the effect of $IP_3$ on a large ensemble of channels, instead of observing the effect on single channels. The rate of channel formation was reflected by the rate of change of membrane conductance ($dg/dt$) and normally reached a steady state dependent on DT concentration, *cis* pH, transmembrane pH

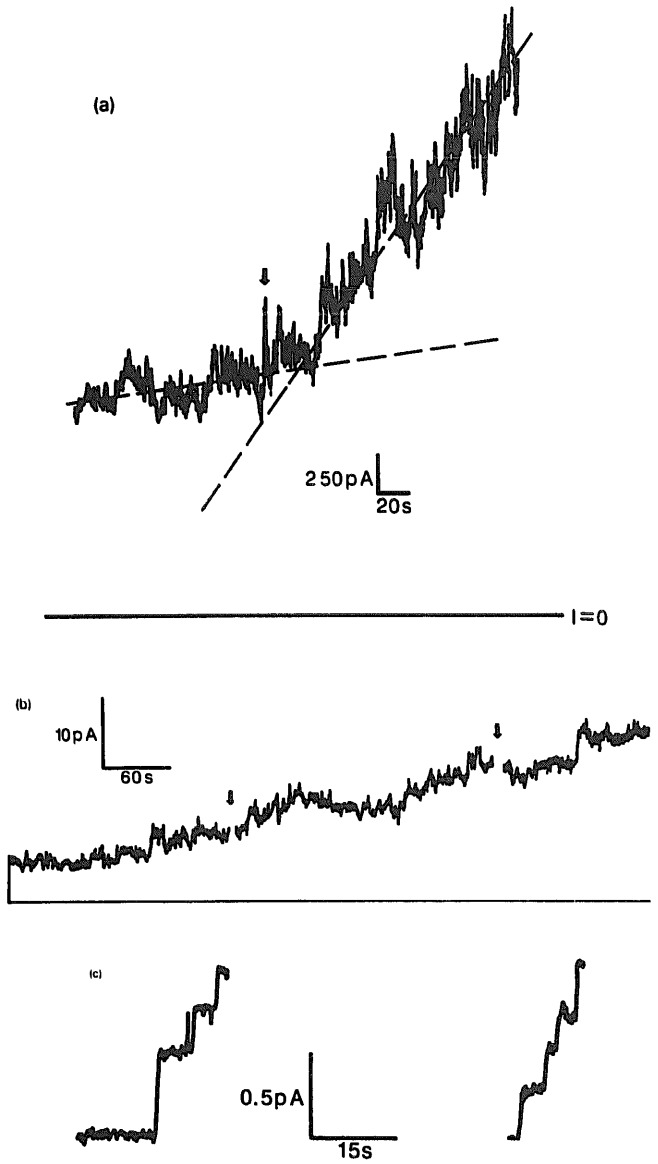


Fig. 1. $IP_3$ stimulation of DT-channel opening. Fig. 1a shows the membrane current as a function of time while the voltage ($V$) was held constant at +30 mV (DT or *cis* side positive). Membrane current ($I$) reflects the conductance ($g = I/V$) due to DT-channel formation in the lipid bilayer. After addition of 40 ng DT to the *cis* side, a steady rise in current (conductance) developed. When the rate of rise reached a steady state (about 3–5 min), $IP_3$ was added to the *trans* solution to a final concentration of 10 $\mu$M (arrow). Within seconds there was a dramatic increase in the rate of conductance rise ($dg/dt$) which soon reached a new steady state which was 9-fold higher than the initial rate. The membrane was composed of 40% phosphatidylethanolamine (soybean), 40% phosphatidylcholine (soybean), and 20% phosphatidylserine (bovine). This *cis* side (to which DT was added) contained 100 mM NaCl, 10 mM DMG, 2 mM $MgCl_2$ and 0.2 mM EDTA, all adjusted to pH 4.7. The *trans* side contained the same solution adjusted to pH 7.2. Fig. 1b shows the blockade of $IP_3$ stimulation by ApUp. Current is shown as a function of time for a membrane treated with DT as in (a), but with ApUp on the *trans* side at a concentration of 1 $\mu$M. At the arrows, $IP_3$ was added to a concentration of 10 $\mu$M and 110 $\mu$M, respectively. Note that there was no change in the rate of current increase due to the presence of ApUp. Conditions were as in Fig. 1a. (Fig. 1c) Single channels of DT before and after $IP_3$ addition. Note that single-channel conductance is unaffected by $IP_3$. The first jump on the left shows two channels opening at once. Conditions were as in Fig. 1a.

gradient, voltage, and membrane lipid composition [10,18,28]. After $IP_3$ addition (arrow), $dg/dt$ increased to a new, higher steady-state value, indicating an increase in the rate of channel formation. Since the single-channel conductance of DT channels was unaffected by $IP_3$ (Fig. 1c) we conclude that $IP_3$ increases the rate of opening of DT channels. The $IP_3$ stimulation could be measured at concentrations as low as 200 nM and was unaffected by the presence or absence of 2 mM $Mg^{2+}$. In over 50 membranes, concentrations of 0.2–20 $\mu$M $IP_3$ caused a dose-dependent stimulation of $dg/dt$ of up to 20-fold. Occasionally, larger stimulations of $dg/dt$ were observed. The quantitative variability in this effect stems from the variability of the baseline $dg/dt$ which is a sensitive function of DT concentration and voltage. $IP_3$ appeared to have a greater effect (20–200-fold increase in $dg/dt$) in membranes which had a lower baseline $dg/dt$ (100 pS/min). Indeed, $IP_3$ can stimulate a measurable $dg/dt$ under conditions where no conductance is normally observed (e.g., in a membrane composed of pure phosphatidyl ethanolamine, data not shown). The variability in baseline $dg/dt$ even under identical conditions stems from the inherent variability of the number of channels inserted in the bilayer. Because only a very small fraction (less than 1 molecule in $10^5$) of the total protein added to the chamber ends up in the bilayer, the baseline $dg/dt$ at a given DT concentration can be quite variable (see Ref. 31 for a more detailed discussion of this point). The effect was only observed at these concentrations when $IP_3$ was added to the *trans* side. When $IP_3$ was added to the *cis* side at identical concentrations no stimulation was observed. When $IP_3$ was added to the *cis* side at much higher concentrations (100 $\mu$M) a small stimulation (less than 3-fold) of DT channel formation was sometimes seen. Since $IP_3$ is small enough to permeate the DT channel [18], this result may indicate crossover of $IP_3$ from the *cis* to the *trans* side. Alternatively, the $IP_3$ binding site might be located in the channel itself, in a site accessible from either *cis* or *trans* sides of the membrane. $IP_3$ preparations from Sigma and Calbiochem, as well as a highly purified preparation from R.F. Irvine (Cambridge University) were all effective in stimulating DT channel opening, making it unlikely that a contaminant is responsible for the effect.

*Voltage dependence of DT-channel opening*

Fig. 2 shows the voltage dependence of DT-channel opening in the absence of $IP_3$. DT-induced currents in response to a series of voltage pulses are shown. Note that the rate of current (and therefore conductance) increase is a steep function of voltage. The rates of conductance increase ($dg/dt$) are plotted semi-logarithmically as a function of voltage in Fig. 3 for a membrane before and after addition of $IP_3$. The linear-

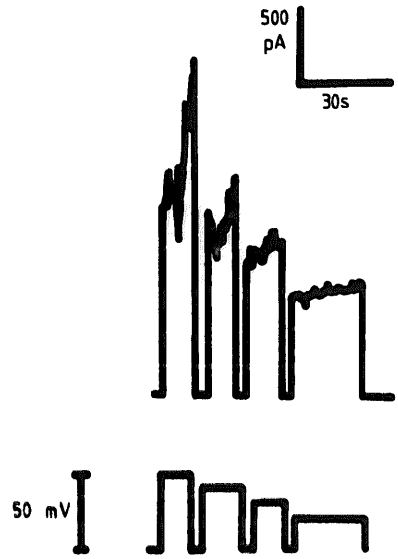


Fig. 2. Voltage dependence of DT-channel opening. Fig. 2 shows membrane currents due to DT at four different voltages (+50, +40, +30, +20 mV). Note that the rate of current (conductance) increase is a steep function of voltage. This reflects the voltage dependence of DT-channel opening.

ity and slope of the semi-logarithmic plot of $dg/dt$ versus voltage suggest that DT-channel gating obeys the Boltzman relation (see Fig. 3 legend) and has an 'apparent gating charge' of approximately 2. That is to say, the channel opens as though it has two formal charges sensing the entire transmembrane voltage. Note that while $IP_3$ appeared to have a small effect on the slope of the line (decreasing the apparent gating charge from 2.2 to 1.6), the major effect of $IP_3$ was to shift the line to the left by about 30 mV on the voltage axis so that the rate of DT-channel opening previously observed at +40 mV was instead seen at +10 mV. In otherwords, $IP_3$ causes DT channels to open as though they had an extra 30 mV positive voltage across the membrane. Thus, the rate of channel opening (and perhaps A chain translocation [22,23]) at 0 mV is nearly 40 times greater in the presence of $IP_3$.

*Pharmacology of DT-channel opening*

Other inositol phosphates could also stimulate DT-channel opening. Table I shows the relative potencies of various DT ligands in stimulating or inhibiting the stimulation of the DT-channel. While inositol 1,3,4,5-tetrakisphosphate ($IP_4$) was somewhat less potent than $IP_3$, and $IP_6$ was much less potent (in agreement with our earlier findings [19]), it is interesting to note that inositol 1,4-bisphosphate ($IP_2$) was equipotent to $IP_3$ in stimulating DT channels. This contrasts with the specificity of $Ca^{2+}$ release observed *in vivo*. In most preparations, $IP_2$ is ineffective in releasing $Ca^{2+}$ from intracellular stores and only inositol phosphates containing phosphate moieties at the 4 and 5 positions are active [1]. However, in at least one preparation from transverse tubules [8], $IP_2$ can substitute for $IP_3$ in activating $Ca^{2+}$ channels. The physiologic role of $IP_4$ is still uncertain, but there is evidence to suggest that $IP_4$ may play a synergistic role with $IP_3$ in the release of $Ca^{2+}$ [32]. While our results do not address this question directly, they do demonstrate that $IP_4$ can directly open DT channels, and this may be relevant to the mode of action of $IP_4$ *in vivo*. The role of higher phosphoinositides such as $IP_5$ and $IP_6$ is even less certain.

*Involvement of the 'P' site in $IP_3$ action*

$IP_3$ does not stimulate channel opening of the DT mutant CRM45. This mutant protein forms channels which are pH and voltage dependent [18] but lacks the 17000 dalton C-terminus of DT which contains the

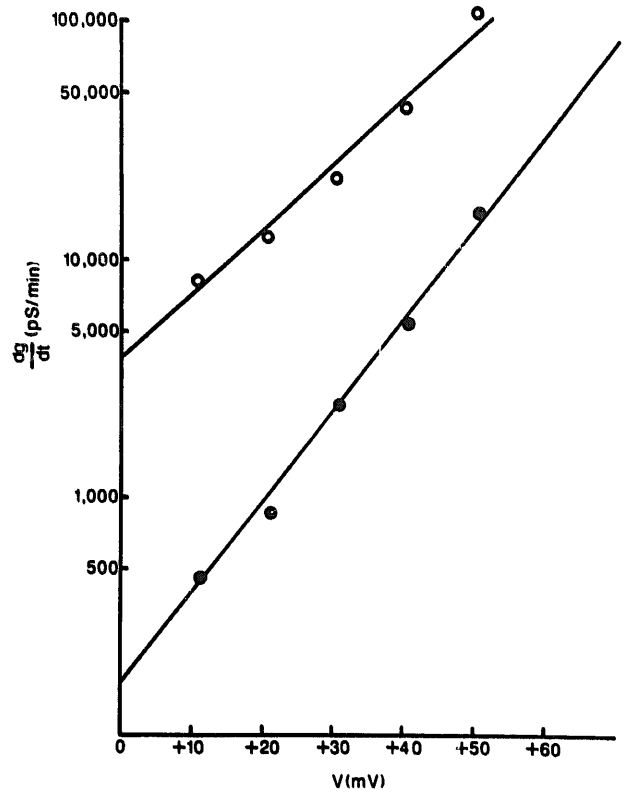


Fig. 3. Effects of $IP_3$ on the voltage dependence of DT-channel opening. The data of Fig. 2 are replotted semi-logarithmically (filled circles). The open circles are data from the same membrane after $IP_3$ was added to a concentration of 2 $\mu$M. All points represent steady state rates of conductance increase ($dg/dt$) measured at different voltages. The linear relation between $dg/dt$ and $V$ indicates that the rate of DT-channel opening is an exponential function of voltage. If channels are assumed to be distributed among open and closed states according to the Boltzman relation,

$$n_o/n_c = \exp(-nq(V-V_0)/kT)$$

where $n_o$ is the number of open channels, $n_c$ is the number of closed channels, $V$ is the membrane voltage, $V_0$ is the voltage at which half the channels are open and $q$, $k$ and $T$ have their usual meanings, then the slope of the line suggests that the DT-channel has about two 'apparent gating charges' which sense the membrane voltage (see Ref. 31 for details of this analysis). Conditions were as in Fig. 1.

TABLE I

*Relative potencies of inositol phosphates on the DT channel*

The concentration required to evoke a stimulatory response of at least 2-fold in $dg/dt$ is shown in the right hand column for a number of inositol phosphate compounds. Also shown are the concentrations of ATP and ApUp needed to block *completely* the stimulation by inositol phosphates. The middle column shows binding constants (where known) for the various compounds [29]. Note that the relative order of potencies of these compounds is preserved between the two measurements.

| Ligand | Effect | $K_d$ binding ($\mu$M) | Threshold ($\mu$M) |
|---|---|---|---|
| $IP_3$ | + | n.d. | 0.2 |
| $IP_2$ | + | n.d. | 0.2 |
| $IP_4$ | + | n.d. | 1.0 |
| $IP_1$ | + | n.d. | 25 |
| $IP_6$ | + | 25 | 30 |
| Inositol | + | n.d. | > 1000 |
| ApUp | − | < 0.001 | 1 |
| ATP | − | 11.7 | 25 |

phosphate binding or 'P' site. Molecules such as adenosyl (3'-5')-uridine 3'-monophosphate (ApUp) and ATP which bind tightly to DT at both its 'P' site and its NAD site [29] were each able to block the effects of $IP_3$ when added to the *trans* side (Fig. 1b). One micromolar ApUp or 25 $\mu$M ATP blocked the effects of 1 $\mu$M $IP_3$ in accord with the relative binding affinities of ApUp and ATP as measured by Lory et al. [29] (Table I). When added alone, neither ATP nor ApUp had any effect on DT-channel opening. Taken together, these results strongly suggest that $IP_3$ directly stimulates DT-channel opening by binding to the 'P' site of DT.

While millimolar concentrations of $Mg^{2+}$ had no influence on $IP_3$ gating of the DT-channel, $Ca^{2+}$ (*trans*, 1 $\mu$M) could block the effect of $IP_3$. $Ca^{2+}$ alone had no effect on DT gating. This finding is consistent with those of Kuno and Gardner [5] and Worley et al. [33] who observed $Ca^{2+}$ blockade of $IP_3$ channel gating and $IP_3$ receptor binding, respectively. Our result suggests that $Ca^{2+}$ may directly block $IP_3$ channel gating *in vivo*.

We believe the ability of $IP_3$ to directly open DT channels provides a good model for understanding $IP_3$-induced $Ca^{2+}$ release from intracellular stores. (1) $IP_3$ opens DT channels at submicromolar concentrations which are considered to be physiologically relevant. (2) $IP_3$ activation occurs through a direct binding of $IP_3$ to a specific site on the DT molecule and cannot occur when this site is blocked (ApUp, ATP) or absent (CRM45). It is thus unnecessary to invoke other intermediate steps or messengers. (3) The DT-channel is poorly selective and shows a significant permeability to $Ca^{2+}$ as well as to other ions [18,23,28,34]. (4) The topological orientation of the DT channel is correct for $IP_3$ activation since the *trans* side of the bilayer corresponds to the cytosolic side of the endosomal membrane. Several other $Ca^{2+}$-permeable channels from lymphocyte plasma membrane [5], sarcoplasmic reticulum [6,7], and transverse tubule [8] have been reported to be gated by $IP_3$. The DT-channel bears a close resemblance to the lymphocyte channel in its low single-channel conductance (6 pS vs. 7 pS), relative lack of ion selectivity, and gating by submicromolar concentrations of $IP_3$. These channels may subserve different regulatory roles of $IP_3$ and other inositol phosphates.

*Relevance to DT intoxication and protein translocation*

The ability of $IP_3$ to open DT channels may also have important implications for the cellular physiology of DT intoxication. Since many eukaryotic cells are sensitive to DT, it should be possible to test for differential DT sensitivity in the presence and absence of agents which induce $IP_3$ production. Recent evidence has shown that a transmembrane pH gradient and/or a membrane potential can accomplish the translocation of DT into cells [21,22], and these are precisely the conditions which favor DT-channel opening in lipid bilayers. Our present results suggest that $IP_3$ in the cytosol would enhance DT translocation and thus speed the intoxication process. Significant influx of $Ca^{2+}$ to the cytosol could occur through the DT-channel, since it rarely closes under physiologic conditions. This $Ca^{2+}$ influx to the cytosol could be the trigger for an endogenous 'suicide' program in some cell lines [35]. Thus, the channel activity of DT could contribute indirectly to cytotoxicity by permitting passage of the ADP-ribosylating A fragment, and directly by allowing unregulated $Ca^{2+}$ influx to the cytosol.

These observations raise the possibility that DT (a viral-coded protein) might be related to a eukaryotic intracellular protein involved in $IP_3$-gated $Ca^{2+}$ release. Since DT has been proposed to act as a 'tunnel protein' involved in the translocation of proteins across membranes [18,34], it is conceivable that other $IP_3$ binding proteins in cells might function in protein translocation. The finding that $IP_3$ receptors in cerebellum are primarily localized to endoplasmic reticulum and nuclear membranes, two intracellular membranes involved in protein translocation, is consistent with this hypothesis. While $IP_3$ has been demonstrated to play a key role in intracellular $Ca^{2+}$ release, a role for $IP_3$ in protein translocation remains speculative.

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