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Activation of intestinal CFTR Cl^- channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase

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Heat-stable enterotoxins (STa) produced by pathogenic bacteria induce profound salt and water secretion in the gut, leading to diarrhea. Recently, guanylin, an endogenous peptide with properties similar to STa, was identified. While STa and guanylin bind to the same receptor guanylyl cyclase and raise cell cGMP, the signaling mechanism distal to cGMP remains controversial. Here we show that STa, guanylin and cGMP each activate intestinal Cl^- secretion, and that this is abolished by inhibitors of cAMP-dependent protein kinase (PKA), suggesting that PKA is a major mediator of this effect. These agents induce Cl^- secretion only in cells expressing the wild-type CFTR, indicating that this molecule is the final common effector of the signaling pathway. The involvement of CFTR suggests a possible cystic fibrosis heterozygote advantage against STa-induced diarrhea.

Key words: cAMP-dependent protein kinase/CFTR/guanylin/heat-stable enterotoxin/intestinal epithelium

Introduction

Enterotoxigenic *Escherichia coli* and other bacteria, by elaborating enterotoxins, cause acute diarrheal disorders, which account for as much as 50% of total infant deaths in developing countries (reviewed in Giannella, 1981). Two major types of enterotoxin are produced by *E.coli*, a large molecular weight, heat-labile enterotoxin (LT) and a small molecular weight, heat-stable enterotoxin (ST). The mechanism of action of LT has been well defined. Similar to cholera toxin, LT exerts its effect via a cAMP-dependent pathway. STa, the most common subtype of ST, has been shown to stimulate a specific receptor, STaR, predominantly found in the apical membranes of the intestinal epithelial cells (Waldman and Murad, 1987; Singh *et al.*, 1988). STaR was recently cloned and its sequence revealed a new member of the guanylyl cyclase receptor family (Schulz *et al.*, 1990; de Sauvage *et al.*, 1991; reviewed in Garbers, 1992). Binding of STa to this receptor leads to stimulation of STaR guanylyl cyclase and elevation of cytosolic cGMP, which in turn induces Cl^- secretion in the small intestine (Field *et al.*, 1978), as well as in a colonic carcinoma cell line, T_{84} (Huott *et al.*, 1988).

An endogenous ligand for STaR, guanylin, has recently been identified (Currie *et al.*, 1992; de Sauvage *et al.*, 1992b; Schulz *et al.*, 1992). Guanylin was originally identified as a 15 amino acid peptide able to bind to and stimulate STaR in T_{84} cells (Currie *et al.*, 1992). Cloning of the guanylin cDNA indicates that guanylin corresponds to the C-terminus of a large inactive precursor, proguanylin (de Sauvage *et al.*, 1992b; Schulz *et al.*, 1992). Northern blot analysis and *in situ* hybridization show high level expression of guanylin mRNA at the base of the intestinal crypt cells (de Sauvage *et al.*, 1992b). These results are consistent with guanylin having a primary role in the regulation of intestinal secretion. However, bioassay and Northern blot analysis suggested that the kidney also contains active guanylin-like material (Currie *et al.*, 1992; Schulz *et al.*, 1992). Coupled with the observation that ^{125}I-STa binding sites exist in epithelial cells in many different organs of the North American opossum (Forte *et al.*, 1988; Krause *et al.*, 1990), this seems to suggest that guanylin-like ligands and STaR subtypes may also regulate epithelial transport in other organs.

Although STa and guanylin both appear to stimulate intestinal Cl^- secretion by increasing intracellular cGMP, the molecular mechanism by which cGMP modifies Cl^- transport is speculative (Lin *et al.*, 1992; Yuen and Garbers, 1992). Previous studies have shown that Cl^- secretion in intestinal epithelial cells can be induced by at least three parallel pathways.

(i) The cAMP pathway in which a receptor-generated increase in cAMP activates cAMP-dependent protein kinase (PKA), which in turn phosphorylates and opens the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels (reviewed in Anderson *et al.*, 1992). A defect in this pathway is the basis for the disease cystic fibrosis (CF). Mutations in the CF gene lead to reduction, altered processing, defective regulation and/or dysfunction of the CFTR protein (reviewed in Welsh and Smith, 1993), which is a Cl^- channel localized to the apical membrane of secretory epithelial cells (reviewed in Anderson *et al.*, 1992; Frizzell, 1993).

(ii) The Ca^{2+} pathway in which receptor-generated increase in intracellular Ca^{2+} activates multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), leading to phosphorylation and opening of Ca^{2+}-dependent Cl^- channels (Wagner *et al.*, 1991; Worrell and Frizzell, 1991). These channels are entirely independent of CFTR (Wagner *et al.*, 1992), and therefore the Ca^{2+} pathway is preserved in CF (Wagner *et al.*, 1991).

(iii) The volume-sensitive pathway which is activated upon exposure of the cell to hypotonic solutions (Worrell *et al.*, 1989). The precise molecular events leading to hypotonicity-induced Cl^- currents are not known, but it has been

suggested that the human multidrug-resistance P-glycoprotein may be the Cl^- channel involved (Valverde *et al.*, 1992). This pathway is also preserved in CF mutant cells (Wagner *et al.*, 1991).

The purpose of this study was to delineate the molecular pathway by which STa/guanylin stimulate Cl^- secretion in intestinal cells and to relate this to previously described pathways. Our results indicate that STa and guanylin, by stimulating STaR guanylyl cyclase and raising intracellular cGMP, mainly exert their effect on Cl^- secretion via PKA, which in turn activates the CFTR Cl^- channel. The implications, including the possibility of CF heterozygote advantage in STa-induced diarrhea, are discussed.

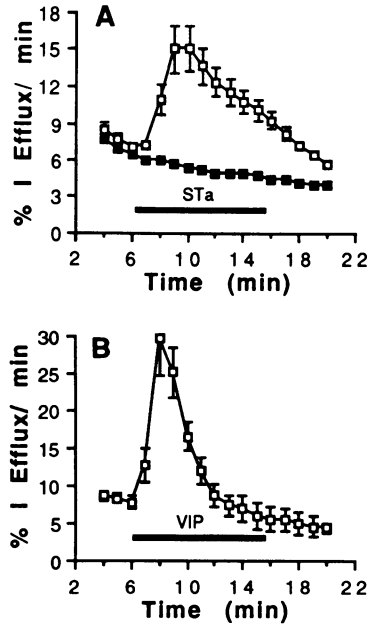

Fig. 1. Effect of STa and VIP on ^{125}I efflux in cultured monolayers of T_{84} cells. STa (**A**; □; 1 μM; N = 5) and VIP (**B**; 0.1 μM; N = 3) were added where indicated by the bars. Control experiments (A; ■; N = 3) received no intervention.

Results

Anion efflux and whole-cell patch-clamp experiments demonstrate that STa, guanylin and CPT-cGMP all stimulate Cl^- secretion in T_{84} cells

^{125}I efflux and whole-cell patch-clamp experiments were performed to examine the effects of STa, human guanylin and the intracellular messenger cGMP on Cl^- transport in T_{84} cells. This cell line expresses the heat-stable enterotoxin receptor (STaR) (Huott *et al.*, 1988; de Sauvage *et al.*, 1992a), and all known types of membrane Cl^- channels implicated in Cl^- secretion, including the PKA-activated CFTR Cl^- channel and the Ca^{2+}-activated Cl^- channel (Cliff and Frizzell, 1990).

In radiotracer flux experiments, addition of STa (1 μM) caused a marked increase in the ^{125}I efflux rate (Figure 1A; baseline, 7.1 ± 0.3%/min, after STa, 15.1 ± 1.8%/min; $P<0.01$ by paired t-test, N = 5). STa-induced Cl^- (I^-) conductive loss was smaller than that induced by vasoactive intestinal peptide (VIP; baseline, 7.8 ± 0.9%/min, after VIP, 29.8 ± 5.0%/min; $P<0.05$, N = 3), which is known to stimulate Cl^- secretion via the cAMP-mediated pathway (Dharmsathaphorn *et al.*, 1985) (Figure 1B). The apparently slower and submaximal response to STa, as compared with VIP, is similar to that noted by other investigators (Guandalini *et al.*, 1982; Huott *et al.*, 1988). These investigators found that the STa-mediated effect on Cl^- transport, although closely resembling the cAMP-mediated mechanism, differed in at least two respects. The time

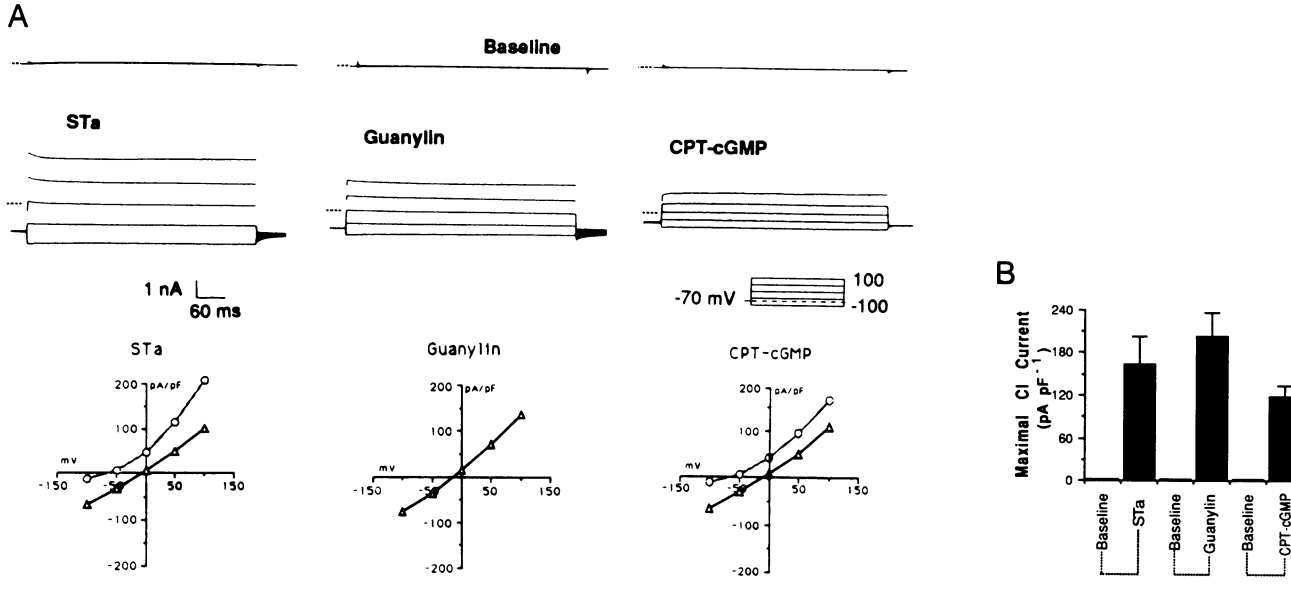

Fig. 2. Effect of STa, human guanylin and CPT-cGMP on whole-cell Cl^- currents in T_{84} cells. (**A**) Whole-cell Cl^- currents typically recorded at baseline (top) and after stimulation (middle) of STa (1 μM), guanylin (20–100 μM) and CPT-cGMP (0.5 mM), respectively. Dotted line indicates the zero level Cl^- current. The resting membrane potential was held at −70 mV. To examine the $I-V$ relation of a recorded current, membrane potential was sequentially altered from −100 to +100 mV in 50 mV steps, using a voltage-clamp protocol (see inset). The voltage steps were of 500 ms in duration and separated by 750 ms intervals. Data were averaged over the final 80 ms of the pulses. Displayed on the bottom are $I-V$ relations of the Cl^- current activated by these agents, with a transmembrane Cl^- concentration ($[Cl^-]$) ratio of 1:1 (△) and 1:12 (○), respectively. Data were normalized by dividing by cell capacitance, an index of cell surface area (Wagner *et al.*, 1992). (**B**) Maximal outward Cl^- current recorded at +100 mV (mean ± SE) at baseline and after addition of STa (n = 11), guanylin (n = 7) and CPT-cGMP (n = 15), respectively.

interval required to observe maximal effects of STa on net Cl^- secretion was longer than that observed with effectors acting through cAMP. In addition, they noted that the maximal effect of STa on either net Cl^- secretion or ^{36}Cl uptake was less than that maximally produced by VIP. Nevertheless, the two agonists were not additive; the combined effect (STa + VIP) approximated that induced by VIP alone.

Whole-cell patch-clamp experiments revealed that STa, human guanylin and 8-(4-chlorophenylthio)-guanosine 3',5' cyclic monophosphate (CPT-cGMP), a membrane-permeant analog of cGMP, all markedly enhanced whole-cell Cl^- current (Figure 2A). In most cells, the activated Cl^- current (typically recorded 10–15 min after stimulation) showed a nearly linear current–voltage ($I-V$) relation and time-independent activation upon voltage steps, similar to that stimulated via cAMP-dependent pathway acting through CFTR (Cliff and Frizzell, 1990). Under these conditions, the recorded current is carried predominantly by Cl^- because K^+ is absent in the internal (pipette) solution and Cs^+, used to replace K^+, is known to block K^+ channels. Furthermore, when pipette [Cl^-] was lowered to generate a transmembrane [Cl^-] ratio of 1:12, the reversal potential of STa- and CPT-cGMP-elicited Cl^- currents shifted to −60 mV, in good agreement with the predicted Nernst equation (−65 mV) assuming a Cl^--selective conductance (Figure 2A). These results (summarized in Figure 2B) demonstrate that STa, the endogenous intestinal peptide guanylin, and CPT-cGMP each stimulate Cl^- secretion by activating a membrane Cl^- channel.

Both ^{125}I efflux and whole-cell experiments show that inhibitors of PKA attenuate STa-stimulated Cl^- transport

The signaling events distal to the action of cGMP were further explored. It has been shown previously that staurosporine inhibits STa action, suggesting the involvement of a serine/threonine kinase (Forte *et al.*, 1992). We next asked which protein kinase(s) is the major intracellular mediator of the effect of cGMP. By using membrane permeant-specific kinase inhibitors, we first tested for the involvement of PKA versus cGMP-dependent protein kinase (PKG). We performed ^{125}I efflux experiments in which we used the inhibitory diastereomers of guanosine or adenosine 3',5'-cyclic phosphorothioate, Rp-8-Br-cGMPS and Rp-8-Br-cAMPS, which competitively inhibit cGMP or cAMP from binding to the regulatory site of the corresponding kinases, with K_i values of ~4 μM (Butt *et al.*, 1990). The effect of these inhibitors on STa-stimulated ^{125}I efflux was tested at a dose ~10 times their K_i values. As seen in the experiments given in Figure 3A, in the control group STa enhanced ^{125}I efflux rate by ~11%/min (before STa, 7.1 ± 0.6%/min, after STa, 17.9 ± 1.4%/min; $N = 3$). In the Rp-8-Br-cAMPS-treated group, however, STa only increased ^{125}I efflux by 1–2%/min (before STa, 6.0 ± 0.2%/min, after STa, 7.5 ± 1.4%/min; $N = 3$). Thus, Rp-8-Br-cAMPS diminished STa-stimulated Cl^- secretion by ~90%. By contrast, Rp-8-Br-cGMPS only attenuated STa-stimulated Cl^- transport by ~40% (before STa, 6.7 ± 0.4%/min, after STa, 12.8 ± 2.6%/min; $N = 3$). These results suggest that cGMP, elevated by STa

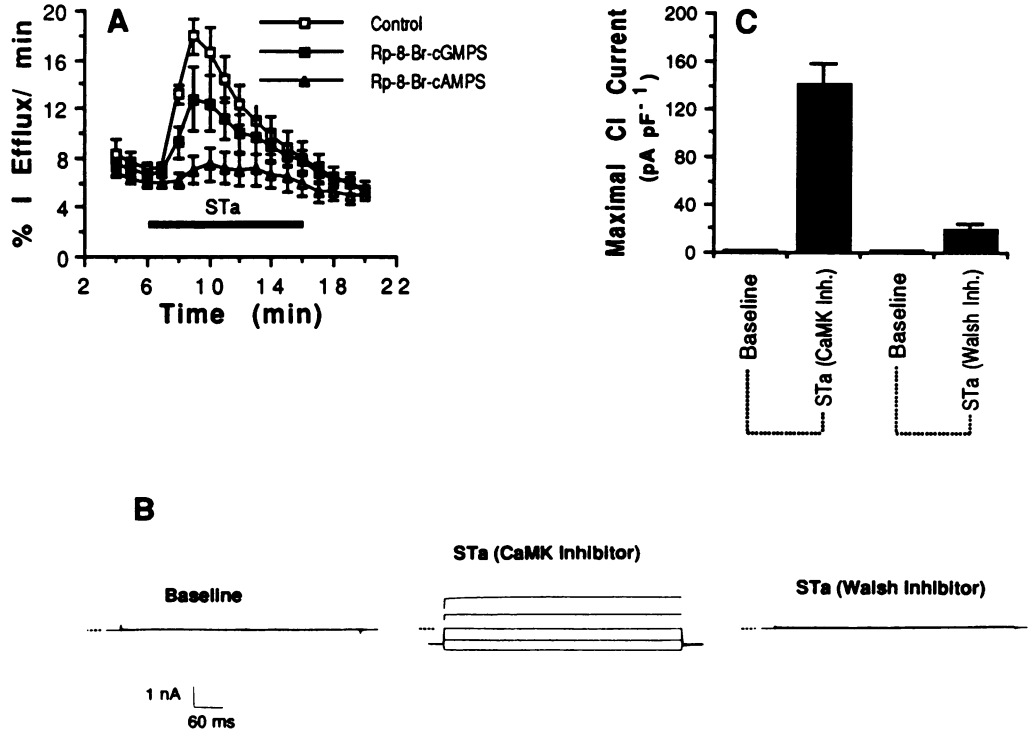

Fig. 3. Effect of addition of protein kinase inhibitors on STa-stimulated Cl^- transport. (**A**) Effect of Rp-8-Br-cGMPS and Rp-8-Br-cAMPS on STa-stimulated ^{125}I efflux ($N = 3$). In the inhibitor groups, cells were first pretreated with an inhibitor (25–50 μM) for ~2 h and were continuously exposed to the inhibitor during the efflux experiment. Data shown were obtained from paired experiments carried out under identical conditions on the same day. (**B**) Representative whole-cell current recorded at baseline (left) and after stimulation of STa, in the presence of the specific peptide inhibitor of CaMKII, CaMK [273–302] (20 μM; middle), and in the presence of Walsh inhibitor (1–20 μM; right), respectively. Cells were internally perfused with the inhibitor by addition to the pipette solution. (**C**) Maximal outward Cl^- current induced by STa in the presence of CaMK [273–302] ($n = 4$) and of Walsh inhibitor ($n = 17$).

primarily acts on PKA in the intact cell. This cannot be through cAMP, however, as might occur if cGMP inhibits a cyclic nucleotide-dependent phosphodiesterase, since we (data not shown) and others (Field *et al.*, 1978; Giannella and Drake, 1979; Huott *et al.*, 1988) find no change in intracellular cAMP concentration after STa stimulation.

To further substantiate a role for PKA in STa action, we performed a series of experiments in which highly specific peptide kinase inhibitors of both PKA and CaMKII, which are mediators of the cAMP- and Ca^{2+}-dependent pathways of Cl^- secretion respectively, were perfused into the cell by means of the patch pipette. A corresponding peptide inhibitor of the particulate PKG which is localized in the apical membrane of T_{84} cells (de Jonge, 1976) is not available to our knowledge. First we tested the effect of intracellular perfusion with Walsh inhibitor, a potent peptide pseudo-substrate inhibitor of the catalytic site of PKA (Cheng *et al.*, 1986). Whole-cell patch-clamp experiments revealed that perfusion of the cell interior with Walsh inhibitor (1−20 μM) dramatically reduced STa-elicited Cl^- current (Figure 3B) from 164 ± 40 pA/pF (see Figure 2B, n = 11) to 19 ± 6 pA/pF (Figure 3C, n = 17; $P<0.001$ by unpaired t-test). In the concentration range tested, Walsh inhibitor is reported to be specific for PKA over PKG and other serine/threonine kinases (Cheng *et al.*, 1986), providing further evidence for a primary role for PKA in STa action. By contrast, a peptide inhibitor of CaMKII, CaMK [273−304] (20 μM) (Malinow *et al.*, 1989), which has been shown to block Ca^{2+}-activated Cl^- current (Wagner *et al.*, 1991), failed to inhibit STa-induced Cl^- current (Figure 3B and C; n = 4). These results support the conclusion that PKA is the primary cGMP-dependent mediator of STa action and, therefore, that the STa/cGMP pathway converges on the cAMP-dependent pathway described in the Introduction.

Antisense oligodeoxynucleotides to CFTR mRNA diminish VIP- and STa-stimulated Cl^- transport

As previously described, CFTR mediates cAMP-dependent Cl^- secretion, a process that is defective in CF. Because the characteristics of the whole-cell Cl^- current induced by STa closely resemble Cl^- currents flowing through CFTR and because the experiments with kinase inhibitors implicated PKA in the STa process, we devised a series of experiments to test for the involvement of CFTR in STa-induced Cl^- secretion. First, we used antisense oligodeoxynucleotides to CFTR mRNA to reduce the expression of CFTR in T_{84} cells. We have previously shown that treatment of T_{84} cells with a pair of antisense oligomers derived from the first 36 bp of the CFTR cDNA sequence leads to a reduction in the amount of measurable CFTR protein, as assessed by immunoprecipitation (Wagner *et al.*, 1992). Oligomer treatment also eliminates cAMP-dependent whole-cell Cl^- currents, as compared with untreated and sense- or misantisense-treated cells (Wagner *et al.*, 1992). As shown in Figure 4A, VIP-stimulated ^{125}I efflux was largely diminished in cells whose CFTR had been depleted by pretreatment with antisense oligodeoxynucleotides. In parallel, STa-induced Cl^- secretion was also greatly inhibited in cells depleted of CFTR by antisense oligomers (Figure 4B). This result demonstrates that STa-stimulated Cl^- secretion can be attenuated by depletion of CFTR and, therefore, supports a role for CFTR in STa action.

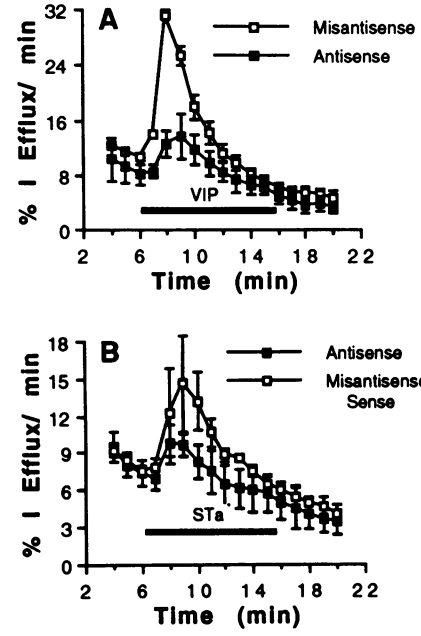

Fig. 4. Effect of pretreatment of antisense oligodeoxynucleotides to CFTR mRNA on VIP- and STa-stimulated Cl^- transport in T_{84} cells. (**A**) Effect of VIP on ^{125}I efflux in cell monolayers pretreated with misantisense (N = 2) and with antisense (N = 3). (**B**) Effect of STa on ^{125}I efflux in misantisense-/sense- and antisense-treated cell monolayers (N = 3 each).

CPT-cGMP increases whole-cell Cl^- current in 3T3-CFTR but not in NIH-3T3 fibroblasts

To further validate our notion that STa/cGMP work via CFTR, we have performed additional experiments to test the effect of CPT-cGMP on whole-cell Cl^- current in NIH-3T3 cells which do not express CFTR (Gregory *et al.*, 1990) and in NIH-3T3 cells stably transfected with CFTR (3T3-CFTR) (Berger *et al.*, 1991). As illustrated in Figure 5, CPT-cGMP augments whole-cell Cl^- current, with the characteristic linear $I-V$ relation and time-independent gating expected of CFTR current, in 3T3-CFTR but not in NIH-3T3, demonstrating that cGMP stimulation of Cl^- secretion is highly dependent on the presence of CFTR. It should also be noted that NIH-3T3 cell lines are originally derived from connective tissue fibroblasts which contain PKA but do not apparently contain PKG (Walter, 1989). This suggests that these cells still possess all the intracellular components required for cGMP-stimulated Cl^- current and, therefore, further supports the conclusion that PKA mediates the STa/cGMP effect.

CPT-cGMP increases whole-cell Cl^- current in normal but not CF-derived airway epithelial cells

As a final approach to determine if cGMP acts through CFTR, we compared whole-cell Cl^- current in normal versus CF-derived epithelial cells. Involvement of CFTR would be substantiated if the cGMP response is present in cells with normal CFTR, but reduced or absent in cells with mutant CFTR. To test this hypothesis we used two simian virus 40-transformed cell lines derived from normal and CF airway epithelium, 56FHTE-8o- and 2CFSMEo-, each of which contains message for CFTR (Cozens *et al.*, 1992a,b). By means of the patch-clamp technique, we have previously characterized the whole-cell Cl^- conductances in these cell

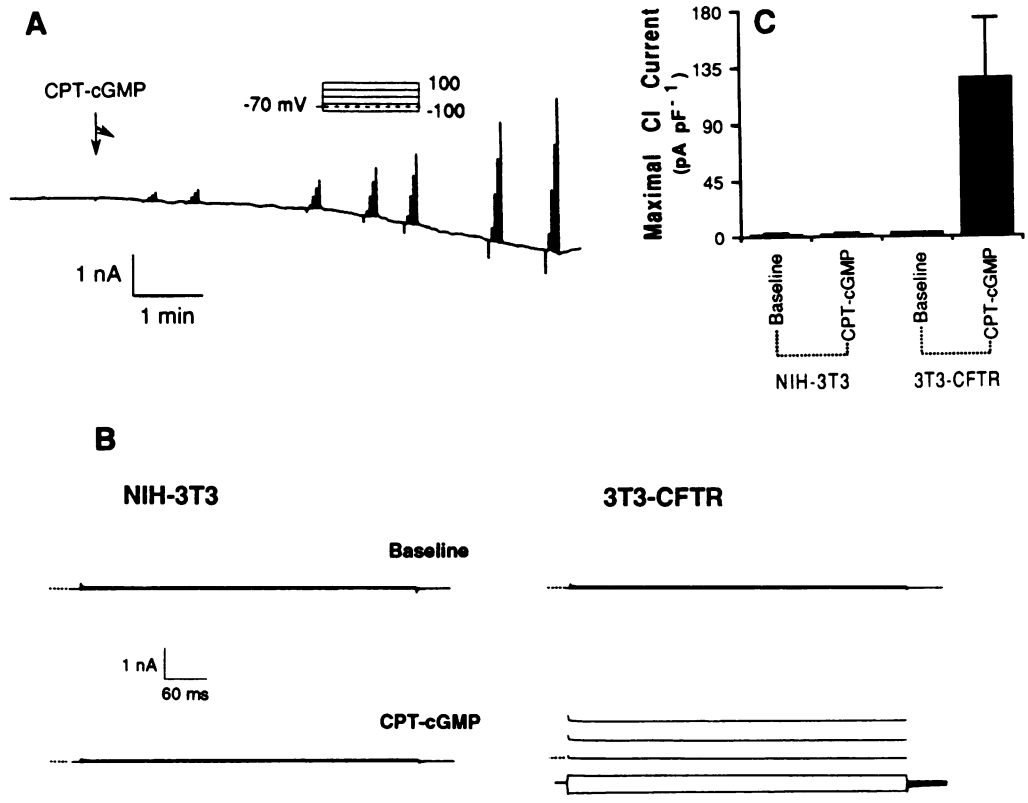

Fig. 5. Effect of CPT-cGMP on whole-cell Cl^- currents in NIH-3T3 and 3T3-CFTR fibroblasts. (**A**) Time-course of the whole-cell Cl^- currents recorded in 3T3-CFTR before and after addition of CPT-cGMP (0.5 mM). The current steps were elicited using the membrane voltage-clamping protocol given in the inset. (**B**) Effect of CPT-cGMP on whole-cell Cl^- currents in NIH-3T3 (left) and in 3T3-CFTR (right). Shown are whole-cell Cl^- currents typically recorded at baseline (top) and after stimulation (bottom) of CPT-cGMP. (**C**) Maximal outward Cl^- current induced by CPT-cGMP in NIH-3T3 (n = 5) and in 3T3-CFTR (n = 7).

lines (Wagner *et al.*, 1991). The two cell lines each possess characteristic Ca^{2+}-dependent and volume-sensitive Cl^- conductances previously described in T_{84} epithelial cells (Cliff and Frizzell, 1990; Worrell and Frizzell, 1991). By contrast, only the normal cell line shows increased Cl^- conductance in response to bath application of a membrane-permeant cAMP analog, CPT-cAMP. Antisense oligomers to CFTR eliminated the cAMP-dependent Cl^- conductance in the normal airway epithelial cell line (Wagner *et al.*, 1992), converting it to the same phenotype as the CF-derived cell line, i.e. absent cAMP-dependent Cl^- conductance with preserved Ca^{2+}-dependent and volume-sensitive Cl^- conductances. Consistent with our hypothesis that cGMP-dependent Cl^- transport also occurs through CFTR Cl^- channels, CPT-cGMP (0.5 mM) activated characteristic whole-cell Cl^- current in the normal human airway epithelial cell 56FHTE-8o-, but not in the CF-derived transformed airway cell 2CFSMEo- (Figure 6).

Discussion

In summary, we have shown that STa stimulates ^{125}I efflux and whole-cell Cl^- currents in the cultured colonic epithelial T_{84} cell line. The effects of STa and guanylin on Cl^- current activation are mimicked by a membrane-permeant analog of cGMP, CPT-cGMP. This fulfils the criteria of Sutherland and co-workers (Robinson *et al.*, 1971) that cellular events modulated by changes in nucleotide levels should be mimicked by the extracellular addition of that cyclic nucleotide. STa effects on ^{125}I efflux are attenuated to a large extent by the membrane-permeant PKA inhibitor Rp-8-Br-cAMPS, while the STa effect on whole-cell Cl^- current is blocked by Walsh inhibitor, the peptide inhibitor of PKA. These observations strongly support a role for PKA in STa action. The characteristics of the whole-cell Cl^- current activated by STa, CPT-cGMP and guanylin, including a linear $I-V$ relation and time-independent activation on steady-state voltage pulse protocols, suggest that the current flows through CFTR Cl^- channels. Experiments employing antisense depletion of CFTR, as well as comparisons of the responses of wild-type versus CFTR-transfected cells and normal versus CF-derived cells, corroborate the involvement of CFTR as the final common downstream mediator of STa, guanylin and/or cGMP action. The discussion below will elaborate on some of these points.

PKA mediates STa-induced Cl^- secretion

The central role of cGMP as the mediator of STa-provoked ion secretion has long been accepted (Field *et al.*, 1978; Hughes *et al.*, 1978; Newsome *et al.*, 1978; Guerrant *et al.*, 1980; Guandalini *et al.*, 1982). Definitive confirmation was provided by the cloning of STaR which is a transmembrane guanylyl cyclase (Schulz *et al.*, 1990; de Sauvage *et al.*, 1991). STa and its physiological ligand guanylin interact with STaR to produce large and persistent increases in cGMP. A paradox, however, was raised by the observation that certain hormones (e.g. α-adrenergic agonists, insulin and cholecystokinin) cause short-lived increases in cGMP but

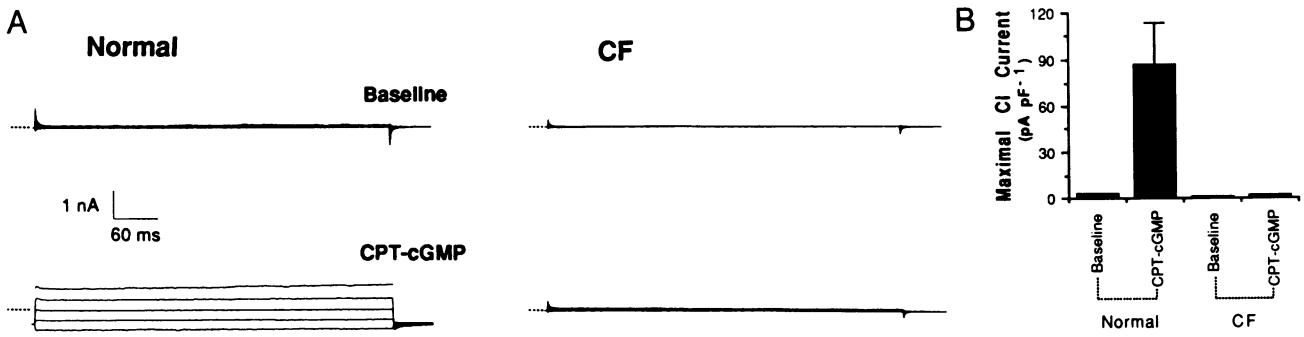

Fig. 6. Effect of CPT-cGMP on whole-cell Cl^- currents in normal and CF-derived airway epithelial cells 56FHTE-8o- and 2CFSMEo-. (**A**) Effect of CPT-cGMP on whole-cell Cl^- current in normal (left) and in CF-derived (right) airway epithelial cells. Shown are whole-cell Cl^- currents typically recorded at baseline (top) and after stimulation (bottom) of CPT-cGMP (0.5 mM). (**B**) Maximal outward Cl^- current induced by CPT-cGMP in normal and CF airway cells (n = 5 each).

do not induce Cl^- secretion (Brasitus *et al.*, 1976). Also, submaximal concentrations of STa that elevate intracellular cGMP levels ~2-fold do not stimulate intestinal Cl^- secretion (Guandalini *et al.*, 1982). Furthermore, the mechanism by which cGMP might exert its action was unknown.

cGMP can exert its actions through a variety of mechanisms, including: (i) activation or inhibition of cGMP-binding phosphodiesterases, which can lead to secondary alterations of cAMP; (ii) inhibition of Na^+/Ca^{2+} exchange, leading to alterations in $[Ca^{2+}]_i$; (iii) activation of cyclic nucleotide-dependent protein kinases, e.g. PKG and PKA; and (iv) direct activation of ion channels (reviewed in Goy, 1991). We and others have found no evidence for changes in intracellular concentrations of Ca^{2+} or cAMP (Field *et al.*, 1978; Giannella and Drake, 1979; Huott *et al.*, 1988), thus effectively ruling out the first two mechanisms. By contrast, both PKG (Lin *et al.*, 1992), a particulate form of which is localized to the apical membrane of intestinal epithelial cells (de Jonge, 1976), and PKA (Forte *et al.*, 1992) have been proposed as the cGMP-dependent mediator of STa action. CFTR is phosphorylated *in vitro* by purified PKG (Berger *et al.*, 1992; Picciotto *et al.*, 1992) at the same serine residues phosphorylated by PKA (Picciotto *et al.*, 1992). Both enzymes theoretically may mediate STa action since STa-induced concentration of cGMP (Huott *et al.*, 1988) exceeds the K_a for both enzymes [K_a^{cGMP} = 1 μM for PKA and 0.02 μM for PKG (Kuo and Greengard, 1970; Takai *et al.*, 1975)]. Our results with specific kinase inhibitors strongly suggest that PKA is the major mediator of cGMP-dependent STa action. Furthermore, we have shown that cGMP stimulates Cl^- currents in NIH-3T3 cells transfected with CFTR. Since PKG is not detected in connective tissue fibroblasts (Walter, 1989), cGMP most likely exerts its effect on PKA in these cells as well.

The conclusion that PKA is the primary cGMP-dependent mediator of STa-induced Cl^- secretion explains several previous experimental observations. STa and VIP effects have been noted not to be additive (Huott *et al.*, 1988), implying that the cGMP- and cAMP-dependent agonists converge on a common downstream mechanism. Also, the STa effect is slower than the VIP response. This may be attributable to the time required to accumulate intracellular cGMP to levels sufficient to activate PKA. Finally, it resolves the previous ambiguity that hormones such as α-adrenergic agonists and insulin induce increases in cell cGMP but do not stimulate Cl^- secretion. Since these agonists only increase cGMP to levels ~30% greater than the baseline level for a relatively short period, they do not generate sufficient amounts to cross-activate PKA.

CFTR is required in STa-stimulated Cl^- secretion

If STa activates PKA via cGMP, it logically follows that CFTR mediates the Cl^- secretion invoked by STa. The evidence that PKA phosphorylates and regulates CFTR in secretory epithelial cells is very strong. Mutations in CFTR result in defective cAMP-regulated Cl^- conductances across apical membranes of secretory epithelial cells lining the ducts of exocrine organs of patients with CF (Riordan *et al.*, 1989; Drumm *et al.*, 1990; Rich *et al.*, 1990; Kartner *et al.*, 1991). cAMP agonists activate CFTR Cl^- channels in heterologous expression systems (Anderson *et al.*, 1991; Bear *et al.*, 1991; Kartner *et al.*, 1991), and the purified catalytic subunit of PKA plus ATP activates CFTR Cl^- channels in excised cell-free membrane patches (Tabcharani *et al.*, 1991). Multiple serines in the R-domain of CFTR are phosphorylated *in vitro* by PKA and *in vivo* when intracellular levels of cAMP are increased (Cheng *et al.*, 1991). Simultaneous mutations in four of these serine residues prevent cAMP-dependent activation of CFTR. Thus, if STa activates PKA in T_{84} cells, CFTR should be phosphorylated and activated.

Our data provide almost irrefutable support for the involvement of CFTR in STa action. We have presented three lines of experimental evidence: (i) STa-enhanced ^{125}I efflux is substantially attenuated in cells depleted of CFTR by pretreatment with antisense oligodeoxynucleotides, but not in cells pretreated with the control (sense or misantisense) oligomers; (ii) CPT-cGMP augments membrane Cl^- current in NIH-3T3 fibroblasts transfected with CFTR, 3T3-CFTR, but has no effect in untransfected NIH-3T3 cells; and (iii) CPT-cGMP is effective in stimulating membrane Cl^- current in transformed airway epithelial cells derived from normal subjects, but not in cells derived from CF patients. Taken together, these results strongly support the conclusion that wild-type CFTR must be expressed for STa (or cGMP) action. In support of this, Baxter and colleagues (1988) have found that intestinal tissue from children with CF did not exhibit an increased short-circuit current, a reflection of net Cl^- secretion, when stimulated by STa.

Our results show that guanylin also stimulates whole-cell Cl^- currents characteristic of CFTR, as would be expected

for a ligand of the STa receptor. The effect of this endogenous ligand is likely to be absent in the disease CF. In general, the intestinal manifestations of CF are meconium ileus in 5–10% of neonates and intestinal obstruction in children and adults, largely due to the accumulation of intestinal concretions that cause mechanical obstruction of the distal small intestine. This is ascribed to defective intestinal salt and water transport with subsequent dehydration of luminal contents. CFTR knock-out mice die shortly after birth from intestinal obstruction, perforation and fatal peritonitis, a syndrome with striking similarity to meconium ileus (Snouwaert *et al.*, 1992). Cytochemical data have localized CFTR to crypt epithelial cells in the small intestine, the cell population responsible for fluid and electrolyte secretion (Crawford *et al.*, 1991; Kartner *et al.*, 1992). It is notable that guanylin also localizes to the crypt cells of the small intestine (de Sauvage *et al.*, 1992b). Defective function of guanylin, as well as cAMP-dependent secretagogues, may contribute to the intestinal manifestations of CF.

Does heterozygote advantage maintain CFTR mutations in the gene pool?

Of the different hypotheses to explain the persistence of the high frequency of CF, the one most consistent with the data is heterozygote advantage (Romeo *et al.*, 1989; Rodman and Zamudio, 1991). Since the pathophysiology of CF is that of defective cAMP-dependent Cl^- secretion across the apical membrane of secretory epithelial cells, it has been proposed that secretory diarrheas, mediated by toxins such as *E.coli* LT and *Vibrio cholerae* toxin, which increase cellular cAMP, meet the criteria for a disease-based cofactor that selects for the CF mutations (Quinton, 1982; Baxter *et al.*, 1988; Bijman *et al.*, 1988; Hansson, 1988; Rodman and Zamudio, 1991). Our results suggest that cGMP-dependent toxins, such as STa, provide further selective pressure to maintain CF mutations in the gene pool. It should be noted, however, that the hypothesis of heterozygote advantage requires testing at the population level (Romeo *et al.*, 1989). This will require the collection of an enormous amount of data, since it has been estimated that an advantage of ~2% would be sufficient to maintain CF gene frequencies at current levels (Knudson *et al.*, 1967).

Materials and methods

Reagents

Synthesis and characterization of STa have been described (de Sauvage *et al.*, 1992a). Proguanylin was expressed in *E.coli* and digested with trypsin to liberate the C-terminal 22 amino acid bioactive fragment (Garcia *et al.*, 1993). CPT-cGMP, Rp-8-Br-cAMPS and Rp-8-Br-cGMPS were purchased from BioLog (Germany). Walsh inhibitor (PKI 5–24) was purchased from Peninsula Laboratories. CaMK [273–304] was a gift from Dr Howard Schulman, Department of Neurobiology, Stanford University.

Cell lines

T_{84} cells were grown as described (Wagner *et al.*, 1992). Transformed normal and CF airway epithelial cells (56FHTE-8o- and 2CFSMEo-) were cultured as described by Cozens *et al.* (1991). NIH-3T3 and 3T3-CFTR fibroblasts were kindly provided by Dr Mulligan's laboratory at the Whitehead Institute; the cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum.

Anion efflux assay

T_{84} cells were plated on 22 × 22 mm square plastic coverslips and used at 80–100% confluency 1–2 weeks after seeding. The efflux solution contained (in mM): 135 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 10 glucose and 10 HEPES (pH 7.4). Cells were first loaded with 20 μCi/ml ^{125}I for ~2 h in a water bath gassed with 100% O_2 at 37°C. Extracellular ^{125}I was eliminated by rapidly rinsing the cells/coverslip three times in efflux solution for a cumulative time of 1 min. The efflux experiment was then carried out by sequentially transferring the cells/coverslip at 1 min intervals through a series of cell culture dishes containing 3 ml efflux solution at room temperature. The cell monolayer/coverslip was retained in the last dish of transfer. ^{125}I effluxed into each dish was counted individually in a gamma radiation counter. Non-efflux counts were determined by counting the cells together with the 3 ml efflux solution in the last cell culture dish. Efflux data is normalized as percent efflux/min (Clancy *et al.*, 1990) and presented as mean ± SE. Sample sizes are presented as N = number of cell monolayers.

Electrophysiology

Whole-cell patch-clamp experiments were performed in single cells grown on glass coverslips 1–2 (for T_{84}, 56FHTE-8o- and 2CFSMEo- cells) or 3–4 (for NIH-3T3 and 3T3-CFTR cells) days following seeding. Cells grown on glass coverslip were placed in a 1 ml acrylic chamber on the stage of a Zeiss IM inverted microscope and bathed in a solution containing (in mM): 170 Tris–Cl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES and 15 glucose (pH 7.4; ~330 mosm), at 25–30°C. The pipette solution contained (in mM): 140 CsCl, 2 $MgCl_2$, 1 EGTA, 2 MgATP, 5 HEPES and 10 glucose (pH 7.35 adjusted with CsOH; ~300 mosm). Reduction of pipette [Cl^-] was achieved by replacement of Cl^- with aspartate$^-$ (internal [Cl^-], 14 mM). The bath solution was made ~30 mosm hypertonic compared with the pipette solution to prevent a volume-induced Cl^- current (Wagner *et al.*, 1991). Micropipettes for whole-cell patch-clamp recordings were made as described by Hamill *et al.* (1981) and had a tip resistance of 2–3 MΩ. Whole-cell voltage-clamp experiments were performed using an Axopatch amplifier (Axon Instruments). Voltage-clamp protocols were run with an aid of a Tecmar 12-bit A/D–D/A converter (Scientific Solution) and an IBM-AT computer. Signals, filtered at 1 kHz, were displayed on a strip-chart recorder and stored on floppy disks. Data were analyzed by means of pClamp, version 5.5 (Axon Instruments). Sample sizes are presented as n = number of whole-cell patches.

Oligodeoxynucleotides

Antisense oligodeoxynucleotides to CFTR mRNA and their corresponding control oligomers were used as described (Wagner *et al.*, 1992). Briefly, the antisense oligomers were a pair of adjoining 18mers that are complementary to nucleotides 1–18 and 19–36 of CFTR mRNA. Corresponding pairs of adjoining 18mers of sense or misantisense oligomers were used as control oligomers. The pair of misantisense oligodeoxynucleotides had the same sequence as the antisense except for the presence of four mismatches in each. T_{84} cells grown on coverslips at 40–70% confluency were pretreated with the desired oligodeoxynucleotides (20 μM) 36–48 h prior to ^{125}I efflux assay (Wagner *et al.*, 1992). The oligomers were replenished every 12 h.

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