DCEBIO stimulates Cl\(^-\) secretion in the mouse jejunum

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Hamilton, Kirk L., and Matt Kiessling. DCEBIO stimulates Cl\(^-\) secretion in the mouse jejunum. Am J Physiol Cell Physiol 290: C152-C164, 2006.—We investigated the effects of 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benimidazol-2-one (DCEBIO) on the Cl\(^-\) secretory response of the mouse jejunum using the Ussing short-circuit current (I\(_{sc}\)) technique. DCEBIO stimulated a concentration-dependent, sustained increase in I\(_{sc}\) (EC\(_{50}\) 41 ± 1 μM). Pretreating tissues with 0.25 μM forskolin reduced the concentration-dependent increase in I\(_{sc}\) by DCEBIO and increased the EC\(_{50}\) (53 ± 5 μM). Bumetanide blocked (82 ± 5%) the DCEBIO-stimulated I\(_{sc}\) consistent with Cl\(^-\) secretion. DCEBIO was a more potent stimulator of Cl\(^-\) secretion than its parent molecule, 1-ethyl-2-benzimidazolinone. Glibenclamide or NPPB reduced the DCEBIO-stimulated I\(_{sc}\) by >80% indicating the participation of CFTR in the DCEBIO-stimulated I\(_{sc}\) response. Clostrimazole reduced DCEBIO-stimulated I\(_{sc}\) by 67 ± 15%, suggesting the participation of the intermediate conductance Ca\(^{2+}\)-activated K\(^+\) channel (IKCa) in the DCEBIO-activated I\(_{sc}\) response. In the presence of maximum forskolin (10 μM), the DCEBIO response was reduced and biphasic, reaching a peak response of the change in I\(_{sc}\) of 43 ± 5 μA/cm\(^2\) and then falling to a steady-state response of 17 ± 10 μA/cm\(^2\) compared with DCEBIO control tissues (61 ± 6 μA/cm\(^2\)). The forskolin-stimulated I\(_{sc}\) in the presence of DCEBIO was reduced compared with forskolin control tissues. Similar results were observed with DCEBIO and 8-BrCAMP where adenylyl cyclase was bypassed. H89, a PKA inhibitor, reduced the DCEBIO-activated I\(_{sc}\), providing evidence that DCEBIO increased Cl\(^-\) secretion via a cAMP/PKA-dependent manner. These data suggest that DCEBIO stimulates Cl\(^-\) secretion of the mouse jejunal mucosa and that DCEBIO targets components of the Cl\(^-\) secretory mechanism.

1-ethyl-2-benzimidazolinone; forskolin; glibenclamide; clostrimazole; H89

THE JEJUNUM is the primary site of fluid secretion of the small intestine (7). In the mouse jejunum, as in other Cl\(^-\)-secreting tissues, electrogenic Cl\(^-\) secretion is the predominant transport mechanism responsible for fluid secretion and occurs within the crypt (71), whereas absorption is a property of the villi. Cl\(^-\) secretion occurs through the interplay of four membrane transporter proteins (Cl\(^-\) channel, Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, K\(^+\) channel, and the Na\(^+\)-K\(^+\)-ATPase). Cl\(^-\) enters the cell via the bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter. A sustained Cl\(^-\) secretory response requires active Cl\(^-\) channels, as well as activated basolateral K\(^+\) channels, which play a crucial role in hyperpolarizing the membrane potential of the cell, thus maintaining a driving force for Cl\(^-\) exit across the apical membrane via Cl\(^-\) channels (68). K\(^+\) channels also aid in recycling K\(^+\) across the basolateral membrane (68). The Na\(^+\)-K\(^+\)-ATPase maintains the Na\(^+\) and K\(^+\) concentration gradients across the membrane. Thus Cl\(^-\) secretion results in Cl\(^-\) movement across the epithelium; Na\(^+\) and water follow via the paracellular pathway, resulting in a hydrated mucosal surface of the epithelium. Variations in the activity of any of these transport proteins can alter the rate of Cl\(^-\) secretion, and thus fluid secretion, which plays a vital role in several diseases, such as cystic fibrosis (CF) (50, 70), distal intestinal obstruction syndrome (DIOS) (17, 51), chronic obstruction pulmonary disease (42, 49), and diarrhea (19).

In particular, CF affects various epithelia of the body by reducing Cl\(^-\) secretion. The CF gene codes for the CF transmembrane conductance regulator (CFTR), which functions as a cAMP-dependent Cl\(^-\) channel (53, 54). Mutations in the CF gene lead to defects in CFTR, resulting in reduced Cl\(^-\) and fluid secretion manifested as dehydration of the respiratory and intestinal epithelia. Intestinal complications of CF include meconium ileus (impacted ileum), malnutrition, and DIOS. Indeed, 10–15% of infants with CF suffer from meconium ileus (48) and have a higher risk of malnutrition (37) or DIOS (51) in later life. In addition, children with CF have severely reduced Cl\(^-\) transport, as determined by jejunal biopsies (45, 64). Pharmacological modulation of ion transport has been suggested as a possible therapeutic treatment of intestinal complications of CF (36, 55).

The benimidazolones are a class of compounds that were first characterized as modulators of K\(^+\) channels. Indeed, Olesen and colleagues (46, 47) first described the effects of 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benimidazol-2-one (NS004) and 1-(2'-hydroxy-5'-trifluoromethylphenyl)-1,3-dihydro-2H-benimidazol-2-one (NS1619) on the activity of large-conductance K\(^+\) channels of bovine smooth muscle cells and mouse cerebellar granule cells. However, Gribkoff et al. (24) demonstrated that NS004 activated both wild-type CFTR and F508-CFTR expressed in Xenopus oocytes. Al-Nakkash et al. (1) likewise reported that NS004 activated phosphorylated F508-CFTR expressed in NIH3T3 mouse fibroblast cells. In addition, Becq and colleagues (12) have reported that NS04 activates phosphorylated (via forskolin) wild-type CFTR and G551D-CFTR expressed in Chinese hamster ovary cells. Recently, a benzimidazolone has been implicated in the modulation of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (63). Therefore, benzimidazolones can modulate several transport proteins involved in the Cl\(^-\) secretory response.

1-Ethyl-2-benzimidazolone (1-BEBO), another benzimidazolone, has been demonstrated to stimulate Cl\(^-\) secretion in several epithelia, including the T84 colonic cell line (14), mouse jejunum (28) and colon (11, 38), rat colon (14, 69), and Calu-3 human airway cells (16), for example. 1-BEBO is a...
known activator of K⁺ channels (34), in particular the intermediate-conductance Ca²⁺-dependent K⁺ channel (IKCa, KCNN4) (14, 28, 33, 69). 1-EBIO has been reported to stimulate intracellular levels of cAMP in native tissue (11, 39); however, others do not concur (67). 1-EBIO increases the activity of CFTR in cultured cells (13, 16) and native epithelia (10, 38).

Recently, 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benimidazol-2-one (DCEBIO), a derivative of 1-EBIO, has been demonstrated to be an extremely potent activator of Cl⁻ secretion in T84 colonic cells (61). However, the effects of DCEBIO are yet to be determined in native epithelia. The aims of this study were twofold: first, to examine the effect of DCEBIO on the short-circuit current (Isc) response of a native epithelium, the mouse jejunum; and second, to determine the mechanism of action of DCEBIO on the Cl⁻ secretory response of the mouse jejunum.

MATERIALS AND METHODS

Animals and tissue preparation. All experiments were performed on the jejunum of male Swiss-Webster mice (20–35 g) and were approved by the University of Otago Animal Ethics Committee. The animals had access to tap water and food ad libitum until the time of the experiment. The mice were euthanized by cervical dislocation, and the intact isolated jejunum was placed in a NaCl Ringer solution composed of (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES/Tris, and 4 pyruvate/glutamine, with pH adjusted to 7.4. HEPES-buffered Ringer solution was used in these experiments to maintain at 37°C by water-jacketed solution reservoirs. Tissues were voltage clamped to 0 mV (Biodesign, South Campus Electronics, University of Otago), and Isc was continuously recorded with a MacLab data-acquisition system (ADInstruments, Castle Hill, Victoria, Australia) was used for curve fitting and determining the EC50, Vmax, and Hill coefficients values of the concentration-dependent response experiments. Data are presented as means ± SE. In some figures, the SE bar is within the symbol. A P value of ≤0.05 was considered significant. The number of tissues for a given protocol and the number of animals used in a series of experiments are provided in the text. Sometimes more than one tissue from an animal was used for a particular protocol within a given series of experiments.

RESULTS

DCEBIO-activated Cl⁻ secretion of the mouse jejunum. Initially, we determined whether DCEBIO could activate an Isc response of the mouse jejunum. DCEBIO (100 μM, serosal) stimulated a sustained increase in Isc (ΔIsc of 74 ± 8 μA/cm², n = 8, N = 6) (Fig. 1A, A and D) compared with control tissues (ΔIsc of −5 ± 10 μA/cm², n = 5) (Fig. 1C and D). DCEBIO was also effective when added from the mucosal side of the tissue. Mucosal addition of DCEBIO (100 μM) (Fig. 1B) activated Isc by 41 ± 1 μA/cm² (n = 3, N = 3) (Fig. 1E), which was greater (P < 0.01) than paired control tissues (−5 ± 5 μA/cm², n = 3).

DCEBIO (1–125 μM, serosal) stimulated a concentration-dependent increase in Isc as shown in Fig. 2A. This relationship exhibited a Vmax of 102 ± 2 μA/cm², an EC50 of 41 ± 1 μM, and Hill coefficient of 1.9 ± 0.1 (Fig. 2, A and C, circles) (n = 6–8 per data point, N = 4). However, 1-EBIO, the parent molecule of DCEBIO (61), was less potent than DCEBIO. 1-EBIO, too, increased Isc in a concentration-dependent manner with a Vmax of 86 ± 8 μA/cm², an EC50 of 86 ± 150 μM and Hill coefficient of 1.6 ± 0.3 (n = 8–11 per data point, N = 7, data not shown) as has been demonstrated by us and others (14, 28).

We used bumetanide, a known blocker of the basolateral Na⁺/K⁺/2Cl⁻ cotransporter the entry step for Cl⁻ in the Cl⁻ secretory response, to establish that the DCEBIO-stimulated Isc was, indeed, Cl⁻ secretion. Bumetanide (20 μM, serosal) caused a rapid reduction of the DCEBIO (100 μM, serosal)-stimulated Isc (ΔIsc of −59 ± 5 μA/cm², n = 8, N = 6) (Fig. 1A, A and D) compared with paired control tissues (ΔIsc of −5 ± 4 μA/cm², n = 7) (Fig. 1D), indicative of Cl⁻ secretion. Bumetanide (20 μM) inhibited 82 ± 5% of the DCEBIO-stimulated Isc. Bumetanide (0.01–50 μM, serosal) exhibited a concentration-dependent inhibition of the DCEBIO (100 μM, serosal)-activated Isc with a Ki of 0.9 ± 0.1 μM (n = 5–10 per data point, N = 8, data not shown). A maximum reduction of DCEBIO-activated Isc was achieved at 10 μM bumetanide. Bumetanide (20 μM, serosal) reduced the Isc (K1) by 55% and the Isc (K2) by 85 ± 16% (n = 3, N = 3) (Fig. 1, B and E).
These data suggest that DCEBIO stimulates a concentration-dependent Cl⁻ secretory response of the mouse jejunum. In addition, DCEBIO is a more effective activator of Cl⁻ secretion than its parent compound, 1-EBIO.

Do CFTR and IKCa participate in the DCEBIO-stimulated Isc response? As shown above, DCEBIO activated a sustained increase in Isc, suggesting that more than one transport protein is involved in the Cl⁻ secretory response. At least an apical Cl⁻ channel and a basolateral IKCa must participate in the DCEBIO-stimulated Isc (52) along with the Na⁺-K⁺-2Cl⁻ cotransporter to maintain a sustained Cl⁻ secretory response.

CFTR is a key Cl⁻ channel involved in Cl⁻ secretory response of many epithelial tissues (19, 52) and is present in the mouse jejunum (2). Therefore, we tested for the participation of CFTR in the DCEBIO-stimulated Isc response with glibenclamide and NPPB, known blockers of CFTR (10, 58, 60, 73). We (3) recently demonstrated that mucosally applied glibenclamide reduced the forskolin-activated Isc of the mouse jejunum by 60%. Similarly, we (27) reported that mucosal rather than serosal applied glibenclamide reduced methoxalen-stimulated Cl⁻ secretion of the mouse jejunum, suggesting that glibenclamide does not have access to the opposite side of the epithelium. Seven animals were used in this series of experiments. For each animal, two tissues were pretreated (30 min) with glibenclamide (100 μM, mucosal), whereas a third tissue was treated with DMSO only. After that, DCEBIO (100 μM, serosal) was added to one of the glibenclamide-treated tissues and to the DMSO tissue. In control tissues, DCEBIO activated Isc by 63 ± 11 μA/cm² (Fig. 3, A and D) (n = 6); however, DCEBIO increased Isc by only 12 ± 8 μA/cm² in the presence of glibenclamide (Fig. 3, B and D) (n = 7) in paired experimental tissues. Therefore, pretreatment of tissues with glibenclamide reduced the action of DCEBIO by 81%. In addition, glibenclamide (100 μM, mucosal) added in the presence of DCEBIO (100 μM, serosal) reduced the DCEBIO-stimulated Isc by 77 ± 10% compared with paired DCEBIO control tissues (n = 5, N = 5, data not shown).

In similar experiments, pretreatment of tissues with NPPB (50 or 100 μM, mucosal), another inhibitor of CFTR (10, 73), greatly reduced DCEBIO-stimulated Isc compared with DCEBIO control tissues (n = 4, N = 4, data not shown).

These results demonstrate that CFTR participates in the DCEBIO-stimulatory Cl⁻ secretory response of the mouse jejunum.

One of the predominant K⁺ channels present in the basolateral membrane of mouse crypt cells appears to be KCNn4, the IKCa channel (6, 28). As far as we are aware, the molecular identity of IKCa in the mouse jejunum has yet to be reported (D. Vandorpe, unpublished observation). However, IKCa has
been demonstrated in mouse stomach, proximal colon, and distal colon (66). We (6, 28) have functionally (patch clamp) demonstrated that 1-EBIO activates IKCa in the mouse jejunum, and others (11, 14) have reported similar findings in native epithelia and cultured cells. On the basis of excised patch experiments, Bridges and co-workers (61) demonstrated that DCEBIO directly activates IKCa expressed in human embryonic kidney (HEK)-293 cells. We (62) have confirmed those findings. So, it was of great interest to determine whether IKCa participated in the DCEBIO-stimulated Isc.

Experimental tissues were pretreated (30 min) with clotrimazole, whereas control tissues were pretreated with DMSO. Pretreatment with clotrimazole significantly reduced the activation of Isc by DCEBIO (ΔIsc of 13 ± 9 μA/cm², n = 6) (Fig. 2). Bumetanide (20 μM, serosal) was added to all tissues at the end of the experiment. C: summary of concentration-dependent change in ΔIsc with DCEBIO or DCEBIO in the presence of forskolin. Data for DCEBIO concentration-dependent experiments are Vmax of 102 ± 2 μA/cm², an EC50 of 41 ± 1 μM and Hill coefficient of 1.9 ± 0.1 (n = 6–8 per data point, N = 4 animals). Data for DCEBIO concentration-dependent experiments in the presence of forskolin are Vmax of 58 ± 4 μA/cm², an EC50 of 53 ± 5 μM and Hill coefficient of 2.0 ± 0.2 (n = 6 per data point, N = 6 animals). Values are means ± SE.

Experimental tissues were pretreated (30 min) with clotrimazole, whereas control tissues were pretreated with DMSO. Pretreatment with clotrimazole significantly reduced the activation of Isc by DCEBIO (ΔIsc of 13 ± 9 μA/cm², n = 6) (Fig. 3). Bumetanide (20 μM, serosal) was added to all tissues at the end of an experiment. D: mean ΔIsc in response to DCEBIO in the absence and presence of clotrimazole. Values are means ± SE; n = 6 tissues each, N = 7 animals. **P < 0.01.
Fig. 4. Effects of pretreatment (30 min) of clotrimazole (CLOT; 3 μM, serosal) on the DCEBIO (100 μM, serosal) stimulated I_{sc} of the mouse jejunum. A: representative trace of the effect of DCEBIO on the I_{sc} of a control tissue. B: representative trace of the effect of DCEBIO on I_{sc} in the presence of clotrimazole. C: representative trace of an overall control tissue. Bumetanide (20 μM, serosal) was added to all tissues at the end of the experiment. D: mean ΔI_{sc} in response to DCEBIO in the absence and presence of clotrimazole. Values are means ± SE; n = 6–8 tissues each, N = 7 animals. **P < 0.01.

4, B and D) compared with paired DCEBIO control tissues (ΔI_{sc} of 55 ± 6 μA/cm², n = 8) (Fig. 4, A and D). Clotrimazole reduced the DCEBIO-stimulated I_{sc} by 67 ± 15%. These data suggest that the Ca^{2+}-activated K⁺ channel participates in the DCEBIO-activated I_{sc} response.

Do DCEBIO and forskolin share a common pathway in activating Cl⁻ secretion? Cuthbert and colleagues (11, 39) have demonstrated that 1-EBIO (600 μM) elevates intracellular cAMP levels of isolated mouse colonic crypts. However, Wallace et al. (67) did not demonstrate an effect of 1-EBIO (600 μM) on intracellular cAMP levels of T84 colonic cells. We have demonstrated that DCEBIO-stimulated Cl⁻ secretion is reduced by glibenclamide (Fig. 3) and NPPB, indicative of the participation of CFTR. Bridges and colleagues (61) reported that DCEBIO (60 μM) activates Cl⁻ currents (via CFTR) in basolaterally nystatin permeabilized T84 monolayers, which suggests that CFTR is activated by DCEBIO. Because CFTR participated in the DCEBIO response in the present study, then in the presence of a maximal concentration of forskolin, an activator of CFTR via adenylyl cyclase, and thus cAMP/PKA, we predict that the DCEBIO-stimulated I_{sc} should be reduced. We used six animals to test this hypothesis. Tissues were either pretreated with maximal forskolin (10 μM, mucosal and serosal; unpublished data), followed by the addition of DCEBIO (100 μM, serosal) or treated with DMSO before DCEBIO. As noted above, DCEBIO stimulated a sustained increase in I_{sc} (61 ± 6 μA/cm², n = 6) in the absence of forskolin as shown in Fig. 5, B and D. Pretreatment of tissues with forskolin increased I_{sc} by 82 ± 13 μA/cm² (Fig. 5C) (n = 6). However, in the presence of a maximal forskolin-stimulated I_{sc} response, the DCEBIO-activated I_{sc} response was biphasic and reduced, reaching a peak response of ΔI_{sc} of 43 ± 5 μA/cm² and then falling to a sustained response of 17 ± 10 μA/cm² above the I_{sc} stimulated by forskolin (Fig. 5, C and D) (n = 6). These experiments are summarized in Fig. 5D. These data suggest that DCEBIO and forskolin may share a common pathway (CFTR).

On the basis of these data, if CFTR participates in the DCEBIO-activated I_{sc} response, then we predict that pretreating tissues with a maximum concentration of DCEBIO should reduce the forskolin-stimulated I_{sc} response of the mouse jejunum. Five animals were used to test this hypothesis. Tissues were subjected to DCEBIO (100 μM, serosal) followed by forskolin (10 μM, mucosal and serosal) or to DMSO followed by forskolin. Pretreatment of tissues with DCEBIO increased the I_{sc} by 35 ± 8 μA/cm² (n = 5) (Fig. 6C). However, in the presence of DCEBIO, the forskolin-stimulated I_{sc} was significantly reduced by 63% (ΔI_{sc} of 31 ± 7 μA/cm², n = 5) (Fig. 6, B and C) compared (P < 0.01) with paired forskolin control tissues (ΔI_{sc} by 84 ± 11 μA/cm², n = 5) (Fig. 6, A and C). DCEBIO stimulated I_{sc} by 45 ± 9 μA/cm² (n = 5) in control tissues.

Again, these results demonstrate that DCEBIO and forskolin increase the I_{sc} of the mouse jejunum via a similar pathway and provide further evidence that CFTR participates in the DCEBIO-stimulated increase in I_{sc}.

It was surprising that DCEBIO and forskolin did not have an additive effect on the stimulated I_{sc} of the mouse jejunum. However, forskolin (10 μM) has been shown to increase intracellular Ca^{2+} levels in epithelial tissues such as mouse tracheal and nasal epithelia (26, 38). However, Greger and
colleagues (4, 23) demonstrated in the rat colon that forskolin (5 μM) led to a reduction in intracellular Ca^{2+} and a suspected decreased activity of IK_{Ca}. In addition, Cuthbert and colleagues (40) demonstrated little effect of forskolin (10 μM) on intracellular Ca^{2+} of cultured human colonic cells (colony 29). Nonetheless, it is possible that forskolin modulates IK_{Ca}, as well as CFTR (via PKA), and thus I_{sc}. To test this hypothesis, we examined the effects of clotrimazole on forskolin-stimulated I_{sc}. We selected a higher concentration of clotrimazole (20 μM, serosal) in these experiments because Devor et al. (15) reported that a higher concentration of clotrimazole was required for cAMP-dependent compared with Ca^{2+}-dependent agonists of Cl^- secretion. Five animals were used in this series of experiments. Two tissues were pretreated with clotrimazole (for 30 min), followed by the addition of forskolin (10 μM, mucosal and serosal), whereas a control tissue was pretreated with DMSO followed by forskolin. As shown in Fig. 7, even in the presence of 20 μM clotrimazole, forskolin still activated I_{sc} by 48 ± 5 μA/cm^2 (n = 5) (Fig. 7, B and C), which was reduced compared (P < 0.01) with forskolin control tissues (ΔI_{sc} of 83 ± 9 μA/cm^2, n = 10) (Fig. 7, A and C). Clotrimazole reduced the forskolin-activated I_{sc} by 48 ± 5%.

Fig. 5. Effects of DCEBIO (100 μM, serosal) in the absence and presence of forskolin (10 μM, mucosal and serosal) on the I_{sc} of the mouse jejunum. A: representative trace of effect of forskolin on the I_{sc} of a control tissue. B: representative trace of the effect of DCEBIO on a control tissue. C: representative trace of the effect of DCEBIO on I_{sc} in the presence of forskolin. Bumetanide (20 μM, serosal) was added to all tissues at the end of the experiment. D: mean ΔI_{sc} for DCEBIO control tissues and DCEBIO peak I_{sc} (DCEBIO-Peak) and DCEBIO at steady-state I_{sc} (DCEBIO-SS) in the presence of forskolin. Values are means ± SE; n = 6 tissues each, N = 6 animals. *P < 0.05, **P < 0.01.

Fig. 6. Effects of forskolin (10 μM, mucosal and serosal) in the absence and presence of DCEBIO (100 μM, serosal) on the I_{sc} of the mouse jejunum. A: representative trace of forskolin control tissue. B: representative trace of the effect of forskolin on I_{sc} of a control tissue. C: representative trace of the effect of DCEBIO on I_{sc} in the presence of forskolin. Bumetanide (20 μM, serosal) was added to all tissues at the end of the experiment. C: mean ΔI_{sc} in response to forskolin in the absence and presence of DCEBIO. The effect of pretreatment of DCEBIO on the I_{sc} before forskolin is also given. Values are means ± SE; n = 5 tissues each, N = 6 animals. *P < 0.05, **P < 0.01.
These data suggest that IK_{Ca} is modulated by forskolin in the forskolin-stimulated I_{sc} response.

**Does DCEBIO stimulate Cl^- secretion via a cAMP-dependent mechanism?** The data presented suggest that DCEBIO and forskolin use a similar pathway(s) to stimulate Cl^- secretion. Therefore, if DCEBIO, in fact, is working via cAMP (PKA) to activate CFTR and also stimulating IK_{Ca}, resulting in a sustained I_{sc} response, then if we prephosphorylated CFTR with a very low concentration of forskolin (0.25 μM), we predict that the DCEBIO concentration-dependent increase of I_{sc} would be reduced (V_{max}) and the EC_{50} would shift to a higher concentration of DCEBIO. To test this hypothesis, we used six animals. As shown in Fig. 2B, pretreatment of tissues with 0.25 μM (mucosal and serosal) forskolin increased ΔI_{sc} by 18 ± 7 μA/cm² (n = 6), after which DCEBIO (1–125 μM, serosal) increased I_{sc} in a concentration-dependent manner (n = 6 per data point) (Fig. 2, B and C, filled triangles). However, as predicted, the EC_{50} for the DCEBIO concentration-dependent response in the presence of 0.25 μM forskolin, was shifted from 41 ± 1 μM (Fig. 2C, filled circles) (DCEBIO alone) to 53 ± 5 μM (Fig. 2C, filled triangles, DCEBIO with forskolin), whereas V_{max} was reduced from 102 ± 2 μA/cm² (Fig. 2C, filled circles) to 58 ± 4 μA/cm² (Fig. 2C, filled triangles) (n = 6, N = 6) (Fig. 2C).

These data suggest that prephosphorylating CFTR with a submaximal concentration of forskolin reduces the effect of DCEBIO on I_{sc}. Again, one could interpret these data to suggest that forskolin, via increasing intracellular Ca^{2+}, has increased the activity of IK_{Ca} before the addition of DCEBIO, thus reducing the potential action of DCEBIO. However, we believe this is not the case for the DCEBIO concentration-dependent experiments that were conducted with a very low concentration of forskolin. Nonetheless, to further examine the hypothesis that the action of DCEBIO involved cAMP, as well as activating I_{sc} via IK_{Ca}, we examined the effects of DCEBIO on I_{sc} in the presence and absence of 8-BrcAMP, thus passing the level of adenyl cyclase. 8-BrcAMP (1–300 μM, mucosal and serosal) stimulated a concentration-dependent increase in I_{sc} with a V_{max} of 78 ± 2 μA/cm² and an EC_{50} of 58 ± 2 μM (n = 4–7 per data point, N = 4, data not shown). The experimental protocols were similar to those used in the DCEBIO and forskolin experiments described above (Figs. 5 and 6). On the basis of the forskolin-DCEBIO experiments (see Fig. 5), we predicted that pretreatment of tissues with a maximum concentration of 8-BrcAMP (300 μM, mucosal and serosal) would reduce the peak and steady-state I_{sc} response of DCEBIO. In this series of experiments, three animals were used to test this hypothesis. One tissue was pretreated with 8-BrcAMP for 30 min, followed by the addition of DCEBIO (100 μM, serosal), whereas a paired tissue was treated with DMSO before DCEBIO. DCEBIO stimulated a sustained increase in I_{sc} (55 ± 17 μA/cm²; n = 3) in the absence of 8-BrcAMP as shown in Fig. 8, A and C. However, in the presence of a maximal 8-BrcAMP-stimulated I_{sc} response (ΔI_{sc} of 74 ± 3 μA/cm², n = 3), the DCEBIO response was once again biphasic with a peak response of ΔI_{sc} of 44 ± 2 μA/cm² and then falling to a sustained response of 8 ± 6 μA/cm² above the I_{sc} stimulated by 8-BrcAMP (Figs. 8, B and C) (n = 3).

Working from these data, if DCEBIO increased intracellular cAMP, then we predicted that pretreating tissues with a maximum concentration of DCEBIO (100 μM, serosal) would reduce the 8-BrcAMP-stimulated I_{sc} response. We used four animals to test this hypothesis. These experiments were conducted with similar protocols as described for the DCEBIO-forskolin experiments (see Fig. 6). Tissues were subjected to DCEBIO, followed by 8-BrcAMP (300 μM, mucosal and serosal) or DMSO, followed by 8-BrcAMP. Pretreatment of tissues with DCEBIO increased I_{sc} by 61 ± 6 μA/cm² (Fig. 9B) (n = 4). However, in the presence of DCEBIO, the 8-BrcAMP-stimulated I_{sc} was significantly reduced by 76%
Our data suggest that DCEBIO may activate \( I_{sc} \) via a cAMP/PKA-dependent mechanism. If this were the case, then we predicted that an inhibitor of PKA would reduce the DCEBIO-activated \( I_{sc} \). H89 is a relatively specific inhibitor of PKA (29) and has been used (10–50 \( \mu \)M) to demonstrate the PKA dependence of several transport proteins of native tissues (18, 41, 65). Therefore, we used H89 (50 \( \mu \)M, serosal) to test the hypothesis that DCEBIO activates \( I_{sc} \) via a cAMP/PKA-dependent manner. Five animals were used in this series of experiments. In addition, with paired tissues from the same animals, we also examined the effect of H89 on forskolin-stimulated \( I_{sc} \). Tissues were pretreated with either H89 (50 \( \mu \)M, serosal) or \( H_2O \) for 30 min before the addition of either DCEBIO (100 \( \mu \)M, serosal) or forskolin (10 \( \mu \)M, mucosal and serosal). H89 did not alter the basal \( I_{sc} \) (Fig. 10). Indeed, H89 reduced the DCEBIO-activated \( I_{sc} \) (\( \Delta I_{sc} \) of 37 \pm 3 \( \mu \)A/cm\(^2\), \( n = 5 \)) (Fig. 10, B and C) compared with (\( P < 0.01 \)) paired tissues.

(\( \Delta I_{sc} \) of 16 \pm 6 \( \mu \)A/cm\(^2\), \( n = 4 \)) (Fig. 9, B and C) compared (\( P < 0.05 \)) with paired 8-Br-cAMP control tissues (\( \Delta I_{sc} \) of 68 \pm 17 \( \mu \)A/cm\(^2\), \( n = 4 \)) (Fig. 9, A and C).

Our data suggest that DCEBIO may activate \( I_{sc} \) via a cAMP/PKA-dependent mechanism. If this were the case, then we predicted that an inhibitor of PKA would reduce the DCEBIO-activated \( I_{sc} \). H89 is a relatively specific inhibitor of PKA (29) and has been used (10–50 \( \mu \)M) to demonstrate the PKA dependence of several transport proteins of native tissues (18, 41, 65). Therefore, we used H89 (50 \( \mu \)M, serosal) to test the hypothesis that DCEBIO activates \( I_{sc} \) via a cAMP/PKA-dependent manner. Five animals were used in this series of experiments. In addition, with paired tissues from the same animals, we also examined the effect of H89 on forskolin-stimulated \( I_{sc} \). Tissues were pretreated with either H89 (50 \( \mu \)M, serosal) or \( H_2O \) for 30 min before the addition of either DCEBIO (100 \( \mu \)M, serosal) or forskolin (10 \( \mu \)M, mucosal and serosal). H89 did not alter the basal \( I_{sc} \) (Fig. 10). Indeed, H89 reduced the DCEBIO-activated \( I_{sc} \) (\( \Delta I_{sc} \) of 37 \pm 3 \( \mu \)A/cm\(^2\), \( n = 5 \)) (Fig. 10, B and C) compared with (\( P < 0.01 \)) paired tissues.
DCEBIO control tissues ($\Delta I_{sc}$ of 58 ± 4 μA/cm², n = 5) (Fig. 10, A and C). In a similar fashion, the presence of H89 reduced the forskolin-stimulated $I_{sc}$ ($\Delta I_{sc}$ of 36 ± 10 μA/cm², n = 5) (Fig. 10, E and F) compared ($P < 0.01$) with paired forskolin control tissues ($\Delta I_{sc}$ of 102 ± 16 μA/cm², n = 5) (Fig. 10, D and F).

These data strongly suggest that DCEBIO activates $I_{sc}$ (Cl⁻ secretion) via a cAMP/PKA-dependent mechanism, as well as the participation of IKCa.

**DISCUSSION**

In this report, we provide the first examination of the effects of the benzimidazolone DCEBIO on the Cl⁻ secretory response of any native epithelium, the mouse jejunum. In addition to the involvement of IKCa, we have demonstrated for the first time that the mechanism of action of DCEBIO includes the cAMP/PKA pathway. DCEBIO stimulated a concentration-dependent, sustained increase in $I_{sc}$ of the mouse jejunum (Figs. 1 and 2). We (27, 28) and others (8, 9, 25) have demonstrated electrogenic Cl⁻ secretion of the mouse jejunum. Therefore, we used bumetanide to confirm that DCEBIO stimulated $I_{sc}$ consistent with Cl⁻ secretion. Indeed, bumetanide reduced the DCEBIO-activated $I_{sc}$ by >80% (Fig. 1), without regard to which side of the tissue DCEBIO was added. In the present study, the IC₅₀ for bumetanide inhibition for DCEBIO-activated Cl⁻ secretion was 0.9 μM, which is very similar to bumetanide inhibition of $I_{sc}$ of the flounder intestine (0.7 μM) (43, 44), T84 cells (2 μM) (22), and mouse jejunum (0.7 μM) (27), for example.

The bumetanide-insensitive, DCEBIO-stimulated $I_{sc}$ may well be HCO₃⁻ secretion (25, 59); however, the present experiments were conducted in HEPES-buffered Ringer solution. Thus, if HCO₃⁻ secretion occurred, the CO₂ source could have been from metabolically generated CO₂.

**DCEBIO: modulation of components of Cl⁻ secretion.** The sustained Cl⁻ secretory response exhibited by DCEBIO suggests that multiple transport proteins of the Cl⁻ secretory pathway are involved in the DCEBIO response. Indeed, Bridges and colleagues (61) reported that DCEBIO activated CFTR in nystatin-permeablized basolateral membranes of T84 monolayers, similarly to NS004 (13). They also reported that DCEBIO stimulated the activity of IKCa expressed in HEK-293 cells (61), which we have confirmed (62).

Several lines of evidence suggest that CFTR and IKCa participate in the DCEBIO response of the mouse jejunum. First, CFTR is expressed in the mouse intestine and in the jejunum in particular (2). Second, a maximum phosphorylation of CFTR via adenylyl cyclase (with forskolin) greatly reduced the action of DCEBIO (Fig. 5). In fact, DCEBIO in the presence of forskolin resulted in a biphasic increase of $I_{sc}$, which had a reduced peak response compared with DCEBIO alone, and a steady-state $I_{sc}$ that remained above the increased $I_{sc}$ level activated by forskolin. The hypothesis for this experiment was twofold: 1) if DCEBIO alters cAMP levels, as some have described for 1-EBIO (11, 39), then maximal stimulation of $I_{sc}$ by DCEBIO, in the presence of forskolin, should be reduced compared with DCEBIO alone; and 2) in the presence of a maximal cAMP signal (via forskolin), DCEBIO could
azole inhibits DCEBIO-stimulated IKCa channel activity with a demonstrated, using excised patch-clamp experiments, that clotrimazole greatly reduced the secretory response of the mouse jejunum, suggesting that the effect of DCEBIO in the Cl− secretion response is just now emerging. The mechanism of action of 1-EBIO is still debatable; nonetheless, there is little doubt that 1-EBIO activates IKCa directly (14, 28, 33, 69) and stimulates CFTR in nystatin-permeabilized preparations (13, 16). Cuthbert and colleagues (11, 39) have demonstrated that 1-EBIO (≥600 μM) increases cAMP via activation of adenylyl cyclase; however, Wallace et al. (67), using the same concentration of 1-EBIO, did not observe an effect of 1-EBIO on cAMP in T84 cells.

In the present study, we have several lines of evidence that suggest that in addition to activating IKCa, DCEBIO appears to increase Cl− secretion in a cAMP-dependent manner. First, pretreating tissues with 0.25 μM forskolin, an activator of adenylyl cyclase, reduced and shifted the DCEBIO concentration-dependence curve (Fig. 2) with a reduction in Vmax and a rightward shift of EC50. It could be interpreted that the modest amount of forskolin used in these experiments may have increased intracellular Ca2+; however, we think that this is not the case. Greger and colleagues (4, 23) demonstrated that forskolin (10 μM) reduced Ca2+ levels in isolated rat colonic crypts, whereas Cuthbert and colleagues (40) reported little effect of forskolin (10 μM) on intracellular Ca2+ of cultured human colonic cells (colony 29). Considering that we used a concentration of forskolin (0.25 μM) that was five times lower, forskolin may not alter intracellular Ca2+ but slightly raise cAMP and thus phosphorylate CFTR. Therefore, we think that in the presence of low forskolin, DCEBIO can further increase intracellular cAMP, thereby increasing the activity of CFTR while activating IKCa, resulting in a sustained Cl− secretory response.

Second, we demonstrated that pretreatment of tissues with 300 μM 8-BrcAMP (a maximal concentration for activating IKCa in the mouse jejunum) reduced the DCEBIO-stimulated Isc (Fig. 8). Likewise, pretreatment of tissues with DCEBIO (100 μM) reduced the 8-BrcAMP-activated Isc (Fig. 9). It could be argued that pretreatment of 300 μM 8-BrcAMP may have increased either intracellular Ca2+ or activated IKCa before the subsequent addition of DCEBIO. However, it has been reported that 1 mM 8-BrcAMP altered neither intracellular Ca2+ levels nor the activity of IKCa in Calu-3 cells (32). Holz et al. (30) reported that 1 mM 8-BrcAMP resulted in a fast transient increase in intracellular Ca2+ in primary cultures of rat pancreatic β-cells. In the present study, we have used a low concentration (300 μM) of 8-BrcAMP compared with these other studies, and therefore we suggest that DCEBIO increases cAMP, which is then augmented by the addition of 8-BrcAMP (Fig. 9). The small increase in Isc with 8-BrcAMP in the presence of DCEBIO was not surprising, because Cuthbert et al. (11) reported that high concentrations of 1-EBIO was not as

Further increase the Isc by the stimulation of IKCa. This appears to be the case; however, we were surprised by the fall in DCEBIO-stimulated Isc to a lower steady-state level (Fig. 5C) in the presence of forskolin. We did not observe this response during similar experiments with 1-EBIO (unpublished data). At present, we are uncertain as to the complex transient activation of Isc stimulated by DCEBIO in the presence of forskolin. Undoubtedly, the transient activation of Isc could result from a further hyperpolarization of the membrane potential (i.e., activation of IKCa). However, the subsequent decline of Isc to a new lower steady state would depend on the following: 1) a reduced hyperpolarization (i.e., slight decrease in K+ conductance), 2) an increase in activity of CFTR or an as yet undescribed component of the Cl− conductance without activation of K+ conductance, or 3) possibly an altered function of the Na+/K+-ATPase. However, Koegel et al. (35) reported that 1-EBIO downregulated IKCa of keratinocytes over an exposure time of hours, whereas they noted a stimulation of IKCa over a time frame of minutes. This would not explain our current results, because we noted a reduction of DCEBIO-stimulated Isc in <5 min after the peak Isc response (Fig. 5C). Third, pretreatment of the mouse jejunum with a maximum concentration of DCEBIO greatly reduced the effect of forskolin (Fig. 6), suggesting that DCEBIO and forskolin might use a common pathway for activating Cl− secretion. Fourth, mucosally applied glibenclamide, a known inhibitor of CFTR (58, 60), reduced the DCEBIO-stimulated Isc by >80% (Fig. 3). We (27) have previously demonstrated that glibenclamide applied from the serosal solution did not affect Cl− secretion of the mouse jejunum, suggesting that the effect of glibenclamide is on a transport protein (e.g., CFTR) that resides in the apical membrane. Finally, mucosally applied NPPB also reduced the DCEBIO-stimulated Isc.

Although IKCa has yet to be verified in the mouse jejunum by molecular techniques, IKCa is present within the mouse stomach and proximal and distal colon (66). IKCa is the predominant K+ channel of the basolateral membrane of the mouse jejunal crypt cell (6, 28). Clotrimazole, a known inhibitor of IKCa (15, 56, 57), was used to demonstrate the participation of IKCa in the DCEBIO-stimulated Isc response. Clotrimazole greatly reduced the secretory response (Isc) of the mouse jejunum by DCEBIO (Fig. 4). We have not examined the effects of DCEBIO and clotrimazole at the single-channel level of IKCa of jejunal crypts. However, we have demonstrated, using excised patch-clamp experiments, that clotrimazole inhibits DCEBIO-stimulated IKCa channel activity with a Ki of 65 nM for IKCa expressed in HEK-293 cells (62). Recently, Fioletti et al. (21) reported a DCEBIO- and clotrimazole-sensitive IKCa channel in a murine skeletal myoblast cell line.

We have not examined the effects of DCEBIO on single-channel recordings of IKCa of isolated jejunal crypts; however, we suspect that DCEBIO directly activates IKCa rather than altering intracellular Ca2+ levels resulting in the activation of IKCa. First, we (62) and others (61) have reported, on the basis of inside-out patch experiments, that DCEBIO directly activates IKCa heterologously expressed in HEK-293 cells or Xenopus oocytes. Second, D. C. Devor (unpublished observation) demonstrated that DCEBIO (using fura-2 measurements) did not alter intracellular Ca2+ concentrations of either T84 colonic cells (containing native IKCa channels) or HEK-293 cells expressing IKCa. Third, Cuthbert et al. (11) reported that 1-EBIO did not alter intracellular Ca2+ levels of isolated mouse colonic crypts. Similarly, D. C. Devor (unpublished observation) determined on the basis of fura-2 experiments that 1-EBIO failed to enhance intracellular Ca2+ levels of T84 colonic cells. Finally, we have reported that 1-EBIO activates IKCa directly using inside-out patch experiments (28).

These data strongly suggest that DCEBIO stimulates a Cl− secretory response of the mouse jejunum. It would appear that in the mouse jejunum, the effect of DCEBIO involves both the activation of IKCa as well as the modulation of CFTR.

**DCEBIO stimulates Cl− secretion via cAMP?** Our understanding of the mechanism of action of DCEBIO in the Cl− secretory response is just now emerging. The mechanism of action of 1-EBIO is still debatable; nonetheless, there is little doubt that 1-EBIO activates IKCa directly (14, 28, 33, 69) and stimulates CFTR in nystatin-permeabilized preparations (13, 16). Cuthbert and colleagues (11, 39) have demonstrated that 1-EBIO (≥600 μM) increases cAMP via activation of adenylyl cyclase; however, Wallace et al. (67), using the same concentration of 1-EBIO, did not observe an effect of 1-EBIO on cAMP in T84 cells.

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effective as forskolin in stimulating cAMP levels of the mouse colon.

Finally, if DCEBIO activates Cl− secretion via a cAMP-dependent manner, as well as by activating IKCa, then a PKA inhibitor should reduce the effect of DCEBIO on Isc. Indeed, H89 significantly reduced the DCEBIO-stimulated Isc by 34 ± 6% (Fig. 10, A–C). Similarly in paired tissues, H89 reduced the forskolin-stimulated Isc by 66 ± 8% (Fig. 10, D–F). In addition, Turner et al. (65) demonstrated that H89 (10 μM) reduced forskolin- and epinephrine-stimulated Isc of rabbit conjunctival epithelium. Similarly, Erlenkamp et al. (18) reported that PKA was stimulated by forskolin and inhibited by H89 (50 μM) in ventricular myocytes of the guinea pig.

Potency of DCEBIO compared with 1-EBIO. The only difference between DCEBIO and its parent molecule 1-EBIO is the presence of chloro groups at positions 5 and 6 on the phenyl ring of the benzimidazolone structure (61). The chloro groups at those positions fulfill an optimal size and electronic character not achieved by bromo or methyl groups (61). Indeed, this slight change in the structure of the benzimidazolone significantly enhances the potency of DCEBIO over 1-EBIO. Bridges and colleagues (61) reported that DCEBIO exhibited an EC50 of 45 μM, whereas 1-EBIO had an EC50 of 1,200 μM in Isc experiments of T84 colonic monolayers. Similarly, with a native epithelium, the mouse jejunum, we report herein the EC50 of DCEBIO and 1-EBIO on Isc were 41 μM and 862 μM, respectively (Fig. 2). We and others have reported that 1-EBIO exhibits an EC50 on Isc for Cl− secretion of between 0.5 and 1 mM for Cl− secretion in cultured cells and native epithelia (11, 14, 16, 28, 61). Similarly, with patch-clamp experiments, the EC50 of DCEBIO on the activation of IKCa channels expressed in Xenopus oocytes is 840 nM (61) and 4 μM for IKCa expressed in HEK-293 cells (62). However, the EC50 for activation of IKCa by 1-EBIO for native IKCa channels of T84 colonic cells (61) and IKCa expressed in HEK-293 cells (33) is ~80 μM. The lower EC50 in patch-clamp experiments of 1-EBIO is not surprising, considering the accessibility of the drug to the channel. Without a doubt, DCEBIO is a more potent activator of Cl− secretion and IKCa than 1-EBIO.

In summary, we have used the Ussing chamber short-circuit current technique to demonstrate for the first time in a native epithelium that DCEBIO stimulates a Cl− secretory response of the mouse jejunum. Both CFTR and IKCa participate in the DCEBIO-activated Cl− secretory response. The action of DCEBIO, as well as activation of IKCa, appears to occur in a cAMP-dependent manner.

NOTE ADDED IN PROOF After our manuscript was accepted, we became aware of the recent article by Ayabe et al. (2a). On the basis of RT-PCR results, they reported the presence of mRNA of mIK1 in the lower portion of the crypt of the mouse small intestine. The specific region of the small intestine was not noted. In addition, Ayabe et al. detected mRNA of mIK1 only in the Paneth cells of the crypt; however, they stated that other cell types of the crypt may express mIK1 at levels below the detection limits of their assay.

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