Original Paper

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Heat-Stable Toxin from *Escherichia coli* Activates Chloride Current via cGMP-Dependent Protein Kinase

Key Words
Diarrhea
Channel
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Intestine

Abstract
Heat-stable toxin (STa) increases cyclic GMP (cGMP) in isolated intestinal cells and in T84 cells, a colonic secretory cell line. Whole-cell current recordings from patch clamp experiments show identical properties for currents activated by either STa or the cystic fibrosis transmembrane conductance regulator (CFTR) channel. STa-activated currents display a linear current-voltage relationship and a relative permeability sequence of Br > Cl > I. STa or 8-Br-cGMP-activated currents remain when 20 μM Walsh inhibitor, a blocker of protein kinase A (PKA), is added in the pipette, suggesting that cGMP-dependent protein kinase (PKG) activates the currents. Intracellular addition of Rp-8-Br-cGMP, an agent that activates PKGII and inhibits PKGI and PKA, causes induction of a chloride conductance identical to that stimulated by STa. We conclude that STa activates CFTR by phosphorylation with cGMP-dependent protein kinase.

Introduction

Heat-stable toxin (STa) produced by *Escherichia coli* is responsible for traveller's diarrhea and is a major cause of death in young children in developing countries. STa and the endogenous intestinal peptide guanylin [1] bind to a luminal intestinal receptor with a guanylate cyclase activity [1] causing formation of cGMP [2]. Elevation of intracellular cGMP accompanying the occupation of the STa receptor coincides with increased fluid secretion, suggesting the two are coupled [3, 4]. Our goal was to determine how STa increased cGMP levels to stimulate intestinal chloride channels, that result in increased chloride secretion.

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T84 cells are convenient for the study of STa-mediated chloride secretion, because they express an apical STa receptor and display net transepithelial chloride secretion [5]. Transepithelial chloride transport can be measured by the short-circuit current that is caused by transepithelial ion movement, which in T84 cells is carried by chloride. In addition, the patch clamp technique can be used to measure the magnitude and properties of whole-cell chloride currents activated by STa in individual cells.

T84 cells have a 10 pS chloride channel [6, 7] exhibiting ion selectivity (Br > Cl > I > F) that is identical to that of cystic fibrosis membrane conductance regulator (CFTR) [8–10]. Since T84 cells express mRNA coding for CFTR, these channels are likely identical [11]. Addition of the catalytic subunit of protein kinase A (PKA) [7] activates the 10 pS chloride channel, in excised patches of T84 cells. Further, cGMP and ATP added to a bath including 10 μ*M* Walsh inhibitor (a cAMP-dependent protein kinase inhibitor), also cause chloride channel activation in excised patches [7]. Since these experiments were performed in the absence of added kinase, this suggests that a particulate cGMP-dependent protein kinase endogenous to the excised patch (PKGII) phosphorylates the channel, causing it to open. Particulate PKGII is expressed in intestinal tissues [12] whereas soluble PKGI is expressed in the cytosol of other tissues, i.e. the lung, heart, liver and platelets [13].

Demonstrating the role played by particulate PKGII in STa-mediated secretion should increase our understanding of how cGMP stimulates chloride secretion in intestinal cells. Thus one purpose of our study was to determine whether cGMP participates in a signal transduction pathway leading to chloride current activation in STa-stimulated cells. A second purpose was to determine whether more than one signal transduction pathway activates the CFTR chloride channel.

Methods

Cell Culture

T84 cells obtained from Dr. Doug Jefferies (Tuft's University, Boston, Mass., USA) were grown in Dulbecco's modified Eagle medium containing 25 m*M* $NaHCO_3$ (Gibco, Gaithersburg, Md., USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, USA), 50 units of penicillin and 50 μg/ml streptomycin. Cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air. For single-channel recording, cells from passages 28–42 were seeded onto small squares of Thomas microcover glass (Swedesboro, N.J., USA) and used 3–6 days from seeding. For whole-cell recordings, cells from passages 28–42 were grown in T_{25} flasks for 5–10 days after seeding. Cells were trypsinized with 0.025% trypsin in Ca^{2+}, Mg^{2+}-free Hanks' solution for less than 15 min, dispersed by trituration 3–4 times, then allowed to settle onto the glass bottom experimental chamber for 10–15 min. This protocol consistently yielded STa-stimulated whole-cell currents.

Channel Recordings

Fabrication and use of pipettes for single-channel recording were performed as previously described [7]. Whole-cell recordings were performed using pipettes fabricated from 1.2 mm diameter glass capillary tubes. The pipettes were pulled twice on a Kopf (Tujunga, Calif., USA) puller and fire polished on a microforge (Narishige MF 83). An isolated cell was touched from above, gentle suction was applied to form a tight seal and finally, abrupt strong suction broke a connection between the pipette and the cell interior. In experiments involving anion substitutions, a 150 m*M* KCl agar bridge was connected between the bath and silver pellet at ground. All recordings were done at 25 °C.

Data Acquisition and Analysis

Whole-cell currents were amplified on an EPC-7 patch clamp amplifier (List Electronics, Darmstadt, FRG) without capacitance compensation, visualized on a Nicolet digital oscilloscope (Nicolet Instruments, Madison, Wisc., USA) and stored on a VCR tape through a Sony PCM-601 digital audio processor set at 44 kHz. The whole-cell currents were stimulated using voltages generated and currents measured on 'P clamp' software version 5.1 (Axon Instruments, Foster City,

Calif., USA) on an AST IBM-PC compatible 386 computer. The membrane potential was held at 0 mV, then depolarized or hyperpolarized for 1 s in 20 mV steps (between −100 and +100 mV) with a 5 second pause between each pulse control.

Solutions

For whole-cell recordings the bath solutions contained (in m*M*): 115 NaCl, 40 N-methyl-*D*-glucamine glutamate, 5 K glutamate, 2 $MgCl_2$, 1 $CaCl_2$, Hepes, pH 7.2, with NaOH. The pipette solutions contained (in m*M*): 75 N-methyl-*D*-glucamine Cl, 40 CsCl, 25 N-methyl-*D*-glucamine glutamate, 1 EGTA, 0.1 $CaCl_2$, 2 $MgCl_2$, 5 Hepes, 2 ATP, 0.5 GTP, pH 7.2, with glutamate. Measurements indicated about 100 n*M* free Ca^{2+} using fura-2; Cs was added to the pipette solution in block potassium channels. In addition, potassium was removed and replaced by a more impermeant cation in an effort to make chloride currents predominate. For anion substition experiments NaCl in the bath was replaced with NaI, NaBr or NaF. The relative permeability ratio (Px/PCl) was calculated using the Goldman-Hodgkin-Katz equation [14], for different bath solutions.

cGMP Accumulation

cGMP accumulation was measured between days 1 and 7, after trypsinization and at 22 or 37 °C to determine the effect of cell confluence and handling procedures on the STa activity. T84 cells seeded in 35-mm dishes, 3 per determination, were grown according to procedures described above and held at 37 °C until just before STa in maximal doses of 1–2 μg/ml was added. cGMP accumulation was measured at 37 or 22 °C, as indicated, in the presence of Hanks' medium containing 1 m*M* isomethylbutylxanthine. At the end of an incubation, buffer was removed and 1 ml of 0.1 *N* HCl was added for 30 min at room temperature to lyse the cells. cGMP was measured with ^{125}I-labeled cGMP using an Amerlex-M magnetic separation kit distributed by Amersham Corp. (Arlington Heights, Ill., USA).

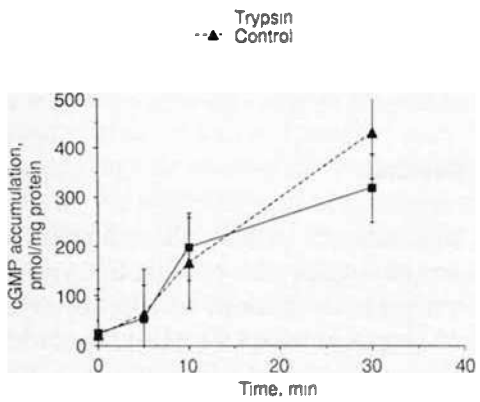
Materials

STa was obtained from Dr. Donald C. Robertson, University of Kansas. In some experiments, STa was purchased from Sigma Chemical Co. (St. Louis, Mo., USA). This STa was about 4-fold less potent than that provided by Dr. Robertson. H8, N-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide, was obtained from Sigma Chemical Co. Rp-8-Br-cGMP (a diastereomer of 8-Br-cGMP phosphorothioate) and Rp-8-Br-cAMP were obtained from Dr. Hugo de Jonge, Erasmus University, Rotterdam, The Netherlands. The Walsh inhibitor was kindly provided by Dr. Richard Huganir, Johns Hopkins University.

Results

STa-induced cGMP accumulation was measured in cells with or without trypsinization (fig. 1a), at 22 or 37 °C (fig. 1b) or after increasing days in culture (fig. 1c) in order to determine whether cGMP levels were altered under these conditions. Trypsinization did not affect cGMP accumulation, as depicted in figure 1a. In contrast, lowering temperature of the assay from 37 to 22 °C dramatically decreased basal cGMP levels (no STa), from 21 ± 2.4 to 1 ± 0.2 pmol/mg protein, respectively, and also decreased STa-mediated increase in cGMP (fig. 1b). A most important factor in cGMP accumulation was found to be the number of days of cell culture. At 7 days after seeding, the capacity to generate cGMP increased 5-fold at 60 min and 10-fold at 90 min (fig. 1c). We found that maximal levels of cGMP occurred in cells that were cultured for at least 7 days, a time which coincides with confluence. Although trypsinization, used to release cells for patch clamp experiments, was less successful in terms of cell viability for confluent versus preconfluent cells, the older cells were used because of their favorable cGMP accumulation.

Short-circuit currents are activated by STa, forskolin and in some experiments by high doses of cGMP [15]. Therefore, in order to better understand how STa activates cellular chloride currents, we measured whole-cell currents stimulated by these agents. Using the whole-cell patch clamp technique in the absence of chemical stimuli (before STa), the current at 100 mV was very small (14.6 ± 1.8 pA; n = 9). Extracellular bath addition of STa (1 μg/ml) activated a linear whole-cell current



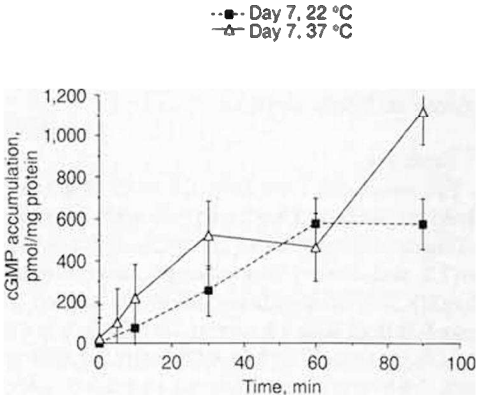
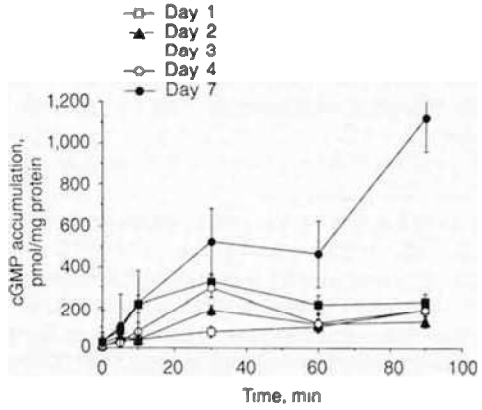
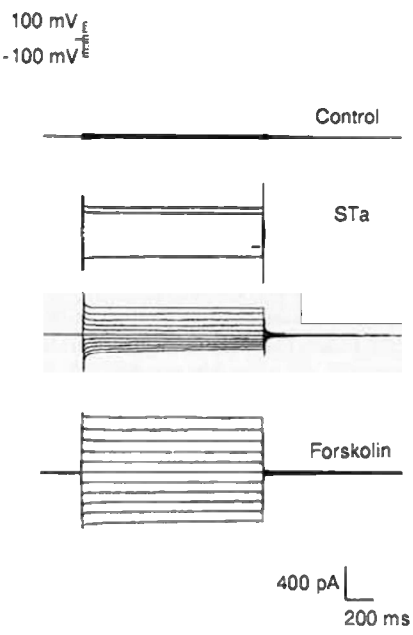


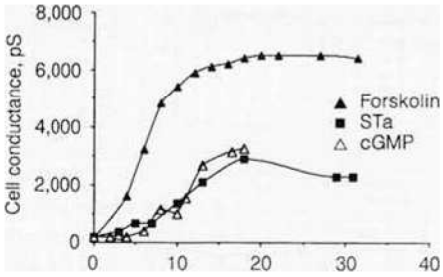
Fig. 1. a Effect of trypsinization on cGMP accumulation. Cells were trypsinized or not trypsinized (control). then cGMP accumulation was measured on the two populations of cells at 37°C. Cells were used on day 7 after seeding. Data were collected from 3 dishes for each time point and experiments repeated on 3 different culture passes. Data are shown as means ± SE (n = 3). **b** Effect of temperature on cGMP accumulation. Assays at 22 and 37°C were compared for cells used 7 days after seeding. cGMP accumulation at 37°C is significantly greater than that at 22°C at 90 min ($p < 0.1$ using Student's t test). **c** cGMP accumulation of cells cultured for increasing numbers of days. cGMP accumulation increases after culturing for 7 days. This coincides with confluence. cGMP accumulation on day 7 is significantly different from that of day 2 ($p < 0.02$ using Student's t test).

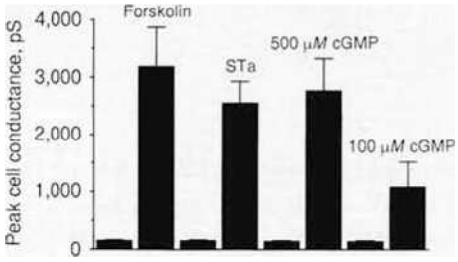


(fig. 2a) resulting in a linear current-voltage relationship (fig. 2b), similar to that activated by 10 μ*M* forskolin or 500 μ*M* 8-Br-cGMP, as shown in figure 2a. As the STa-mediated current increased, the reversal potential approached 0 mV (the chloride equilibrium potential), because chloride concentrations were equal in the cell and bath (fig. 2b). When the bath solution was replaced with NaI in the presence of STa, the iodide current was less than the chloride current, and the reversal potential was more positive (fig. 2b). As shown in table 1 the reversal potentials for different ion replacements had a sequence of Br < Cl < I. Therefore the relative ion permeabilities (Px/PCl) have a sequence of PBr/PCl > PCl/PCl > PI/PCl.

A representative example of the times required to reach peak conductance (at 100 mV) is shown in figure 2c. Figure 2d shows the average peak conductance for each treatment. The 10 μ*M* forskolin-activated







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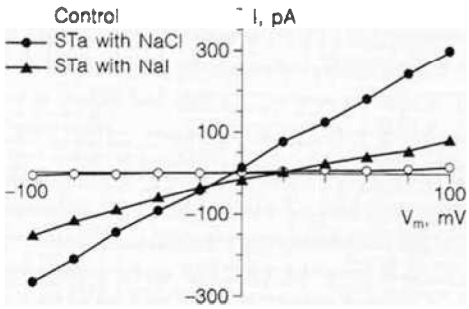

Fig. 2. a Activation of whole-cell chloride currents with STa, cGMP or forskolin. The bath surrounding the cells was exchanged with the same type of bath solution containing either 1 μg/ml STa, 10 μ*M* forskolin or 500 μ*M* 8-Br-cGMP. Under control conditions the reversal potential was negative, when STa was added the reversal potential approached zero as predicted for a chloride current, when NaI bath was added the potential reversed to slightly positive since the channel is less permeable to I than Cl. When chloride was returned to the bath, the reversal potential reversed to the equilibrium potential for Cl (not shown). **b** Current-voltage relationships in chloride or iodide medium. STa with Cl in the bath or STa-treated cells with I replacing Cl in the bath all yielded linear currents. V_m = Membrane potential. **c** Activation of whole-cell conductance with time. Cells were stimulated with forskolin 10 μ*M*, STa (1 μg/ml; Sigma) or 500 μ*M* 8-Br-cGMP. Three individual cells trypsinized from the same culture are shown. **d** Average peak whole-cell conductances from several experiments. Forskolin (10 μ*M*), low- and high-potency STa (1 μg/ml) and low (100 or 200 μ*M*) 8-Br-cGMP or high (500 μ*M* to 1 m*M*) 8-Br-cGMP each caused increased chloride conductance. Each bar represents the data from 9 individual cells from different days and cultures. The lower bar on the left of each bar represents the data from controls before adding any agents. Cell capacitances were uniformly about 20 pF.

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