Activation of the Cystic Fibrosis Transmembrane Conductance Regulator by cGMP in the Human Colonic Cancer Cell Line, Caco-2

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Intestinal chloride (Cl⁻) secretion can be induced by the heat-stable enterotoxin (STa) from Escherichia coli via generation of cGMP. We investigated the regulatory pathway responsible for cGMP-mediated Cl⁻ secretion in the human colonic carcinoma cell line Caco-2 using whole-cell voltage clamp techniques. Cyclic GMP or cAMP induced a 5-fold increase in Cl⁻ conductance (gCl) in the presence of intracellular ATP and 3-isobutyl-1-methylxanthine. Current activation by cGMP persisted in the presence of the type I cGMP-dependent protein kinase (PKG) inhibitor, KTS823, but was inhibited by the specific peptide inhibitor of the PKA-dependent protein kinase A (PKA), PKI₄₋₆. The stimulatory effects of cGMP and cAMP on gCl were not additive. The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl⁻ channel that is regulated by intracellular ATP and by cAMP-dependent phosphorylation. In order to determine whether CFTR was involved in the cGMP-dependent increase in gCl, we tested the effect of intracellular injected anti-CFTR peptide antibodies previously shown to inhibit CFTR function. Antibodies introduced into individual cells via the patch pipette completely inhibited cGMP-dependent current activation. Cyclic GMP also failed to activate gCl in cystic fibrosis cells. Taken together, these studies demonstrate that activation of the CFTR via PKA-dependent phosphorylation accounts for the cGMP-mediated increase in Cl⁻ secretion in Caco-2 cells.

Guanosine 3',5'-cyclic monophosphate (cGMP) is a ubiquitous intracellular second messenger that is involved in the mediation of secretagogue-induced intestinal Cl⁻ secretion (Forte et al., 1992, 1993; Wong and Garbers, 1992). Guanylin, a 15-amino acid polypeptide recently isolated from the rat small intestine, has a structure similar to the heat-stable enterotoxin (STa) from Escherichia coli. An endogenous activator of the membrane-associated guanylate cyclase C present in the intestine and other epithelial tissue (Currie et al., 1992; Garbers, 1992; Schuls et al., 1989; Vaandragers et al., 1992; Wiegand et al., 1992; Wong and Garbers, 1992), guanylin has been shown to stimulate Cl⁻ secretion in cells from the colonic cell line T84. Both guanylin and STa enhance Cl⁻ secretion via guanylate cyclase C activation leading to an increase in intracellular cGMP (Forte et al., 1992, 1993). It has not been well established as to whether the cyclic nucleotide acts to gate epithelial Cl⁻ channels directly, as has been shown for the cation channel in the retinal rod and cone outer segment (Fesenko et al., 1986; Matesic and Liebman, 1987) or whether activation occurs via a kinase-dependent phosphorylation step.

Increases in intracellular cAMP, intracellular Ca²⁺, or cellular volume activate three separate Cl⁻ conductances (gCl) in the Cl⁻ secretory epithelia of the intestine and upper airways (Chen et al., 1992a, 1992b; Cliff and Frizzell, 1990). The molecular basis for the conductance increase in the case of the cAMP-induced current has been characterized extensively. The absence of a cAMP-inducible Cl⁻ permeability in the apical membranes of Cl⁻ secretory epithelia from patients with cystic fibrosis (CF) is associated with a reduction in fluid secretion. Genetic linkage analysis led to the discovery of the CF gene, and the Cl⁻ permeability defect has been attributed to a single amino acid mutation (ΔFhe-508) in the protein product of the CF gene, the cystic fibrosis transmembrane regulator (CFTR) (Anderson and Welsh, 1991). The expression of CFTR in heterologous expression systems or in cells expressing the mutant gene product is associated with the appearance of a Cl⁻ conductance that is regulated by cAMP (Anderson et al., 1991b; Bear et al., 1992; Dalemans et al., 1991; Drumm et al., 1991; Kartner et al., 1991; Rich et al., 1991). Mutagenesis studies of selected amino acids in the transmembrane domains of the cloned protein demonstrated a concomitant change in the ionic selectivity of the conductance strongly suggesting that CFTR was the cAMP-regulated Cl⁻ channel itself and not an associated regulatory protein (Anderson et al., 1991a). Purification of CFTR to homogeneity and reconstitution into planar bilayers revealed a Cl⁻ channel with properties similar to that observed in intact cells (Bear and Reyes, 1992) arguing strongly that the regulated channel activity is a property of the molecule itself. Regulation of CFTR by phosphorylation may occur via a number of consensus sites for PKA-, protein kinase C (PKC)-, and/or cGMP-dependent protein kinase (PKG)-dependent phosphorylation present within a central cytoplasmic domain of CFTR termed the regulatory (R) domain (Picciotto et al., 1992).

Recent biochemical studies of synthetic peptides derived from portions of the sequence of CFTR within the R-domain containing the most likely consensus sites for PKA-, PKC-, or PKG-dependent phosphorylation confirmed that they were indeed substrates (Picciotto et al., 1992). The work of Picciotto

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and co-workers (Picciotto et al., 1992) established, however, that not all consensus sequence sites were phosphorylated. Thus, while regulation of CFTR is likely to occur through direct phosphorylation of the regulatory domain via protein kinases stimulated by a number of second messenger pathways, it remains of interest to determine which pathways are capable of activating the molecule directly as has been shown for PKA or whether phosphorylation of some sites by PKC potentiates the phosphorylation of CFTR by other kinases, e.g. PKA or PKG. In this regard, PKC-dependent phosphorylation of CFTR has been shown to amplify its activation by PKA (Tabcharani et al., 1991). While phosphorylation of the synthetic peptides derived from the sequence of CFTR yields useful information with respect to the kinetics of the biochemical reaction mechanisms, analysis of the signal transduction pathways in the intact preparation remains the only functional assay in the determination of the interplay between a multiplicity of pathways all potentially capable of generating current activation via kinase stimulation.

While several reports have shown that cGMP is an important mediator of intestinal Cl− secretion induced by various secretagogues, such as E. coli STa and guanylin (Forte et al., 1992), the transport proteins involved in secretory response activated by cGMP to date remain uncertain. The aim of this study was the determination of the channels involved in the secretory response stimulated by cGMP, the determination of whether Gs activation involved a phosphorylation event, and if so, whether the activation mechanism involved synergism between converging kinase regulatory pathways.

EXPERIMENTAL PROCEDURES

Materials—8-(4-Chlorophenylthio)-adenosine 3':5'-cyclic monophosphodiester (CPT-CAMP), 8-bromo-guanosine 3':5'-cyclic monophosphate (8-Br-cGMP), 3-iodo-1-thyrotropin (8-Br-8T), and ATP were obtained from Sigma. PKA-24, a specific peptide inhibitor of PKA, was purchased from Peninsula Laboratories Inc. (Belmont, CA). KT5823, a type 1 PKG inhibitor, was obtained from LC Services Corp. (Woburn, MA). All other chemicals were reagent grade.

Electrophysiological Studies—Caco-2 cells, a human colon carcinoma cell line originally cloned by Zweibaum and co-workers (Pinto et al., 1983), were grown as previously described (Tien et al., 1993). Cells were plated on non-coated 35-mm tissue culture dishes. Electrophysiological studies were conducted on single cells, 1–3 days postplating. Cells dissociated from CF nasal polyops were prepared as described previously (Chan et al., 1992a). The whole-cell current recordings and data acquisition used in these studies were carried out according to previously described methods (Chan et al., 1992a). In brief, whole-cell currents were elicited by applying hyperpolarizing and depolarizing voltage pulses to the voltage clamped cell from a holding potential of −40 mV. Voltage pulses over the range of −110 mV were applied in 10-mV steps using an EPC-7 patch clamp amplifier (List Electronics, Darmstadt, Eberstadt, Germany). The voltage commands were provided via the output of a Metrabyte digital-to-analog converter; currents were sampled with a Data Translation DT2918 analog-to-digital converter. Data were stored and analyzed on an IBM-AT compatible computer. Currents were sampled at 2 kHz and filtered at 1 kHz. Currents were not leak or capacity corrected. Cell capacitance was measured by integrating the current during a 10-mV voltage step and subtracting a baseline established approximately 15 ms after the step. All studies were performed using pipette and bath solutions containing N-methyl-D-glucamine (NMDG) as a Na+ and K+ replacement, and C1− as the major permeant ion. Experiments were performed in asymmetrical Cl− solutions (the calculated Nernst potential for Cl− was −31 mV), thereby allowing the identification of an increase in the leak current as a shift of the current reversal potential in the depolarizing direction. The pipette solution contained (in mM): 40 NMDG-Cl, 100 NMDG-glutamate, 10 HEPES, 2 MgCl2, 5 mM ATP, 5 EGTA, and 0.5 CaCl2 pH 7.2. The bath solution contained (in mM): 140 NMDG-Cl, 10 HEPES, 2 MgCl2, and 2 CaCl2 pH 7.2. Solution osmolarities were monitored using a vapor pressure osmometer (model 5500, Wescor, Logan, UT).

A site-directed antibody against a synthetic peptide, corresponding to amino acids 505–511 of CFTR was produced and purified as described previously (Chan et al., 1992b). In the experiments using the anti-

RESULTS AND DISCUSSION

Phosphorylation of the R-domain of CFTR by PKG has been reported (Picciotto et al., 1992). The functional consequences of CFTR phosphorylation by the kinase have not been fully characterized. Whole-cell voltage clamp experiments were carried out in Caco-2 cells in order to examine Cl− current activation in the presence of cGMP. The magnitude of the whole-cell Cl− current at +110 mV in unstimulated cells was 120 ± 20 pA (n = 25). Cell capacitance and, therefore, cell size were relatively uniform among the cells studied (27 ± 4 picofarads, a = 19). When 1 mM 8-Br-cGMP was added to the pipette solution in the absence of exogenous ATP, no current activation was observed (mean peak current at +110 mV was 187 ± 26 pA, n = 7). The addition of 5 mM ATP and 1 mM IBMX to the intrapipette solution in the presence of 8-Br-cGMP, however, induced current activation that was dependent upon the intrapipette concentration of 8-Br-cGMP (Fig. 1). The increase in membrane current as a function of increasing cGMP concentration (100 µM to 5 mM) was well fitted by a single-site binding curve with an EC50 value of 694 ± 282 µM and a predicted maximal cGMP-stimulated current of 957 ± 143 pA, which was about a 5-fold increase over basal levels (see above). In that the fit to the data could be adequately described by a single-site model, it was not necessary to invoke a more complicated current activation pathway than a single phosphorylation event.

We found that intrapipette application of 8-Br-cGMP gave

![Fig. 1. Concentration-dependent activation of Cl− conductance by 8-Br-cGMP in Caco-2 cells.](image-url)
more consistent and higher levels of current activation as compared with external exposure of the cells to 8-Br-cGMP. Cyclic-GMP was, therefore, applied to the cell via the intrapipette solution for all the experiments described in this study. Fig. 2 illustrates representative current recordings from an unstimulated cell and a cell dialyzed with 1 mM 8-Br-cGMP. The current in the 8-Br-cGMP-stimulated cells was Cl\(^-\) selective. The zero-current potential following 8-Br-cGMP activation was \(-26 \pm 1.3\) mV (n = 7) in agreement with the theoretically predicted value for a perfectly Cl\(^-\) selective conductance. IBMX (1 mM), which was included in the intrapipette solutions in order to prevent breakdown of the cyclic nucleotide via cytoplasmic phosphodiesterase activity, was ineffective in bringing about an increase in current above that observed under basal conditions (basal, 120 \pm 20\,\mu A, n = 25; IBMX, 138 \pm 12\,\mu A, n = 15).

The time course of current activation in the presence of increasing concentrations of 8-Br-cGMP was constant over a wide concentration range and was accelerated only at the highest concentration (5 mM) that was examined in the study. The time required to reach stable maximum current activation for 100 \(\mu\)M, 300 \(\mu\)M, and 1 mM cGMP was 7.5 \pm 3.3 (n = 6), 7.8 \pm 2.5 (n = 6), and 8.9 \pm 2.2 (n = 11) min, respectively. In the presence of 5 mM cGMP, however, the time required to reach maximum current activation was significantly decreased to 2 \pm 1 (n = 4) min.

Cyclic nucleotide specificity was evaluated using CPT-cAMP, a cell-permeant non-hydrolyzable analogue of cAMP, to induce current activation. CPT-cAMP (1 mM), like 8-Br-cGMP, gave rise to a significant increase in current amplitude over that observed in unstimulated cells. The mean peak current produced upon the exposure of cells to CPT-cAMP was 1102 \pm 135, n = 16. Cells dialyzed via the pipette solution with the specific peptide inhibitor of PKA, PKI\(_{16}\) (5 \(\mu\)M), failed to respond with an increase in current to either CPT-cAMP or 8-Br-cGMP in the presence of ATP as shown in Table 1. PKI\(_{16}\) alone had no effect on the magnitude of basal current levels.

In order to examine the additivity of the cyclic nucleotides in inducing anion current stimulation, cells dialyzed with 8-Br-cGMP (1 mM) via the pipette solution were allowed to attain a stable level of current activation. Cell were then exposed to CPT-cAMP (1 mM) via bath solution exchange. In three cells, the amplitude of the peak current following 8-Br-cGMP stimulation was 815 \pm 100\,\mu A, which then increased to 1017 \pm 111\,\mu A. Although there was a small increase in current following exposure of the cells to the second nucleotide, the fact that the response to the two stimuli was not additive suggested that the cyclic nucleotides activate the same conductance. These results are in agreement with the previous findings of Forte and coworkers (Forte et al., 1992) in T84 cells demonstrating that STa-stimulated Cl\(^-\) secretion, as assessed by short-circuit current techniques, was not due to activation of PKG but rather to activation of PKA via cGMP.

Cyclic GMP failed to activate a conductance increase in cultured, dissociated cells derived from CF nasal polyps (basal, 119 \pm 5, n = 4; 8-Br-cGMP, 153 \pm 29, n = 4). CPT-cAMP (1 mM) also failed to activate a \(g_{\text{Cl}}\) in the CF cells (133 \pm 2, n = 2), in agreement with our previous findings (Chan et al., 1992a). The absence of either cGMP- or cAMP-mediated current activation in the CF cells suggested the involvement of CFTR in the secretory response. Exposure of the cyclic nucleotide-stimulated CF cells to a hypotonic solution (~180 milliosmoles) resulted in current activation (mean peak current amplitude in two cells was 1536 \(\mu\)A) indicating that the defect was specific to the cyclic nucleotide response.

To confirm that the cGMP-inducible current was attributable to CFTR, we examined current activation in the presence of a functional antibody introduced into the cell via the patch pipette. The antibody had been previously shown to specifically and significantly inhibit CPT-cAMP-induced \(g_{\text{Cl}}\) in T84 cells (Chan et al., 1992b). A similar antibody-inhibited inhibition of the CPT-cAMP-evoked current was observed in the Caco-2 cells (see Table I) as had been previously reported for T84 cells (Chan et al., 1992b). As observed for CPT-cAMP, 8-Br-cGMP (1 mM) failed to induce an increase in \(g_{\text{Cl}}\) in the presence of anti-CFTR505-511 (Fig. 3), showing the cyclic nucleotide-evoked current was due exclusively to the activation of CFTR. An examination of the volume-regulated \(g_{\text{Cl}}\) in the cells dialyzed with the antibody established the specificity of the current inhibition. In the absence of an increase in current following stimulation of the antibody-dialyzed cell with 8-Br-cGMP, current activation was obtained in the presence of a hypotonic extracellular solution (~180 milliosmoles) (Fig. 3C). The solution was made hypotonic by a decrease in the concentration of NMDG-Cl. Peak current magnitude following the hypotonic stimulus in the cell depicted in Fig. 3B was 1300 \(\mu\)A.

In order to establish whether the activation of CFTR involved PKG-dependent phosphorylation of the channel, activation experiments were carried out in the presence of the kinase inhibitor KT5823. Cytoplasmic PKG occurs in cells in two forms, type I and type II (Butt et al., 1993). Type I PKG is

### Table I

| Condition | Peak current (\(\mu\)A at \(+110\) mV) |
|-----------|----------------------------------|
| Basal (n = 15) | 138 \pm 12 |
| PKI\(_{24}\) (5 \(\mu\)M) (n = 8) | 148 \pm 28 |
| 8-Br-cGMP (1 mM) (n = 11) | 708 \pm 67 |
| CPT-cAMP (1 mM) (n = 16) | 1102 \pm 135 |
| 8-Br-cGMP (1 mM) + CPT-cAMP (1 mM) (n = 5) | 1108 \pm 130 |
| PKI\(_{24}\) + 8-Br-cGMP (n = 4) | 243 \pm 19 |
| PKI\(_{24}\) + CPT-cAMP (n = 4) | 202 \pm 57 |
| Anti-CFTR505-511 (Ab) | 187 \pm 57 |
| Ab + 8-Br-cGMP (n = 3) | 153 \pm 24 |
| Ab + CPT-cAMP (n = 3) | 146 \pm 16 |
In summary, the present studies provide the first direct evidence that the CFTR in Caco-2 cells is regulated in part by PKA and cAMP, in addition to PKG. The activation of CFTR by cGMP, we believe this possibility is unlikely since 1) the PKA inhibitor PK16-24 maximally inhibits 8-Br-cGMP-induced current activation (833 pmol) and 2) the stimulatory effects of cGMP and cAMP were non-additive. Our observations do not rule out the possibility that PKG-dependent phosphorylation of CFTR may potentiate the phosphorylation of CFTR by other kinases such as calcium/calmodulin-dependent kinase II or PKC, both of which have been shown to phosphorylate synthetic peptides derived from the R-domain of CFTR (Picciotto et al., 1992).

In summary, the present studies provide the first direct evidence that the CFTR in Caco-2 cells is regulated in part by cGMP via PKA-dependent phosphorylation. The specific peptide inhibitor of PKA, PKIA-24, and the functional anti-CFTRG6-51 antibody inhibited the 8-Br-cGMP as well as cGMP-induced cGMP-GTP activation (333 pmol), showing that activation was exclusively through CFTR consistent with the observation that cGMP also failed to activate PKC in CF cells. An increase in cGMP could lead to altered secretory function by a multiplicity of pathways including activation of PKG or PKA directly or activation of adenylate cyclase elevating cAMP and thereby stimulating PKA. Huot and co-workers (Huot et al., 1988) have demonstrated that cAMP levels do not increase following stimulation of T84 cells with StA making it unlikely that cGMP leads to an increase in cAMP via activation of adenylate cyclase. The efficacy of PKA for cAMP is approximately 450-fold higher than for cGMP (9.5 versus 4,200 nm (Shabb et al., 1991)) suggesting that direct activation of PKA by cGMP leading to phosphorylation of the target protein CFTR is the most parsimonious mechanism to account for the observations in this study. This mechanism suggests that cAMP and cGMP effector pathways in the intestine converge at the level of PKA leading to the phosphorylation and subsequent activation of CFTR accounting for secretagogue-enhanced salt and water secretion.

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