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European Journal of Pharmacology 299 (1996) 161–170

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Modulation of Cl^- secretion in rat distal colon by genistein, a protein tyrosine kinase inhibitor

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Received 21 August 1995; revised 20 November 1995; accepted 1 December 1995

Abstract

The protein tyrosine kinase inhibitor, genistein, caused an increase of short-circuit current (*Isc*) across the rat distal colon in forskolin-pretreated tissues, suggesting a synergistic interaction of the drug with cAMP-dependent secretion. In the absence of forskolin, genistein had a dual effect on *Isc*, it increased *Isc* in tissues with a low baseline, but decreased *Isc* in tissues with a high baseline *Isc*. The secretory effect of genistein was dependent on the presence of Cl^- and was blocked by inhibitors of Cl^- secretion like bumetanide, an inhibitor of the Na^+-K^+-Cl^- cotransporter, or 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), a Cl^- channel blocker. Unidirectional flux measurements revealed that genistein inhibited Na^+ and Cl^- absorption and induced net Cl^- secretion. The protein tyrosine phosphatase inhibitor vanadate suppressed the secretory effect of genistein. In contrast, genistein caused an inhibition of carbachol-induced, i.e. Ca^{2+}-mediated secretion. Whole-cell patch-clamp experiments confirmed the synergistic effect of genistein on cAMP-induced Cl^- currents. In the presence of forskolin, genistein caused a depolarization concomitant with an increase in membrane inward current. In addition, genistein caused an inhibition of a basal K^+ conductance and inhibited the Ca^{2+}-dependent K^+ conductance stimulated by carbachol. These results suggest a complex role of the protein tyrosine kinase pathway in the control of colonic Cl^- secretion, an antagonistic action on the cAMP pathway and a synergistic action on the Ca^{2+} pathway as revealed by the opposing effects of genistein. The physiological importance of this regulation remains to be clarified.

Keywords: Colon, rat; Cl^- channel; K^+ channel; Ca^{2+}; Genistein; Protein tyrosine kinase

1. Introduction

Classically, Cl^- secretion is under the control of three cell signalling systems, i.e. the cAMP pathway, the Ca^{2+} pathway and the cell volume (Worell et al., 1989; Cliff and Frizzell, 1990). There are differences in the mode of action among the three signalling pathways in colonic tumour cells and native colonic epithelium. For example, in colonic tumour cells, a direct activation of Cl^- channels by intracellular Ca^{2+} has been shown (Morris and Frizzell, 1993), whereas in intact rat colonic epithelium the action of Ca^{2+} is restricted to the activation of a Ca^{2+}-dependent K^+ conductance stimulating Cl^- secretion indirectly by increasing the driving force for Cl^- exit (Böhme et al., 1991; Strabel and Diener, 1995). Differences exist also in the localization of the volume-sensitive Cl^- channels, i.e. mainly apical in colonic tumour cells like T84 cells (MacEwan et al., 1992), and mainly basolateral in the native rat colonic epithelium (Diener et al., 1992). In principle, however, Cl^- currents across the apical or the basolateral membrane can be driven by all three pathways in native or cultured colonic epithelial cells.

Recently, it was found that also the protein tyrosine kinase pathway is involved in the regulation of ion currents. Genistein, an inhibitor of protein tyrosine kinases (Akiyama and Ogawara, 1991), stimulated J^- efflux, an indirect measure for Cl^- permeability, and Cl^- currents in 3T3 fibroblasts expressing the cystic fibrosis transmembrane regulator (CFTR) Cl^- channel (Illek et al., 1995). The inhibitor greatly enhanced the sensitivity of the cells to forskolin, a stimulator of the adenylate cyclase, pointing to an inhibitory effect of the protein tyrosine kinase on the cAMP pathway, which is removed by genistein. In addition, genistein has been shown to inhibit Ca^{2+} entry stimulated by carbachol in HT29/B6 cells (Bischof et al., 1995). Nothing is known about the effect of genistein on

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SSDI 0014-2999(95)00832-2

normal colonic epithelium. Therefore, in the present study, the effect of genistein on short-circuit current, unidirectional ion fluxes and whole-cell ion currents across the rat distal colon was studied.

2. Materials and methods

2.1. Solutions

For the experiments with isolated crypts the following buffers were used. The EDTA solution for the crypt isolation contained (mmol · l^{-1}): NaCl 107, KCl 4.5, NaH_2PO_4 0.2, Na_2HPO_4 1.8, $NaHCO_3$ 25, EDTA (ethylenediaminotetraacetic acid) 10, glucose 12, with 0.1% bovine serum albumin (w/v). The pH was adjusted to 7.4 by Tris-base (tris(hydroxymethyl)aminomethane). The high K^+ Tyrode for the storage of the crypts consisted of (mmol · l^{-1}): K gluconate 100, KCl 30, NaCl 20, $CaCl_2$ 1.25, $MgCl_2$ 1, Hepes (*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid) 10, glucose 12, Na pyruvate 5, 0.1% bovine serum albumin (w/v). The solution was adjusted with KOH to a pH of 7.4. The medium for the superfusion of the crypts was a Ringer solution containing (mmol · l^{-1}): NaCl 140, KCl 5.4, $CaCl_2$ 1.25, $MgSO_4$ 1, Hepes 10. The pipette solution was a K gluconate/KCl solution, which contained (mmol · l^{-1}): K gluconate 100, KCl 30, NaCl 10, $MgCl_2$ 2, EGTA (ethyleneglycol bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid) 0.1, Tris 10, ATP (adenosine 5'-triphosphate disodium salt) 5; pH was 7.2.

The Ussing chamber experiments were carried out in a bathing solution containing (mmol · l^{-1}): NaCl 107, KCl 4.5, $NaHCO_3$ 25, Na_2HPO_4 1.8, NaH_2PO_4 0.2, $CaCl_2$ 1.25, $MgSO_4$ 1 and glucose 12. The solution was gassed with carbogen (5% CO_2 in 95% O_2) and kept at a temperature of 37°C; pH was 7.4. For the Cl^--free buffer, NaCl was replaced with Na gluconate; the Ca^{2+} concentration in this solution was elevated to 5.8 mmol · l^{-1} in order to compensate the Ca^{2+}-buffering properties of gluconate (Kenyon and Gibbons, 1977).

2.2. Tissue preparation

Female SIVZ-50 rats (Institut für Labortierkunde, Universität Zürich, Switzerland) were used with a weight of 180–220 g. The animals had free access to water and food until the day of the experiment. Animals were stunned by a blow on the head and killed by exsanguination. The serosa and muscularis propria were stripped away by hand to obtain the mucosa-submucosa preparation of the distal part of the colon descendens.

2.3. Short-circuit current measurement

The tissue was fixed in a modified Ussing chamber (Andres et al., 1985), bathed with a volume of 4 ml on each side of the mucosa and short-circuited by a voltage clamp (Aachen Microclamp, AC Copy Datentechnik, Aachen, Germany) with correction for solution resistance. The exposed surface of the tissue was 1 cm^2. Short-circuit current (Isc) was continuously recorded and tissue conductance (Gt) was measured every minute. From these values the open-circuit potential difference (Pd) was calculated according to the law of Ohm.

The baseline in electrical parameters was determined as the mean over 3 min just before administration of a drug. In the tables, the maximal increase in Isc induced by genistein was expressed as difference from the former baseline (ΔIsc).

2.4. Measurement of unidirectional ion fluxes

After an equilibration period of 60 min, ^{22}Na (59 kBq) and ^{36}Cl (29 kBq) were added to one side (= labelled side) of the epithelium. After an additional 20 min to allow isotope fluxes to reach a steady state, unidirectional ion fluxes were determined over 3 sequential 20 min periods starting 20 min after administration of the drug to be studied. From the measured unidirectional fluxes net ion fluxes were calculated according to: $J_{net} = J_{ms} - J_{sm}$. Residual ion flux, i.e. the sum of the movement of all ions other than Na^+ and Cl^-, was calculated according to: $J^R_{net} = Isc - (J^{Na}_{net} - J^{Cl}_{net})$. A positive J^R_{net} indicates either the absorption of a cation or the secretion of an anion.

2.5. Crypt isolation

The mucosa-submucosa was fixed on a plastic holder with tissue adhesive and transferred for 8 min in the EDTA solution. The mucosa was vibrated once for 30 s in order to isolate intact crypts. They were collected in an intracellular-like high K^+ Tyrode buffer (Böhme et al., 1991). The mucosa was kept at 37°C during the isolation procedure. All further steps including the patch experiments were carried out at room temperature.

2.6. Patch-clamp experiments

The crypts were pipetted into the experimental chamber, a silicon ring attached to a glass slide. The volume of the chamber was about 0.4 ml. The crypts were fixed to the glass bottom of the chamber with the aid of poly-L-lysine (0.02%, w/v). The preparation was superfused hydrostatically throughout the experiment, perfusion rate was about 1 ml · min^{-1}. The chamber was mounted on the stage of an inverted microscope (Olympus IMT2-F).

Patch pipettes were pulled from thick-walled borosilicate glass capillaries (Jencons Scientific, Bedfordshire, UK, outer diameter 2 mm, inner diameter 1–1.25 mm) on a two-stage puller (H. Ochotzki, Homburg/Saar, Germany). After fire-polishing, the tips had resistances of 5–10 MΩ, when filled with the standard pipette solution.

To obtain a conventional whole-cell recording, the membrane patch under the tip of the pipette was broken by a stronger suction pulse after formation of the seal. In the experiments with carbachol, when chemically perforated patches were used, the pipette tip was prefilled with the normal pipette solution by dipping it into this solution for 8–10 s. The pipette was then backfilled with a solution containing nystatin (300 $\mu g \cdot ml^{-1}$) using a syringe (Böhme et al., 1991). Opening of the patch was indicated by an increase of the capacitance, a decrease of the resistance and a stable membrane potential under current-clamp conditions. Seal resistances were 2–10 GΩ. The capacitance of the patch membrane was compensated for.

Patch-clamp currents were recorded on an RK-400 amplifier (Biologics, Meylan, France). Current and voltage signals were digitized at 48 kHz and stored on a modified digital audio recorder (DTR-1200, Biologics, Meylan, France). The reference point for the patch potentials was the extracellular side of the membrane assumed to have zero potential. Current-voltage (I-V) curves were obtained by clamping the cell to a holding potential of −80 mV and stepwise depolarization for 30 ms. After each depolarization, the cell was clamped again to the holding potential for 1 s. For statistical comparison of membrane currents, outward current was measured at the end of a pulse depolarizing the cell for 30 ms from −80 to +60 mV, inward current was measured at the holding potential of −80 mV.

2.7. Drugs

Daidzein (Biomol, Hamburg, Germany), genistein, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; gift from R. Greger, Physiologisches Institut, Freiburg, Germany), and nystatin were dissolved in dimethyl sulfoxide (DMSO; final maximal concentration 0.25%, v/v). Bumetanide, forskolin, and indomethacin were added from ethanolic stock solutions (final maximal concentration 0.25%, v/v). Tetrodotoxin was dissolved in citrate buffer (5 mg per mg tetrodotoxin; pH 4.3). Carbachol and sodium orthovanadate (Anawa, Wangen, Switzerland) were dissolved in aqueous stock solutions diluted in salt buffer just before use. If not indicated differently, drugs were from Sigma, Buchs, Switzerland. Radioisotopes were obtained from NEN, Dreieich, Germany, and specific activities were 3.9 TBq/g ^{22}Na and 550 MBq/g ^{36}Cl.

2.8. Statistics

Results are given as means ± one standard error of the mean (S.E.). The significance of differences was tested by analysis of variances and, if indicated, by paired or unpaired two-tailed Student's *t*-test or an *U*-test, respectively. An *F*-test was applied to decide which test method was to be used. The quality of linear regressions was checked by the linear correlation coefficient (*r*). Standard errors for calculated values, i.e. net ion transport, were calculated according to the law of error propagation.

3. Results

3.1. Synergistic action of genistein and forskolin on Isc

The effect of genistein was tested in the absence and presence of forskolin. Tissues were pretreated with indomethacin (10^{-6} mol · l^{-1} at the mucosal and the serosal side) in order to suppress the production of prostaglandins, which are known to stimulate colonic cAMP formation (see e.g. Craven and DeRubertis, 1983a). Orientating experiments revealed that all effects of genistein could be induced by serosal as well as mucosal administration.

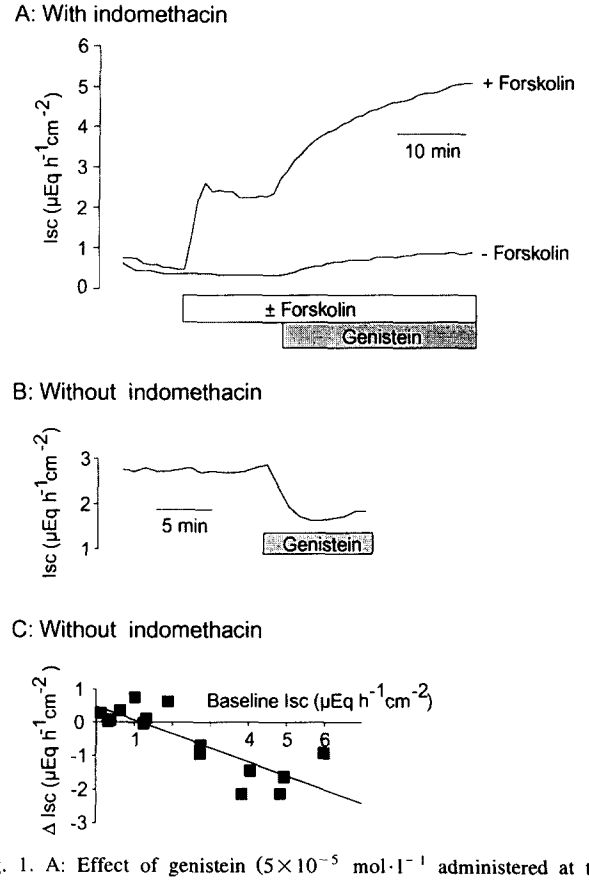

Fig. 1. A: Effect of genistein (5×10^{-5} mol·l^{-1} administered at the mucosal and the serosal side) on Isc in the absence (lower tracing) and presence (upper tracing) of forskolin (2×10^{-7} mol·l^{-1} administered at the mucosal and the serosal side). Tissues were pretreated with indomethacin (10^{-6} mol·l^{-1} at the mucosal and the serosal side) in order to inhibit the endogenous production of prostaglandins. The tracings are representative for 5–6 experiments with similar results; for statistics see Table 1. B: Effect of genistein on control tissue without indomethacin pretreatment. The tracing is representative for 7 out of 15 tissues, in which genistein induced a decrease in *I*sc. C: Dependence of the genistein effect in the absence of indomethacin on the baseline *I*sc just before administration of genistein. The solid line gives the linear correlation according to: $\Delta Isc = 0.48 - 0.41 \times \text{baseline}$ ($r = 0.82$; $n = 15$; $P < 0.05$).

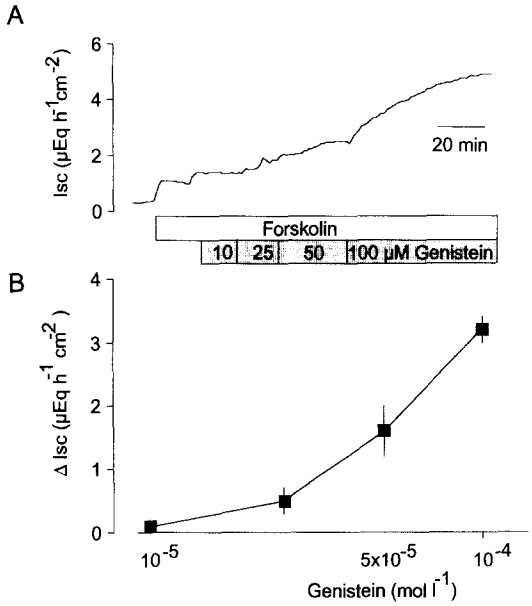

Fig. 2. Concentration dependence of the effect of genistein (administered at the mucosal and the serosal side) on *I*sc. Tissues were pretreated with indomethacin (10^{-6} mol·l^{-1} at both sides) and forskolin (2×10^{-7} mol·l^{-1} at both sides). Original tracing (A) and means ± S.E. (B) expressed as differences from the baseline just before administration of the genistein, $n = 7$.

Therefore, the experiments were performed by administration of the drug on both sides of the tissue.

In the absence of forskolin, genistein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side) induced only a small increase in *I*sc, which developed slowly over 20–30 min (Fig. 1). *I*sc increased finally to a value of 0.3 ± 0.1 μEq·h^{-1}·cm^{-2} ($P < 0.05$, $n = 6$) above baseline (Table 1). The effect of genistein was dramatically enhanced in the presence of a low concentration of forskolin. Forskolin (2×10^{-7} mol·l^{-1} at the mucosal and the serosal side) itself increased *I*sc by 3.4 ± 0.6 μEq·h^{-1}·cm^{-2} ($P < 0.05$, $n = 5$) to a stable plateau (Fig. 1). When genistein was administered in the combined presence of indomethacin and forskolin, it further increased *I*sc by 3.1 ± 0.7 μEq·h^{-1}·cm^{-2} ($P < 0.05$ versus effect in the absence of forskolin, $n = 5$). The increase in *I*sc was paralleled by an increase in *P*d and *G*t (Table 1).

Table 1
The effect of genistein depends on the pretreatment

Pretreatment	ΔIsc (μEq·H^{-1}·cm^{-2})	ΔGt (mS·cm^{-2})	ΔPd (mV)	n
Control	-0.5 ± 0.3	-0.3 ± 0.3	-0.9 ± 0.6	15
Tetrodotoxin	1.3 ± 0.3 [a,b]	1.4 ± 0.8	2.9 ± 0.8 [a,b]	6
Indomethacin	0.3 ± 0.1 [a]	2.8 ± 1.0 [a]	0.3 ± 0.1	6
Indomethacin/ forskolin	3.1 ± 0.7 [a,b]	1.7 ± 0.5 [a]	5.4 ± 1.2 [a,b]	5

Genistein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side) was administered under control conditions, in the presence of tetrodotoxin (10^{-6} mol·l^{-1} at the serosal side), indomethacin (10^{-6} mol·l^{-1} at the mucosal and the serosal side), and in the combined presence of indomethacin and forskolin (2×10^{-7} mol·l^{-1} at the mucosal and the serosal side). Values are given as differences to the baseline just before genistein administration and are means ± S.E., [a] $P < 0.05$ versus baseline, [b] $P < 0.05$ versus genistein response in the presence of indomethacin.

When genistein was administered to untreated control mucosae, the drug exerted quite variable effects. In control tissue, i.e. in the absence of indomethacin, baseline *I*sc was higher (2.4 ± 0.5 μEq·h^{-1}·cm^{-2} in the absence and 0.6 ± 0.2 μEq·h^{-1}·cm^{-2} in the presence of indomethacin, $P < 0.05$, $n = 13$–15). Under these conditions, genistein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side), induced an increase in *I*sc in only 6 out of 15 tissues. In contrast, 7 out of 15 tissues responded with a decrease (Fig. 1B), and in 2 out of 15 tissues no response was observed. The overall response, a decrease in *I*sc of -0.5 ± 0.3 μEq·h^{-1}·cm^{-2}, was not statistically different from zero (Table 1). However, the response to genistein in the absence of indomethacin showed a dependence on the baseline *I*sc just before administration of the drug. Tissues with a low baseline responded with an increase in *I*sc, tissues with a high baseline with a decrease. Both parameters could be linearly correlated ($r = 0.82$, $P < 0.05$; Fig. 1C).

The effect of genistein was unaffected by the neurotoxin, tetrodotoxin. In tissues pretreated with tetrodotoxin (10^{-6} mol·l^{-1} at the serosal side), genistein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side caused a homogenous increase in *I*sc, which amounted to 1.3 ± 0.3 μEq·h^{-1}·cm^{-2} (Table 1).

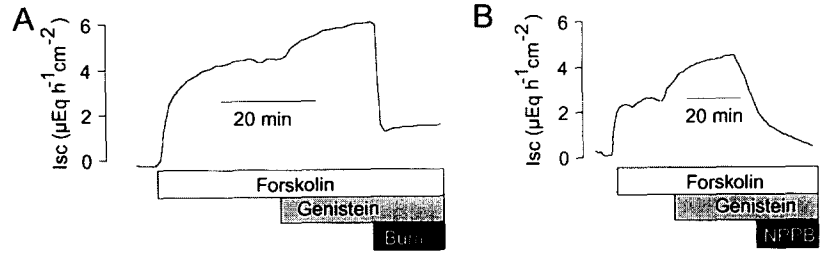

Fig. 3. Inhibition of the effect of genistein (5×10^{-5} mol·l^{-1}) by bumetanide (10^{-4} mol·l^{-1} at the serosal side; A) and NPPB (10^{-4} mol·l^{-1} at the mucosal side; B). Tissues were pretreated with indomethacin (10^{-6} mol·l^{-1} at the mucosal and the serosal side) and forskolin (2×10^{-7} mol·l^{-1} at the mucosal and the serosal side). The tracings are representative for 6–7 experiments with similar results; for statistics see Table 2.

Table 2
Effect of inhibitors on the genistein response

	ΔIsc ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$)	ΔGt ($mS \cdot cm^{-2}$)	ΔPd (mV)	n
Genistein	2.5 ± 0.9 [a]	2.9 ± 1.4	3.1 ± 1.0 [a]	7
+ Bumetanide	-4.7 ± 0.8 [b]	0.1 ± 0.9	-8.3 ± 1.2 [b]	7
Genistein	1.7 ± 0.2 [a]	2.3 ± 0.9 [a]	2.4 ± 0.5 [a]	6
+ NPPB	-3.6 ± 0.2 [b]	2.0 ± 2.1	-7.7 ± 1.8 [b]	6
Genistein	2.0 ± 0.6 [a]	2.0 ± 0.3 [a]	2.4 ± 0.8 [a]	6
+ Tetrodotoxin	0.5 ± 0.1 [b]	1.1 ± 0.5	0.2 ± 0.3	6
Genistein	2.6 ± 0.9 [a]	1.9 ± 0.5 [a]	4.1 ± 1.2 [a]	6
+ Vanadate	-2.5 ± 0.7 [b]	4.9 ± 4.3	-5.6 ± 1.2 [b]	6
Genistein Cl^--free	0.2 ± 0.1 [c]	0.6 ± 0.3	0.3 ± 0.3 [c]	8
Genistein after vanadate	0.6 ± 0.1 [c]	0.6 ± 0.4	1.1 ± 0.1 [c]	8

Response to genistein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side) in tissues pretreated with indomethacin (10^{-6} mol·l^{-1} at the mucosal and the serosal side) and forskolin (2×10^{-7} mol·l^{-1} at the mucosal and the serosal side). Inhibitors used were: bumetanide (10^{-4} mol·l^{-1} at the serosal side), NPPB (10^{-4} mol·l^{-1} at the mucosal side), tetrodotoxin (10^{-6} mol·l^{-1} at the serosal side), vanadate (10^{-4} mol·l^{-1} at the mucosal and the serosal side). In the first 4 series, the inhibitor was administered during the genistein-induced plateau in Isc (cf. Fig. 3A), in the last two series, the inhibitor (or the ion substitution) was administered in pretreatment. Values are differences from the Isc just before administration of the respective drug and are means ± S.E., [a] $P < 0.05$ versus baseline, [b] $P < 0.05$ versus genistein, [c] $P < 0.05$ versus genistein response after pretreatment with indomethacin/forskolin without any inhibitor.

3.2. Concentration dependence of the secretory effect of genistein

All experiments designed to characterize the secretory effect of genistein were conducted in tissues pretreated with indomethacin (10^{-6} mol·l^{-1}) and a low concentration of forskolin (2×10^{-7} mol·l^{-1}) to obtain optimal conditions for the stimulatory effect of genistein on Isc. When genistein (10^{-5}–10^{-4} mol·l^{-1}) was administered cumulatively in increasing concentrations during the plateau in Isc induced by forskolin, a concentration-dependent increase in Isc was observed (Fig. 2A and B). In parallel, Gt and Pd increased. The threshold concentration, at which the first significant effects were observed, was 2.5×10^{-5} mol·l^{-1}. The response in Isc increased until the highest concentration tested (10^{-4} mol·l^{-1}). For all further experiments, an intermediate concentration of genistein (5×10^{-5} mol·l^{-1}) was chosen.

3.3. Ionic nature of the secretory response

The effect of genistein was suppressed by bumetanide (10^{-4} mol·l^{-1} at the serosal side), an inhibitor of the basolateral Na^+-K^+-Cl^- cotransporter, when the drug was administered during the genistein-induced plateau in Isc (Fig. 3A; Table 2). It was also suppressed by NPPB (10^{-4} mol·l^{-1} at the mucosal side; Fig. 3B; Table 2), a Cl^- channel blocker (Wangemann et al., 1986). Both inhibitors decreased Isc at nearly the level of the pre-forskolin period. When Cl^- was substituted with an impermeant anion, gluconate, the effect of genistein was also greatly reduced. In the absence of Cl^-, forskolin (2×10^{-7} mol·l^{-1} at the mucosal and the serosal side) still induced a small increase in Isc (0.5 ± 0.2 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$, $n = 8$, $P < 0.05$). Administration of genistein (5×10^{-5} mol·l^{-1} at both sides), however, had no more effect on Isc (Table 2).

3.4. Unidirectional flux measurements

In the presence of indomethacin (10^{-6} mol·l^{-1} at the mucosal and the serosal side) the mucosa-to-serosa fluxes (J_{ms}) of Na^+ and Cl^- exceeded the corresponding serosa-to-mucosa fluxes (J_{sm}) leading to a net absorption of both ions (Table 3). Forskolin (2×10^{-7} mol·l^{-1} at the mucosal and the serosal side) appeared to inhibit J_{ms}^{Na} and J_{ms}^{Cl}, while J_{sm}^{Cl} appeared to increase; none of these effects reached statistical significance at this low concentration of forskolin. Genistein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side), administered in the presence of forskolin, significantly reduced J_{ms}^{Na} and J_{ms}^{Cl}. At the same time, J_{sm}^{Cl} was stimulated, i.e. genistein inhibited Na^+ and Cl^- absorption and induced a net Cl^- secretion (Table 3).

3.5. Mediation of the genistein effect

When the neurotoxin tetrodotoxin (10^{-6} mol·l^{-1} at the serosal side) was administered during the plateau of the Isc response induced by genistein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side), no decrease in Isc was observed (Table 2), confirming that the drug does not exert its secretory action by a stimulation of enteric neurons. In contrast, the action of genistein was inhibited by vanadate (10^{-4} mol·l^{-1} at the mucosal and the serosal side), a protein tyrosine phosphatase inhibitor (Gordon, 1991). Inhibition was observed when vanadate was used in pretreatment as well as when the blocker was administered during the genistein-induced plateau in Isc (Table 2). In the latter case, a paradoxical increase in Isc preceded the inhibition, a phenomenon which was not investigated further due to its transient nature.

Daidzein, a genistein analogue without activity on the protein tyrosine kinase (Akiyama and Ogawara, 1991), was completely ineffective. Daidzein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side), administered with the same protocol as genistein, i.e. after pretreatment of the tissue with indomethacin and forskolin, caused a decrease in Isc of -0.3 ± 0.2 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ and in Gt of -0.2 ± 0.5 $mS \cdot cm^{-2}$ ($n = 7$, not significant).

3.6. Interaction with carbachol

The above results suggest that genistein enhances the effect of cAMP-induced Cl^- secretion. In order to investi-

Table 3
Effect of genistein on unidirectional $^{22}Na^+$ and $^{36}Cl^-$ fluxes

	J_{ms}^{Na} (μEq·h^{-1}·cm^{-2})	J_{sm}^{Na} (μEq·h^{-1}·cm^{-2})	J_{net}^{Na} (μEq·h^{-1}·cm^{-2})	J_{ms}^{Cl} (μEq·h^{-1}·cm^{-2})	J_{sm}^{Cl} (μEq·h^{-1}·cm^{-2})	J_{net}^{Cl} (μEq·h^{-1}·cm^{-2})	Isc (μEq·h^{-1}·cm^{-2})	J_{net}^{R} (μEq·h^{-1}·cm^{-2})	Gt (mS·cm^{-2})
Indomethacin	18.1 ± 2.6	5.3 ± 0.6	12.8 ± 2.6	23.8 ± 2.6	11.4 ± 1.7	12.4 ± 3.1	1.2 ± 0.2	0.7 ± 4.0	9.3 ± 0.5
Forskolin	13.3 ± 1.4	7.3 ± 0.9	6.1 ± 1.7	19.3 ± 2.5	12.4 ± 0.9	6.9 ± 2.7	3.9 ± 0.4 [a]	4.7 ± 3.2	12.3 ± 1.3 [a]
Genistein	9.3 ± 0.8 [a]	6.6 ± 0.8 [a]	2.7 ± 1.1	13.6 ± 1.5 [a]	16.1 ± 2.8 [a]	−2.6 ± 3.2	6.6 ± 0.4 [a]	6.4 ± 3.4	14.2 ± 1.6 [a]

Concentrations of drugs were: indomethacin (10^{-6} mol·l^{-1} at the mucosal and the serosal side), forskolin (2×10^{-7} mol·l^{-1} at the mucosal and the serosal side), genistein ($5 \times x10^{-5}$ mol·l^{-1} at the mucosal and the serosal side). Isc and Gt values are means of the complete 20 min period. Values are means ± 1 S.E., $n = 10$. [a] $P < 0.05$ versus indomethacin period.

Table 4
Interaction of genistein with carbachol

	ΔIsc ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$)	ΔGt ($mS \cdot cm^{-2}$)	ΔPd (mV)	n
Carbachol	11.4 ± 1.8	11.0 ± 2.3	10.1 ± 1.4	6
Carbachol after genistein	6.2 ± 1.0 [a]	6.2 ± 0.3	11.1 ± 2.2	7

Effect of carbachol (5×10^{-5} mol·l^{-1} at the serosal side) in the absence and presence of genistein (5×10^{-5} mol·l^{-1} at the mucosal and the serosal side). Values are differences from the baseline just before administration of carbachol and are means ± S.E., [a] $P < 0.05$ versus carbachol response under control conditions.

gate the interaction of genistein with Ca^{2+}-induced secretion, the effect of the cholinergic agonist, carbachol, was tested. In the absence of genistein, carbachol (5×10^{-5} mol · l^{-1} at the serosal side) caused an increase in *I*sc, which amounted to 11.4 ± 1.8 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ above baseline ($n = 6$, $P < 0.05$). The effect of carbachol was diminished by about 40%, when carbachol was applied in the presence of genistein ($n = 7$, $P < 0.05$; Table 4).

3.7. Whole-cell patch-clamp recordings

In order to investigate the effect of genistein on Cl^- currents directly, whole-cell patch-clamp recordings were performed at isolated colonic crypts. The protocol was the same as for the Ussing chamber experiments with the exception that the pretreatment with indomethacin was omitted, because the prostaglandin synthesis only takes place quantitatively in the submucosa (Craven and DeRubertis, 1983b), which is not present in the isolated crypt preparation.

Basal membrane potential (pooled from cells at different locations along the crypt axis) amounted to −57.4 ± 4.2 mV ($n = 7$). Forskolin (2×10^{-7} mol · l^{-1}) induced a depolarization to −38.5 ± 4.0 mV ($P < 0.05$; Fig. 4A). This depolarization has been shown to be due to the opening of apical Cl^- channels (Böhme et al., 1991). In accordance with previous data (Böhme et al., 1991), measurements of I-V relations revealed an increase in membrane inward current induced by forskolin. Membrane inward current, measured at −80 mV, i.e. close to the K^+ equilibrium (−84 mV with 130 mmol · l^{-1} K^+ in the pipette and 5.4 mmol · l^{-1} in the perfusion solution), where K^+ currents will not contribute to membrane current, increased by 92.1 ± 47.1 pA ($n = 6$), although this effect did not reach statistical significance for this low concentration of forskolin (Fig. 4B and C). Membrane outward current, however, was only slightly enhanced by forskolin, probably because of the inhibitory action of the drug on basolateral K^+ conductance (Diener et al., unpublished observations).

When genistein (5×10^{-5} mol · l^{-1}) was administered in the presence of forskolin, a further depolarization to −30.0 ± 3.8 mV was observed ($n = 7$; $P < 0.05$ versus forskolin alone; Fig. 4A). This depolarization was associated with a complex change in membrane current. Inward current (measured at −80 mV), which was already increased by forskolin, further increased by 43.1 ± 14.1 pA ($n = 6$, $P < 0.05$ versus forskolin alone), whereas membrane outward current (measured at +60 mV) decreased by 207.3 ± 71.5 pA ($n = 6$, $P < 0.05$; Fig. 4B and C). These effects are not compatible with the assumption that the only action of genistein consists in an enhancement of cAMP stimulation of a Cl^- conductance, but suggests an additional inhibition of a K^+ conductance by the drug.

In order to investigate this assumed effect on K^+ conductance further, the interaction of genistein with carbachol, which opens a Ca^{2+}-dependent K^+ conductance in

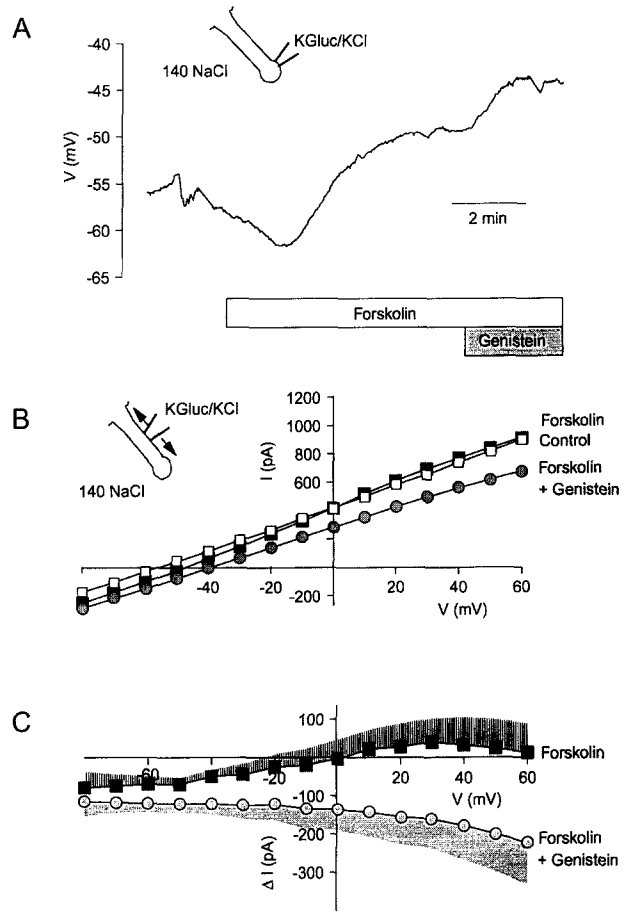

Fig. 4. A: Effect of forskolin (2×10^{-7} mol·l^{-1}) and genistein (5×10^{-5} mol·l^{-1}) on the membrane potential of a crypt cell. The cell was located at the lower third of an isolated crypt as indicated by the schematic (conventional whole-cell recording). The tracing is representative for 7 experiments with similar results. The line interruptions in the voltage tracing are caused by the measurements of I-V relations in the voltage-clamp mode. B: I-V relation under control conditions (open squares), in the presence of forskolin (2×10^{-7} mol·l^{-1}; black squares), and the combined presence of forskolin and genistein (5×10^{-5} mol·l^{-1}; grey circles). Values are means pooled from 7 cells at different positions along the crypt axis as indicated by the schematic. C: Forskolin-stimulated (black squares) and genistein-induced current (grey circles) obtained after subtraction of the current just before administration of the respective drug. Values are means (symbols) ± S.E. (shaded area), $n = 7$.

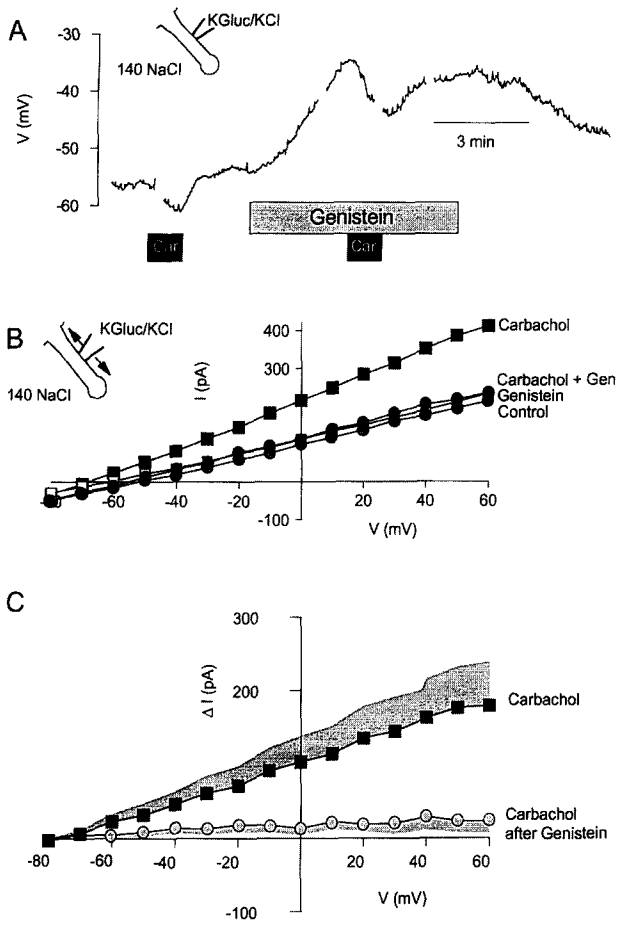

Fig. 5. A: Effect of carbachol (5×10^{-5} mol·l^{-1}) and genistein (5×10^{-5} mol·l^{-1}) on the membrane potential of a crypt cell. The cell was located at the middle of an isolated crypt as indicated by the schematic (nystatin-permeabilized patch). The tracing is representative for 8 experiments with similar results. The line interruptions in the voltage tracing are caused by the measurements of I-V relations in the voltage-clamp mode. B: I-V relation under control conditions (open squares), in the presence of carbachol (5×10^{-5} mol·l^{-1}; black squares), genistein (5×10^{-5} mol·l^{-1}; grey circles), and the combined presence of forskolin and genistein (black circles). Values are means pooled from 8 cells at different positions along the crypt axis as indicated by the schematic. C: Carbachol-stimulated current in the absence (black squares) and presence (grey circles) of genistein obtained after subtraction of the current during the period just before administration of carbachol. Values are means (symbols) ± S.E. (shaded area), $n = 8$.

the isolated colonic crypts (Böhme et al., 1991; Diener, 1994), was tested. Carbachol (5×10^{-5} mol·l^{-1}) caused a membrane hyperpolarization of -8.4 ± 1.6 mV ($n = 8$, $P < 0.05$; Fig. 5A). This hyperpolarization was associated with an increase in membrane outward current, which increased by 178.8 ± 59.4 pA at $+60$ mV ($n = 8$, $P < 0.05$). Membrane inward current (measured at -80 mV) was not changed, a result which is in accordance with previous results from our laboratory that carbachol has no direct effect on Cl^- conductance (Diener, 1994; Strabel and Diener, 1995). The effects of carbachol were reversible after washing out (Fig. 5). Genistein (5×10^{-5} mol·l^{-1}), when administered after washing away the cholinergic agonist, caused a depolarization of 12.2 ± 4.6 mV ($n = 8$, $P < 0.05$; Fig. 5A). This depolarization was associated with an increase in membrane inward current, which increased by 20.1 ± 7.7 pA at -80 mV ($n = 8$, $P < 0.05$; Fig. 5B). Membrane outward current, however, showed an inhomogeneous response: both increases as well as decreases were observed and the resultant decrease in membrane current at $+60$ mV of -19.9 ± 38.6 pA was not significantly different from zero.

When carbachol was administered in the presence of genistein, the drug still induced a significant hyperpolarization of -6.0 ± 1.9 mV ($n = 8$, $P < 0.05$; Fig. 5A), which was not significantly smaller than the voltage response induced by carbachol in the absence of genistein. However, the increase in membrane outward current was reduced by more than 85% ($P < 0.05$ versus the response to carbachol under control conditions; Fig. 5B and C). The effect of genistein was reversible after washing out the inhibitor (Fig. 5A). Consequently, genistein inhibits the activation of the Ca^{2+}-dependent K^+ conductance by carbachol.

4. Discussion

Genistein, a protein tyrosine kinase inhibitor competing with ATP at the enzyme (Casnellie, 1991), caused a concentration-dependent increase in *I*sc in tissues pretreated with a low concentration of forskolin (Fig. 1A and Fig. 2). Ion substitution experiments and sensitivity to bumetanide (Fig. 3A), an inhibitor of the Na^+-K^+-Cl^- cotransporter, or NPPB (Fig. 3B), a Cl^- channel blocker, suggest that this increase in *I*sc was due to Cl^- secretion. This was confirmed by the measurement of unidirectional fluxes. These experiments revealed that genistein in addition inhibited the absorptive, mucosa-to-serosa fluxes of Na^+ and Cl^- (Table 3) as shown already in the rabbit small intestine (Donowitz et al., 1994). Consequently, inhibition of the protein tyrosine kinase by genistein potentiates the effect of a stimulation of the cAMP pathway. In the absence of forskolin, genistein had quite variable effects. When baseline *I*sc was spontaneously low or was lowered pharmacologically with indomethacin, the protein tyrosine kinase inhibitor caused a small increase in *I*sc (Fig. 1A). However, in tissues with a high spontaneous *I*sc, genistein decreased *I*sc (Fig. 1B and C) suggesting that the drug can also exert antisecretory effects (see below). Secretion induced by genistein was blocked by vanadate, a protein tyrosine phosphatase inhibitor (Gordon, 1991; Table 2). Daidzein, a genistein analogue without activity on the protein tyrosine kinase, was ineffective, suggesting that the effects of genistein are indeed mediated by an inhibition of protein tyrosine kinase(s), although nonspecific actions of the drug like e.g. inhibition of topoisomerases or of other enzymes (see Akiyama and Ogawara, 1991) cannot be completely ruled out.

The effects of genistein were not mediated by enteric secretomotor neurons, because the *I*sc induced by genistein was resistant to the neurotoxin, tetrodotoxin (Table 2), suggesting a direct epithelial site of action of the drug.

Patch-clamp experiments at isolated colonic crypts confirmed that genistein had a direct effect on the colonic epithelial cells. In unstimulated cells, i.e. under control conditions, genistein induced a membrane depolarization (Fig. 5A) concomitant with a small increase in membrane inward current (Fig. 5B), suggesting an increase in Cl^- conductance. Consequently, genistein has a stimulatory effect on Cl^- currents also independent from the cAMP pathway, an observation which is in accordance with the results of Illek et al. (1995) obtained at transfected 3T3 fibroblasts. However, when the cAMP pathway was stimulated, the effect of genistein was markedly enhanced. When the crypts were pretreated by a low concentration of forskolin, which itself induces Cl^- secretion by the opening of apical Cl^- channels mediated by the protein kinase A (Böhme et al., 1991), the drug induced a further depolarization concomitant with an increase in membrane inward current (Fig. 4). This increase in membrane inward current is consistent with the assumption that genistein activates a Cl^- conductance. Membrane outward current, measured at +60 mV, i.e. at a potential where both Cl^- and K^+ currents contribute to the current, was reduced (Fig. 4C), suggesting a decrease in K^+ conductance by the drug.

This inhibitory action of genistein on K^+ conductance was proven directly, when studying the effect of genistein on the response to carbachol. The cholinergic agonist induces a biphasic increase in intracellular Ca^{2+} in the isolated crypts (Diener et al., 1991) and thereby opens a Ca^{2+}-dependent K^+ conductance (Böhme et al., 1991). In the presence of genistein, the hyperpolarization induced by carbachol was only slightly reduced, indicating that even in the presence of genistein the K^+ conductance becomes the dominating membrane conductance after stimulation with carbachol (Fig. 5A). However, the increase in membrane outward (K^+) current was strongly reduced (Fig. 5B and C). Consequently, genistein inhibits the activation of the Ca^{2+}-dependent K^+ conductance by carbachol. A plausible explanation may be the reduction of Ca^{2+} influx by genistein as observed in human platelets (Sargeant et al., 1993) or colonic HT29/B6 cells (Bischof et al., 1995), although a direct effect on the K^+ channels cannot be excluded. This inhibitory action on K^+ conductance is probably the reason for the reduction of membrane outward current by genistein, e.g. in the presence of forskolin (Fig. 4C).

The inhibitory effect of genistein may probably also be responsible for the apparently paradoxical *I*sc response in those tissues that exhibited a high spontaneous *I*sc, which was decreased by the protein tyrosine kinase inhibitor (Fig. 1B). Baseline *I*sc in the rat distal colon has been shown to be stimulated by the continuous activity of secretomotor neurons of the submucosal plexus (Andres et al., 1985). These transmitters, among them mainly acetylcholine, may continuously cause the stimulation of Ca^{2+}-dependent K^+ channels and thereby support a basal Cl^- secretion, an effect which is inhibited by genistein. The secretomotor neurons of the submucosal plexus are stimulated by a continuous release of prostaglandins, e.g. prostacyclin (Diener et al., 1988). Consequently, the inhibitory action of genistein is not observed after blockade of the secretomotor neurons by tetrodotoxin or after suppression of their stimulation by indomethacin (Table 1).

In conclusion, genistein exerts a dual action on Cl^- secretion in the rat colon: it interacts synergistically with the cAMP pathway and antagonistically with the Ca^{2+} pathway, suggesting a negative control of cAMP-mediated and an enhancement of Ca^{2+}-mediated secretion by protein tyrosine kinase(s). What is the physiological relevance of this modulatory effect of the protein tyrosine kinase pathway? Different cytosolic or membrane-associated non-receptor forms as well as transmembrane receptor forms of protein tyrosine kinases have been described (Cantley et al., 1991; Gordon, 1991; Thuveson et al., 1995). It is not known which were actually responsible for the observed effects on electrolyte transport. These enzymes are involved in the regulation of cell growth, differentiation and other processes (Carpenter and Cohen, 1990; Glenney, 1992; Heruth et al., 1995). It turns out, now, that this pathway has in addition a modulatory role on electrolyte transport, i.e. protein tyrosine kinase inhibits cAMP-mediated secretion, increases NaCl absorption, and stimulates Ca^{2+}-dependent secretion as indicated by the opposing effects of genistein, the inhibitor of this pathway. However, a natural agonists of this pathway, which regulates ion secretion via protein tyrosine kinases, is not yet known. Potential candidates may be e.g. growth factors (Donowitz et al., 1994), insulin or interleukin-3, which are known to exert their action mediated by protein tyrosine phosphorylation (see e.g. Carpenter and Cohen, 1990; Heruth et al., 1995). Consequently, the physiological importance of the regulation of Cl^- secretion by the protein tyrosine kinase pathway remains to be elucidated.

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