Genistein stimulates electrogenic Cl\(^{-}\) secretion via phosphodiesterase modulation in the mouse jejunum

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Chao PC, Hamilton KL. Genistein stimulates electrogenic Cl\(^{-}\) secretion via phosphodiesterase modulation in the mouse jejunum. Am J Physiol Cell Physiol 297: C688–C698, 2009. First published July 1, 2009; doi:10.1152/ajpcell.00152.2009.—Previously, we demonstrated that genistein stimulated Cl\(^{-}\) secretion in the mouse jejunum (Baker MJ and Hamilton KL, Am J Physiol Cell Physiol 287: C1636–C1645, 2004); however, the mode of action of genistein still remains unclear. Here, we examined the activation of Cl\(^{-}\) secretion by the modulation of phosphodiesterases (PDEs) by genistein (75 μM) in the mouse jejunum with the Ussing short-circuit current (I\(_{sc}\)) technique. Drugs tested included theophylline (10 mM), a nonspecific PDE inhibitor; 8-methoxymethyl-3-isobutyl-1-methylxanthine (8-MM-IBMX; 100 μM), erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA; 40 μM), milrinone (100 μM), and rolipram (40 and 100 μM), which are specific inhibitors of PDE1–PDE4, respectively. Theophylline stimulated a bumetanide-sensitive I\(_{sc}\), indicative of Cl\(^{-}\) secretion, and abolished genistein’s stimulatory action on I\(_{sc}\). Neither 8-MM-IBMX nor EHNA altered the basal I\(_{sc}\) nor did these PDE inhibitors affect the stimulatory action of genistein on the I\(_{sc}\) of the mouse jejunum. Rolipram had no effect on basal I\(_{sc}\), but it reduced the genistein-stimulated I\(_{sc}\) compared with time-matched control tissues. Milrinone stimulated a concentration-dependent increase in I\(_{sc}\). Bumetanide (10 μM) inhibited 60 ± 4% of milrinone-induced I\(_{sc}\). Pretreating tissues with milrinone prevented genistein from stimulating I\(_{sc}\) and pretreatment with genistein reduced the effect of milrinone on I\(_{sc}\). H89 (50 μM), a PKA inhibitor, reduced the milrinone-stimulated I\(_{sc}\). Likewise, H89 reduced the genistein-stimulated I\(_{sc}\). Here, we demonstrate, for the first time, that genistein activates Cl\(^{-}\) secretion of the mouse jejunum via inhibition of a PDE3-dependent pathway.

PDE3; cystic fibrosis transmembrane conductance regulator; Ca\(^{2+}\)-activated Cl\(^{-}\) channels; milrinone

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lecular mechanisms have been proposed, including a direct activation of CFTR (25, 68), modulation of tyrosine kinases (37, 61), protein phosphatases (37, 55), or protein kinases (61).

The crypt region of the jejunum is the primary site for epithelial fluid and electrolyte secretion by the intestine, including Cl\(^{-}\) secretion (13). Recently, we provided the first report on the effects of genistein on the mouse jejunum (4). In that study, recording short-circuit currents \(I_{sc}\) and using a pharmacological approach, we demonstrated that genistein stimulated a sustained increase in Cl\(^{-}\) secretion and that the action of genistein involved, at a minimum, both CFTR and the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (4). We proposed that genistein acted by a phosphorylation-dependent mechanism, and to test that, we examined the effect of forskolin, an activator of adenylyl cyclase, on \(I_{sc}\) in the presence of genistein. The \(I_{sc}\) forskolin-stimulated response was significantly reduced in the presence of genistein. Additionally, pretreating the jejunum with forskolin eliminated the action of genistein on the \(I_{sc}\). We concluded that genistein regulated Cl\(^{-}\) secretion of the mouse jejunum by a phosphorylation-dependent pathway (4).

O’Grady et al. (52) demonstrated that modulation of PDEs altered the cAMP-dependent Cl\(^{-}\) secretion in human colonic epithelial cells (T84). They reported that PDE4 accounted for >70% of the total cAMP hydrolysis in both cellular and membrane fractions from these cells. However, PDE1 and PDE3 also played crucial roles in modulating Cl\(^{-}\) secretion of T84 cells (52). Additionally, Steagall and Drumm (64) demonstrated the presence of PDE1, PDE3–PDE5 in the epithelium of the mouse intestine. They (64) also demonstrated that inhibition of these four PDEs stimulated small increases of \(I_{sc}\) of the mouse jejunum, suggestive of a cAMP-phosphorylation-dependent increase in current by the jejunum. Recently, Ko et al. (40) and Nichols and Morimoto (50) have demonstrated that genistein is an effective inhibitor of PDE1–4.

To date, a systematic examination of the role of genistein in the modulation of PDEs in the Cl\(^{-}\) secretory response of the mouse jejunum has not been carried out. Therefore, the aims of the present study were first, to examine the effects of selective inhibitors of PDEs on the \(I_{sc}\) of the mouse jejunum, and second, to determine the role of PDEs in the mechanism of action of genistein on the Cl\(^{-}\) secretory response of the mouse jejunum.

MATERIALS AND METHODS

Animals and tissue preparation. All experiments were carried out on the jejunum of male Swiss-Webster mice (20 to 35 g) and were approved by the University of Otago Animal Ethics Committee. The animals had access to tap water and food ad libitum. Mice were killed by cervical dislocation. The isolated jejunum was placed in and flushed with a NaCl Ringer solution [in mM: 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES/tris(hydroxymethyl)methylamine (Tris), and 4 pyruvate/glutamine, with pH adjusted to 7.4]. The HEPES-buffered Ringer was used for examining HCO\(_3\)\(^{-}\)-independent Cl\(^{-}\) secretory responses.

Electrophysiological measurements-Ussing chamber experiments. Ussing chamber \(I_{sc}\) experiments were conducted as previously described (4, 11, 31). Once the tissues were pinned, they were glued (Locute 454, Herts Garden City, UK) to plastic annuli (0.7 cm\(^2\)) and mounted in modified Ussing chambers. In all experiments, tissues were bathed on both mucosal and serosal sides with 10 ml of the NaCl Ringer solution. Solutions were aerated (100% O\(_2\)) and maintained at 37°C by water-jacketed solution reservoirs.

Tissues were voltage clamped to 0 mV (Biodesign; South Campus Electronics, University of Otago). \(I_{sc}\) was continuously recorded with a MacLab data acquisition system and analyzed with MacLab Chart (version 3.6.3, AD Instruments, Castle Hill, Australia). Initially, tissues were rinsed three times with NaCl Ringer. Then, all tissues were pretreated (1 h) with indomethacin (1 \(\mu\)M, mucosal and serosal) to reduce prostaglandin production (24) and tetrodotoxin (TTX, 1 \(\mu\)M, serosal) to decrease enteric nerve activity (34) to reduce the rate of transepithelial transport to basal conditions. The basal \(I_{sc}\) was 130 ± 3 \(\mu\)A/cm\(^2\) \(\pm\) 339; not different from our previous studies (4, 30, 31). The change in \(I_{sc}\) (\(\Delta I_{sc}\)) induced by a treatment was expressed as the difference from the former baseline to the steady-state value. Effects on \(I_{sc}\) were compared against changes in \(I_{sc}\) of time-matched control tissues. Data from concentration-dependent response experiments were fitted with the Hill equation (for details of the Hill equation, see Ref. 69). The presence of the circular and longitudinal muscle layers outside the epithelium did not impede access of chemicals to the epithelium, as illustrated by very rapid responses of \(I_{sc}\) with the addition of theophylline, milrinone, or bumetanide (e.g., see Figs. 1–4, 6).

We were concerned that the basal \(I_{sc}\) of a tissue might influence the response of genistein on \(I_{sc}\). However, the amount of genistein-induced \(I_{sc}\) was not correlated with the magnitude of the basal \(I_{sc}\) present \((P = 0.7, n = 40, \text{data not shown})\).

Chemicals. Bumetanide, erythro-9-(2-hydroxyl-3-nonyl)-adenine (EHNA), forskolin, genistein, HEPES, indomethacin, milrinone, 8-methoxy-methyl-3-isobutyl-1-methylxanthine (MM-IBMX), rolipram, and theophylline were purchased from Sigma Chemical (St. Louis, MO). H89 was obtained from Calbiochem (La Jolla, CA). TTX was purchased from Alomone Labs (Jerusalem, Israel), and Tris-(hydroxymethyl)aminoethane (Tris) was obtained from BDH Chemicals (Poole, UK). EHNA, genistein, milrinone, MM-IBMX, and rolipram were dissolved in dimethyl sulfoxide (DMSO), bumetanide was dissolved in ethanol, and indomethacin was dissolved in methanol. H89, HEPES, Tris, and TTX were dissolved in distilled water. Theophylline was added as anhydrous crystals. Drugs were added as a small volume of a stock solution unless otherwise stated.

Statistical analysis. Statistical significance was assessed using Student’s t-test (paired and unpaired). A P value of <0.05 was considered statistically significant. MacCurveFit (Kevin Raner Software, Mt. Waverley, Victoria, Australia) was used for curve fitting and determining the EC \(_{50}\) apparent \(V_{max}\) value, and a Hill coefficient for the concentration-dependent response experiments. Data are presented as means ± SE. The number \((n)\) of tissues and number \((N)\) of animals for a given protocol and a series of experiments, respectively, 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

Genistein and theophylline activation of Cl\(^{-}\) secretion by the mouse jejunum. Previously, we demonstrated that genistein stimulated a sustained concentration-dependent Cl\(^{-}\) secretory response of mouse jejunum (4). Initially, we confirmed our previous findings (4). As can be seen in Fig. 1, genistein (75 \(\mu\)M, mucosal and serosal) activated an increase in \(I_{sc}\) of 36 ± 9 \(\mu\)A/cm\(^2\) (Fig. 1C, \(n = 7\), \(N = 7\)). The genistein-stimulated \(I_{sc}\) was significantly higher than the change in \(I_{sc}\) of the DMSO control tissues (\(\sim 8 ± 4 \mu\)A/cm\(^2\), \(n = 4\)) that received vehicle only (Fig. 1B and C). We used bumetanide, an inhibitor of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter, to confirm that the genistein-activated \(I_{sc}\) was, indeed, due to Cl\(^{-}\) secretion. Bumetanide (20 \(\mu\)M, serosal) significantly reduced (28 ± 7 \(\mu\)A/cm\(^2\), \(n = 7\)) the genistein-stimulated \(I_{sc}\) by 63 ± 16% (\(n = 7\)) consistent with the activation of Cl\(^{-}\) secretion (Fig. 1, A and C). The remainder
of the genistein-stimulated $I_{sc}$ may be due to HCO$_3^-$ secretion (28); in this case, the HCO$_3^-$ must have been generated from a cellular source since these experiments were conducted in HEPES-buffered Ringer.

It has been reported that genistein can regulate Cl$^-$ secretion by altering the phosphorylation state of CFTR through inhibition of protein kinases (37, 61) or protein phosphatases (36, 37, 55). Additionally, modulation of phosphodiesterases (PDEs) would result in elevated levels of cAMP leading to activation of CFTR. At present, the modulation of Cl$^-$ secretion by genistein’s actions on PDEs of native tissues is not well understood. Initially, we used theophylline [a nonselective inhibitor of PDEs (10)] to establish an action of genistein on PDEs that resulted in the Cl$^-$ secretory response by the mouse jejunum.

We predicted that genistein by itself would increase the $I_{sc}$ of the mouse jejunum based on the fact that Cl$^-$ secretion, via CFTR, is through a cAMP-dependent pathway. To test this hypothesis, first tissues were subjected to theophylline (10 mM, serosal) or received no drug followed by the addition of bumetanide (10 μM, serosal) to all tissues. Theophylline stimulated a sustained increase in $I_{sc}$ (24 ± 3 μA/cm$^2$, n = 12) in the absence of theophylline as shown in Fig. 3, A and D. Pretreatment of tissues with theophylline increased $I_{sc}$ by 62 ± 9 μA/cm$^2$ (n = 7) (Fig. 3B). Now, in the presence of theophylline, genistein did not increase $I_{sc}$. However, there was a small reduction of $I_{sc}$ (−9 ± 4 μA/cm$^2$, n = 12), which was not significantly different from the effects of DMSO on the control tissues or of DMSO added to the theophylline-stimulated tissues (Fig. 3D).

![](image1.png)

**Fig. 1.** Effects of genistein (75 μM, mucosal and serosal) and bumetanide (10 μM, serosal) on short-circuit current ($I_{sc}$) of the mouse jejunum. A: representative trace of the effect of genistein on $I_{sc}$. B: representative trace of a control tissue. C: mean changes of $I_{sc}$ ($\Delta I_{sc}$) of the matched control group of genistein in response to the addition of genistein or in response to bumetanide (Bumet) in the presence (genistein + Bumet) or absence (Bumet control) of genistein. Values are means ± SE; n = 4–7, N = 7. *P ≤ 0.05, **P ≤ 0.01.

**Fig. 2.** Effects of theophylline (10 mM, serosal) and bumetanide (10 μM, serosal) on $I_{sc}$ of the mouse jejunum. A: representative trace of the effect of theophylline on $I_{sc}$. B: representative trace of a control tissue. C: mean $\Delta I_{sc}$ of the controls of genistein in response to the addition of theophylline or in response to bumetanide in the presence (theophylline + Bumet) or absence (Bumet control) of theophylline. Values are means ± SE; n = 8–16, N = 8. ***P ≤ 0.001.

**PDE-DEPENDENT GENISTEIN-STIMULATED Cl$^-$ SECRETION**

**Fig. 1.** Effects of genistein (75 μM, mucosal and serosal) and bumetanide (10 μM, serosal) on short-circuit current ($I_{sc}$) of the mouse jejunum. A: representative trace of the effect of genistein on $I_{sc}$. B: representative trace of a control tissue. C: mean changes of $I_{sc}$ ($\Delta I_{sc}$) of the matched control group of genistein in response to the addition of genistein or in response to bumetanide (Bumet) in the presence (genistein + Bumet) or absence (Bumet control) of genistein. Values are means ± SE; n = 4–7, N = 7. *P ≤ 0.05, **P ≤ 0.01.

**Fig. 2.** Effects of theophylline (10 mM, serosal) and bumetanide (10 μM, serosal) on $I_{sc}$ of the mouse jejunum. A: representative trace of the effect of theophylline on $I_{sc}$. B: representative trace of a control tissue. C: mean $\Delta I_{sc}$ of the controls of genistein in response to the addition of theophylline or in response to bumetanide in the presence (theophylline + Bumet) or absence (Bumet control) of theophylline. Values are means ± SE; n = 8–16, N = 8. ***P ≤ 0.001.
One possible action of genistein is to inhibit PDE1, thus resulting in the stimulation of Cl⁻ secretion. To test this hypothesis, we used MM-IBMX (100 μM, mucosal and serosal), a selective inhibitor of PDE1 (21), which has been used to inhibit PDE1 activity of mouse jejenum and T84 cell monolayers (52, 64). Six animals were used in the series of experiments. Tissues received genistein (75 μM, mucosal and serosal) in the absence (DMSO added to the control tissue) or presence of MM-IBMX, followed by bumetanide (10 μM, serosal). MM-IBMX (n = 6) alone did not alter the basal Iₛₑ of the tissues compared with time-matched DMSO control tissues (data not shown). The stimulation of Iₛₑ by genistein was not significantly different in the presence (ΔIₛₑ = 19 ± 3 μA/cm², n = 6; N = 6) or absence (ΔIₛₑ = 16 ± 3 μA/cm², n = 6) of MM-IBMX. Bumetanide reduced (P ≤ 0.05) the genistein-stimulated Iₛₑ under both experimental instances (8 ± 3 μA/cm² and 12 ± 1 μA/cm² in the absence and presence of MM-IBMX, respectively; n = 6 for both). Steagall and Drumm (64) reported that MM-IBMX (100 μM) activated a “small” increase in Iₛₑ (~15 μA/cm²) of the mouse jejunum. However, it should be noted that in Steagall and Drumm’s study, tissues

On the basis of those results, we predicted that pretreating tissues with genistein would reduce the effect of theophylline on Iₛₑ. Tissues were pretreated with either genistein (75 μM, mucosal and serosal) or DMSO, and then both tissues received theophylline (10 mM, serosal) followed by bumetanide (20 μM, serosal). In the absence of genistein, theophylline stimulated a sustained increase in Iₛₑ of 67 ± 14 μA/cm² (n = 7, N = 7) (Fig. 4, A and C). However, in the presence of genistein, the theophylline-stimulated Iₛₑ was reduced (P ≤ 0.05) by 51% (34 ± 7 μA/cm², n = 6; N = 6) (Fig. 4, B and C).

These results suggest that genistein and theophylline share a common pathway in activating Cl⁻ secretion in the mouse jejunum and that this is potentially via a PDE-sensitive pathway.

**Genistein-stimulated Cl⁻ secretory response is independent of PDE1.** Genistein has been shown to be an effective inhibitor of PDE1–4 (40, 50). To examine the participation of specific PDEs in the genistein-stimulated Cl⁻ secretory response in the mouse jejunum, we examined the effects of genistein in the presence of specific inhibitors of various isoforms of PDEs.

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**Fig. 3.** Effects of genistein (75 μM, mucosal and serosal) on Iₛₑ of the mouse jejunum in the absence and presence of theophylline (10 mM, serosal). Bumetanide (10 μM, serosal) was added to all tissues at the end of an experiment. A: representative trace of the effect of genistein on Iₛₑ in the presence of theophylline. B: representative trace of the effect of theophylline alone on Iₛₑ. C: representative trace of the effect of theophylline alone on Iₛₑ in the presence of genistein alone, in response to genistein in the presence of theophylline (Theo), or in response to genistein’s control in the presence of theophylline (DMSO after Theo). Values are mean ± SE; n = 7–12, N = 13. ***P ≤ 0.001.

**Fig. 4.** Effects of theophylline (10 mM, serosal) on Iₛₑ of the mouse jejunum in the absence and presence of genistein (75 μM, mucosal and serosal). Bumetanide (20 μM, serosal) was added to all tissues at the end of an experiment. A: representative trace of the effect of theophylline on Iₛₑ of a control tissue. B: representative trace of the effect of theophylline on Iₛₑ in the presence of genistein. C: mean ΔIₛₑ in response to theophylline in the absence or presence of genistein. Values are means ± SE; n = 6–7, N = 7. *P ≤ 0.05.
were bathed in HCO₃⁻-containing Ringer rather than HEPES Ringer and were not pretreated with indomethacin or TTX.

These data suggest, under our experimental conditions, that MM-IBMX does not stimulate \( I_{sc} \). Additionally, these data suggest that the genistein-activated Cl\(^-\) secretory response was not altered in the presence of MM-IBMX, strongly suggesting that the mechanism of genistein-induced Cl\(^-\) secretion is independent of PDE1.

**Genistein-activated a PDE2-independent Cl\(^-\) secretory response.** Blockers of PDE2 have been used to demonstrate cAMP-dependent transport in tissues. For example, EHNA, a selective blocker of PDE2 (21), prevented nitric oxide from inhibiting cAMP-activated NaCl transport of the rat thick ascending limb (54). Therefore, we wanted to test the hypothesis that genistein increased \( I_{sc} \) via inhibition of PDE2. To test our hypothesis, we examined the effect of genistein (75 \( \mu \)M, mucosal and serosal) on the \( I_{sc} \) of two tissues bathed in NaCl Ringer in the absence or presence of EHNA while a third tissue received DMSO (vehicle) alone. Initially, we used 100 \( \mu \)M EHNA (mucosal and serosal), which had no effect on basal \( I_{sc} \) (data not shown). However, the combination of 100 \( \mu \)M EHNA and genistein (or vice versa) led to deleterious effects on the transport properties of the mouse jejunal (\( n = 8 \), data not shown). Subsequently, we conducted all experiments with 40 \( \mu \)M EHNA. Two tissues received EHNA while another two tissues received DMSO, after which all tissues were subjected to genistein (75 \( \mu \)M, mucosal and serosal) followed by bumetanide (10 \( \mu \)M, serosal). Addition of 40 \( \mu \)M EHNA did not alter the basal \( I_{sc} \) (\( n = 6 \)) when compared with the effect of DMSO (vehicle) on \( I_{sc} \) of control tissues (\( n = 6 \)), suggesting no effect of PDE2 on basal \( I_{sc} \) (data not shown). Additionally, the genistein-stimulated \( I_{sc} \) was the same in the absence (13 ± 2 \( \mu \)A/cm²) or presence (16 ± 1 \( \mu \)A/cm²) of EHNA. The genistein-stimulated \( I_{sc} \) was significantly (\( P \leq 0.05 \)) blocked by bumetanide. Similarly, with the reverse experiment, EHNA did not reduce the genistein-stimulated \( I_{sc} \) (\( n = 4 \), data not shown). These data suggest that under these experimental conditions, genistein stimulates \( I_{sc} \) by a PDE2-independent mechanism.

**Genistein-activated Cl\(^-\) secretory response involves inhibition of PDE3.** On the basis of the presence of PDE3 in the mouse jejunal (64) and the report by O’Grady and colleagues (52) that Cl\(^-\) secretion is PDE3 dependent in T84 cells, we tested the hypothesis that genistein stimulates \( Cl^{-} \) secretion in the mouse jejunal by inhibition of PDE3. Initially, we wanted to advance the work of Steagall and Drumm (64) by determining the concentration-dependent effect of milrinone (0.1–300 \( \mu \)M, mucosal and serosal), a known inhibitor of PDE3 (47), on \( I_{sc} \) of the mouse jejunal. The increase in \( I_{sc} \) stimulated by milrinone was dose dependent with an \( EC_{50} \) of 46 ± 9 \( \mu \)M, an apparent \( V_{max} \) of 81 ± 7 \( \mu \)A/cm², and a Hill coefficient of 1.1 ± 0.1 (Fig. 5, \( n = 6 \)–13 per concentration; \( N = 6 \)). Again, DMSO had no effect on basal \( I_{sc} \). Addition of bumetanide (10 \( \mu \)M, serosal) significantly (\( P \leq 0.05 \)) decreased the milrinone-induced \( I_{sc} \) (60 ± 4%, \( n = 5 \)), which was consistent with a Cl\(^-\) secretory response (data not shown).

If genistein increases \( I_{sc} \) by inhibiting PDE3, then we predicted that genistein might still increase \( I_{sc} \) in the presence of a submaximal concentration of milrinone (100 \( \mu \)M), unless milrinone is a more potent inhibitor of PDE3. To test this hypothesis, we examined the effect of genistein on \( I_{sc} \) in the presence of 100 \( \mu \)M milrinone (8 animals). One tissue was pretreated with milrinone (mucosal and serosal) followed by the addition of DMSO, the second and third tissues received either milrinone or DMSO followed by genistein (75 \( \mu \)M, mucosal and serosal), while the fourth tissue was a time-matched control receiving only DMSO. All tissues received bumetanide (10 \( \mu \)M, serosal) at the end of the experiment. Milrinone increased the \( I_{sc} \) by 106 ± 11 \( \mu \)A/cm² (\( n = 13 \)) (Fig. 6A, C, and E). As seen in Fig. 6B, genistein stimulated an increase in \( I_{sc} \) (13 ± 7 \( \mu \)A/cm², \( n = 8 \)) in the absence of milrinone (Fig. 6F, \( n = 8 \)). However, milrinone failed to increase \( I_{sc} \) in the presence of milrinone, and indeed, the milrinone-induced \( I_{sc} \) was reduced by genistein (Fig. 6C, D, and F, \( n = 6 \)). The reduction in \( I_{sc} \) by genistein (−19 ± 3 \( \mu \)A/cm², \( n = 6 \)) (Fig. 6F) in the presence of milrinone was greater (\( P \leq 0.01 \)) compared with either the effect of DMSO on the time-matched control tissue (\( \Delta I_{sc} = -5 \pm 4 \mu \)A/cm², \( n = 3 \), Fig. 6D) or on the milrinone control tissue (\( \Delta I_{sc} = 6 \pm 4 \mu \)A/cm², \( n = 3 \), Fig. 6A). These data strongly suggest that milrinone and genistein share a common pathway by inhibiting PDE3 resulting in Cl\(^-\) secretion in the mouse jejunal.

On the basis of these results (Fig. 6), we hypothesized that genistein would reduce the effect of milrinone on \( I_{sc} \) but not eliminate milrinone’s effect on \( I_{sc} \). Therefore, similar experiments were conducted as described above (7 animals). Two tissues were pretreated with genistein (75 \( \mu \)M, mucosal and serosal) while the third tissue received DMSO, after which milrinone (100 \( \mu \)M, mucosal and serosal) was added to one of the two genistein-treated tissues and DMSO was added to the other tissue. Bumetanide (10 \( \mu \)M, serosal) was added to all tissues at the end of the experiment. Milrinone increased \( I_{sc} \) by 100 ± 10 \( \mu \)A/cm² (Fig. 7A and D; \( n = 7 \)). However, in the presence of genistein, the milrinone-stimulated \( I_{sc} \) (55 ± 8 \( \mu \)A/cm²) was reduced by 48 ± 9% (\( P \leq 0.01 \)) (Fig. 7B, D; \( n = 7 \)) compared with milrinone alone while DMSO had no effect (\( \Delta I_{sc} = 1 \pm 1 \mu \)A/cm²) on the genistein-stimulated tissue (Fig. 7D, C, D; \( n = 5 \)).

These data demonstrate, for the first time, that genistein stimulates Cl\(^-\) secretion via a PDE3-dependent manner in native epithelium.
Genistein-stimulated $I_{sc}$ response was reduced by the presence of a PDE4 inhibitor. Using PDE assays, O’Grady and colleagues (52) reported that PDE4 accounted for the major fraction of the total cAMP hydrolytic activity of T84 colonic epithelial cells. However, PDE4 was not involved in $I_{sc}$ secretion as assessed by $I_{sc}$ experiments. Rolipram is a specific inhibitor of many isoforms of PDE4 (12, 16) and has been used to determine the role of PDE4 in a number of tissues (7).

We used rolipram to test the hypothesis that genistein stimulated $I_{sc}$ of the mouse jejunum by inhibition of PDE4. Ten animals were used in the series of experiments. Two tissues were pretreated with either 40 μM or 100 μM rolipram (mucosal and serosal) while another two tissues received DMSO, after which all tissues received genistein (10 μM, mucosal and serosal). Genistein stimulated the $I_{sc}$ by 24 ± 4 μA/cm² (Fig. 8, A, D, and E; $n = 10$), while the genistein vehicle, DMSO, reduced the $I_{sc}$ by 10 ± 5 μA/cm² ($n = 14$; trace not shown). Rolipram did not affect the basal $I_{sc}$ ($-9 ± 4 μA/cm²$, $n = 14$, data not shown) when compared with time-matched control tissues ($-9 ± 3 μA/cm²$, $n = 11$, data not shown), which suggests that PDE4 did not contribute to the basal $I_{sc}$.

Similarly, 100 μM rolipram did not affect the basal $I_{sc}$ ($-13 ± 4 μA/cm²$, $n = 12$) when compared with time-matched control tissues ($-8 ± 3 μA/cm²$, $n = 12$) (data not shown). In the presence of 40 μM rolipram, genistein stimulated $I_{sc}$ by only 8 ± 4 μA/cm² (Fig. 8, A and E; $n = 10$), and genistein activated the $I_{sc}$ by 10 ± 7 μA/cm² in the presence of 100 μM rolipram (Fig. 8, C and E; $n = 9$). Hence, there was a reduction in the genistein-stimulated $I_{sc}$ in the presence of both 40 μM (P $= 0.01$) and 100 μM (P $= 0.05$) rolipram (Fig. 8E). These data suggest that rolipram did not alter the basal $I_{sc}$. However, the pretreatment of the tissues with rolipram reduced the genistein-stimulated $I_{sc}$, thus suggesting a regulatory link between PDE4 and genistein’s effect on $I_{sc}$.

Is the milrinone-stimulated $I_{sc}$ secretion PKA dependent? The level of intracellular cAMP is a fine balance between the activities of adenylyl cyclase and PDEs. Obviously, if a particular PDE is downregulated, then the level of cAMP can be increased, resulting in the activation of PKA. Subsequently, this increased PKA can phosphorylate CFTR, resulting in $I_{sc}$ secretion (62). Recently, for the mouse jejunum, we (30) have used H89, a PKA inhibitor (32), to demonstrate that the forskolin- and 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimi-
dazol-2-one (DCEBIO)-stimulated Cl\(^{-}\)/H\(_{11002}\) secretory responses were PKA dependent. Therefore, we have taken a similar approach to determine whether the milrinone- and genistein-stimulated Cl\(^{-}\)/H\(_{11002}\) secretory responses are PKA dependent.

We hypothesized that milrinone inhibits PDE3 to activate I\(_{sc}\) by a PKA-dependent pathway (Fig. 6E), and then pretreatment of tissues with H89 should attenuate the stimulatory action of milrinone on I\(_{sc}\). D: mean ΔI\(_{sc}\) of the DMSO controls for milrinone in the presence of genistein or in response to milrinone alone or in response to milrinone in the presence of genistein. Values are means ± SE; n = 5–7, N = 7. **P ≤ 0.01.

dazol-2-one (DCEBIO)-stimulated Cl\(^{-}\) secretory responses were PKA dependent. Therefore, we have taken a similar approach to determine whether the milrinone- and genistein-stimulated Cl\(^{-}\) secretory responses are PKA dependent.

We hypothesized that milrinone inhibits PDE3 to activate I\(_{sc}\) by a PKA-dependent pathway (Fig. 6E), and then pretreatment of tissues with H89 should attenuate the stimulatory action of milrinone on I\(_{sc}\). To test this hypothesis, our experimental protocol consisted of pretreating one tissue with H89 (50 μM, serosal) followed by milrinone (100 μM, mucosal and serosal) while water (vehicle for H89) was added to the control tissue followed by milrinone. All tissues received bumetanide (10 μM, serosal) at the end of the experiment. Similar to the water control tissues (Fig. 9A), H89 did not affect the basal I\(_{sc}\) (Fig. 9B) compared with water, as we previously reported (30). In the absence of H89, milrinone stimulated an increase in I\(_{sc}\) of 60 ± 6 μA/cm\(^2\) (Fig. 9, A and C, n = 8, N = 8). However, in the presence of H89, the increase in milrinone-activated I\(_{sc}\) was reduced (P ≤ 0.01, 33% block) to only 40 ± 4 μA/cm\(^2\) (Fig. 9, D and E, n = 5–7, N = 7. **P ≤ 0.01.
since genistein activates Cl⁻ secretory process via a PKA-dependent pathway. Thus, we hypothesize that the action of these two modulators on Cl⁻ secretion is by a shared cAMP-dependent pathway. The milrinone and genistein data (Figs. 6 and 7) indicate that genistein stimulated an increase in I_{sc} of 19 ± 6 μA/cm² in the presence of H89 compared with that in the presence of H89. These results clearly demonstrate that there is no basal PKA activity and that, furthermore, the milrinone-stimulated I_{sc} is dependent on PKA. These results strongly suggest that the Cl⁻ secretory response is attributed to the inhibition of PDE3 by milrinone.

Is the genistein-stimulated Cl⁻ secretion PKA dependent? The milrinone and genistein data (Figs. 6 and 7) indicate that the action of these two modulators on Cl⁻ secretion is by a shared cAMP-dependent pathway. Thus, we hypothesize that since genistein activates Cl⁻ secretion via a PKA-dependent pathway, then genistein’s action on I_{sc} should be reduced in the presence of H89. To test this hypothesis, we examined the action of genistein in the absence and presence of H89. Five animals were used in this series of experiments. Two tissues were either pretreated with water or H89 (50 μM, serosal) followed by the addition of genistein (75 μM, mucosal and serosal). Bumetanide (10 μM, serosal) was added to both tissues at the end of an experiment. As can be seen in Fig. 10, genistein stimulated an increase in I_{sc} of 19 ± 6 μA/cm² (Fig. 10, A and C, n = 5). However, the genistein-stimulated I_{sc} was significantly (P ≤ 0.05) reduced to 7 ± 3 μA/cm² (63% reduction) in the presence of H89 (Fig. 10, B and C, n = 5). Furthermore, the bumetanide-sensitive, genistein-stimulated I_{sc} was greater (89%) for the tissues that were exposed to genistein in the absence (P ≤ 0.05, n = 5) rather than the presence of H89.

To our knowledge, this is the first evidence that genistein activates Cl⁻ secretion of the mouse jejunum by inhibition of PDE3 and that genistein’s action is via a PKA-dependent pathway.

DISCUSSION

Genistein-PDE3-dependent modulation of Cl⁻ secretion. The present study extends our previous work on the action of genistein on the Cl⁻ secretory process of the mouse jejunum (4). The aims of our current study were to examine the effects of selective inhibitors of PDEs on the I_{sc} of the mouse jejunum and to examine the action of genistein on PDEs in the modulation of Cl⁻ secretion of the mouse jejunum. Initially, we used theophylline, a generic PDE inhibitor, to demonstrate that the basal I_{sc} and genistein-stimulated I_{sc} were PDE dependent (Figs. 2–4), after which we used specific inhibitors of PDEs to...
establish which PDE(s) was involved in the genistein-stimulated Cl\(^-\) secretory response.

We demonstrated that MM-IMBX and EHNA did not alter the basal \(I_{sc}\) of the jejunum or reduce the action of genistein on \(I_{sc}\), suggesting that PDE1 and PDE2 were not involved in the basal \(I_{sc}\) or genistein-stimulated \(I_{sc}\). On the other hand, even though rolipram, an inhibitor of PDE4, failed to alter basal \(I_{sc}\), the genistein-activated \(I_{sc}\) was reduced in the presence of rolipram (Fig. 8). While this was an intriguing observation, this was, however, not pursued in the current study. Here, we describe, for the first time in the mouse jejunum, that milrinone stimulates a bumetanide-sensitive, concentration-dependent increase in \(I_{sc}\), indicative of Cl\(^-\) secretion. More importantly, we report, for the first time, that genistein stimulates Cl\(^-\) secretion by a PDE3- and PKA-dependent manner.

Several lines of evidence suggest that the activation of Cl\(^-\) secretion by genistein is via a PDE3- and PKA-dependent mechanism. First, pretreatment of the jejunum with a submaximal concentration (100 \(\mu\)M) of milrinone prevented a further increase in \(I_{sc}\) by genistein (Fig. 6, C and F). Interestingly, genistein reduced \(I_{sc}\) in the presence of milrinone and is discussed below. Second, pretreatment of the jejunum with genistein reduced the milrinone-stimulated \(I_{sc}\) by nearly 50% (Fig. 7, B and D). These data suggest that genistein and milrinone share a common pathway (via PDE3) for increasing Cl\(^-\) secretion of the mouse jejunum. Third, we have previously reported that pretreating the jejunum with a maximal concentration of forskolin prevented genistein from stimulating \(I_{sc}\) of the mouse jejunum, suggesting that genistein increases Cl\(^-\) secretion by resulting in the phosphorylation of CFTR via a PKA-dependent pathway. Also in that study, we demonstrated that genistein decreased the forskolin-stimulated \(I_{sc}\). Lastly, in our present study, H89, an inhibitor of PKA, significantly reduced the genistein-stimulated \(I_{sc}\) and the genistein-stimulated bumetanide-sensitive \(I_{sc}\) (Fig. 10). These data strongly suggest that genistein’s activation on Cl\(^-\) secretion in the mouse jejunum is via a PDE3- and PKA-dependent mechanism.

We strongly believe that CFTR is the apical Cl\(^-\) channel involved in the genistein-activation Cl\(^-\) secretion. However, two Ca\(^{2+}\)-activated Cl\(^-\) channels (CLCA) have been recently identified in the mouse jejunum, CLCA3 and CLCA6. CLCA3 is located in the goblet cells of the small intestine (villi and crypts) (45), while CLCA6 is isolated only in the villi of the small intestine (8, 23). We do not believe CLCA3 plays a role in the genistein response for a number of reasons. First, we have evidence that pretreating mouse jejunal tissues with genistein did not alter the effect of activation of \(I_{sc}\) by carbachol (CCH, a Ca\(^{2+}\) and PKC-dependent acetylcholine analog); thus, CCH had a synergistic effect on \(I_{sc}\) in the presence of genistein (Hamilton KL and Baker MJ, unpublished observations, \(n = 6–12\)). These data suggested that genistein did not stimulate a Ca\(^{2+}\)-dependent Cl\(^-\) secretory response in the mouse jejunum. Second, we demonstrated that the effect of genistein was not affected by a Ca\(^{2+}\)-activated K\(^+\) channel blocker, suggesting that genistein may not influence intracellular Ca\(^{2+}\), or the activation of Ca\(^{2+}\)-activated K\(^+\) channel (KCa3.1), also suggesting that the action of genistein may not involve CLCA channels. Third, Sears et al. (61) demonstrated a similar synergistic effect of genistein and CCH on Cl\(^-\) secretion in T84 cells, suggesting different pathways of Cl\(^-\) secretion (61). Fourth, Cliff and Frizzell (14) demonstrated in T84 cells that two separate Cl\(^-\) conductances were activated by cAMP and Ca\(^{2+}\) segretagogues, suggesting separate Cl\(^-\) channels involved in Cl\(^-\) secretion depending on the second messenger. And lastly, we have not located any references suggesting that genistein can modulate any CLCA channel. Thus, it is our view that the genistein-stimulated \(I_{sc}\), in the current study, is Cl\(^-\) secretion via CFTR.

We are the first to demonstrate that genistein regulates Cl\(^-\) secretion via PDE3 in the mouse jejunal epithelium; however, others have demonstrated that PDE3 modulates Cl\(^-\) secretion. For epithelia, Kelley et al. (39) first described the effect of inhibition of PDEs on CFTR-dependent Cl\(^-\) transport in epithelial cells (Calu-3 cells, a human airway-derived epithelial cell line). Indeed, they noted that various inhibitors of PDE3 and PDE4 caused only meager changes in cAMP levels, even though milrinone activated a significant Cl\(^-\) efflux and bumetanide-sensitive Cl\(^-\) secretion. Kelley et al. (39) suggested that the inconsistency between the effects of inhibitors of PDEs on cAMP and on transport function may reflect the compartmentalized action of PDEs, and that PDE3 was located where it could have the greatest influence on CFTR, for example. In their study, Kelley et al. (39) did not identify the compartment(s) (cytosolic or membrane) where PDE3 was located. However, PDE3 has been localized within the cellular membrane of other epithelia (52, 58). For example, O’Grady and coworkers (52) demonstrated that PDE3 only inhibited 25% of the cAMP-phosphodiesterase activity of the membrane fraction. But PDE3 played a significant role in the Cl\(^-\) secretory response of T84 cells. Also, Cobb et al. (15) reported that inhibition of PDE3 increased \(I_{sc}\) of Calu-3 cells in the absence of changes in cAMP, again, suggesting compartmentalization of the function of PDE3. So, modulation of Cl\(^-\) secretion by inhibition of PDE3 clearly occurs in epithelia.

From our results, one might contend that the genistein-stimulated \(I_{sc}\) is dependent on an elevation of cAMP. This may be the case; however, the ability of genistein to alter cellular levels of cAMP has been debated over the years. In epithelia, many have demonstrated that elevated levels of cAMP are not required for a sustained genistein-stimulated Cl\(^-\) secretory response (35–37, 44, 61). However, some have reported that genistein increased cAMP levels (9). The studies referenced here have been conducted in cultured cell lines where there is ease of isolation and quantity of isolated cells. Unfortunately, the anatomical structure of the jejunum is complex, with an elaborate arrangement of the crypts and villi (13). The crypt region of the jejunum is the primary site for Cl\(^-\) secretion (70). Therefore, to determine the effects of genistein on the cAMP levels of the cellular and membrane fractions of the crypt cells, it would require the isolation of large numbers of viable crypts from the villi. In the past, we have isolated mouse crypts for patch-clamp studies (11, 29, 31). In our hands, the crypt yield was adequate for the patch-clamp experiments; however, the quantity of isolated crypts would not be sufficient to determine cAMP levels. Therefore, at this time, we are unable to determine whether genistein alters cAMP levels within the crypt cell.

**Genistein reduction of \(I_{sc}\) in the presence of milrinone?** Since we proposed that genistein and milrinone shared a common pathway in activating Cl\(^-\) secretion, we were perplexed when genistein reduced the \(I_{sc}\) in the presence of
milrinone, suggesting that genistein might be inhibiting Cl− secretion (Fig. 6, C and F). Here, we discuss two possible interpretations of the effect of genistein on Isc in the presence of milrinone.

First, it has been reported that genistein can have both stimulatory and inhibitory effects on CFTR depending on the concentrations of genistein used (43, 66, 68). Our milrinone-genistein results could be interpreted to suggest that genistein binds directly to nucleotide-binding domain 1 of CFTR in the presence of milrinone, thus, reducing the opening rate of CFTR, as described by Hwang and colleagues (66). They (66) first demonstrated that genistein enhanced the channel current through CFTR in a concentration-dependent manner up to 35 μM, while channel current fell with higher concentrations. Such an action by genistein could result in the reduced Isc observed in the presence of milrinone (Fig. 6, C and F), although, in our study, this may not be the case, for the following reasons. First, we have previously reported that genistein increased Isc in a concentration-dependent (1–125 μM) manner with no indication of a reduction in the Isc for the mouse (4). Additionally, we have used genistein at concentrations as high as 200 μM with no indication of an inhibitory effect on Isc (Chao PC and Hamilton KL, unpublished observations), suggesting that, under our experimental conditions, genistein may not be directly inhibiting the action of CFTR, and thus, does not explain the reduced Isc in the presence of milrinone.

Alternatively, a simple interpretation for the reduced Isc by genistein in the presence of milrinone would be that genistein inhibited basolateral K+ channels. For instance, if K+ channels were inhibited by genistein, then the cellular potential would become more positive and this effect would reduce the driving force for Cl− exit via CFTR, resulting in a decrease in Cl− secretion. However, we have previously demonstrated that KCa3.1 was not involved in the genistein-stimulated Cl− secretory response of the mouse jejunal (4). Unfortunately, we were unable to locate any reports of the action of genistein on the cAMP-dependent K+ channel (Kc;7,1) in epithelia. Nonetheless, genistein did not alter swelling-induced currents via Kc;7,1 channels, which were expressed in COS7 cells (41).

At present, our milrinone-genistein results are intriguing and require further study to elucidate the functional interactions of milrinone and genistein on the Cl− secretory response of the mouse jejunal.

In summary, we describe, for the first time, that genistein’s stimulatory action on Cl− secretion in the mouse jejunal is PDE3 and PKA dependent. Our study provides additional basic functional information of the action of genistein in modulating the Cl− secretory response in a native epithelium. We need to understand the basic action of genistein on ion transport in epithelia to further the potential pharmacotherapeutic development and use of genistein in the treatment of diseases of epithelia.

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REFERENCES


