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STIMULATION OF SECRETION BY THE T_{84} COLONIC EPITHELIAL CELL LINE WITH DIETARY FLAVONOLS

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Abstract—Flavonols are dietary compounds widely distributed in plants and characterized by a 2-phenyl-benzo(α)pyrane nucleus possessing hydroxyl and ketone groups at positions 3 and 4, respectively. Kaempferol, quercetin, and myricetin are flavonols that are further mono-, di-, or trihydroxylated on the phenyl ring, respectively. To test whether these ingested flavonols might exert a direct secretory effect on intestinal epithelial cells, monolayers of the T_{84} colonocyte cell line were mounted in Ussing chambers and examined for ion transport response. Twenty minutes after addition of 100 μM quercetin to either the serosal or mucosal side, the short-circuit current change was maximal at 16.6 $\mu A/cm^2$. Kaempferol was less potent than quercetin, while myricetin and glycosylated quercetin (rutin) did not induce secretion. The secretion induced by quercetin did not seem to be mediated by the reactive oxygen species generated by quercetin through auto-oxidation and/or redox cycling (superoxide, hydrogen peroxide, and the hydroxyl radical) because it was neither enhanced by iron, nor inhibited by desferroxamine B or catalase (alone or in combination with superoxide dismutase). Like vasoactive intestinal peptide, quercetin induced a secretory response that was inhibited by barium chloride and bumetanide, and which exhibited synergism with carbachol. Quercetin also stimulated a modest increase in intracellular cAMP levels and the phosphorylation of endogenous protein substrates for cAMP-dependent protein kinase. Thus, quercetin is a potent stimulus of colonocyte secretion that resembles secretagogues which act via a cAMP-mediated signaling pathway.

Flavonoids constitute a class of compounds which contain the basic structural feature of a 2-phenyl-benzo(α)pyrane nucleus (Fig. 1). Either as free aglycones or more commonly glycosylated at carbons 3, 4, or 7, these compounds are universally distributed among vascular plants where they may serve as natural transport regulators for the plant growth substance auxin [1]. Flavonols are a subgroup of the flavonoids, characterized by a hydroxyl group at position 3 and a ketone at position 4. Flavonols can be further hydroxylated at positions 3′, 4′ or 5′ on the β phenyl ring to yield the 4′-monohydroxy-flavonol kaempferol, the 3′,4′-dihydroxy-flavonol quercetin, and the 3′,4′,5′-trihydroxy-flavonol myricetin (Fig. 1). Both quercetin and myricetin produce reactive oxygen species (superoxide, hydrogen peroxide, and hydroxyl radical) through auto-oxidation and redox cycling [2–4]. Since reactive oxygen species may induce intestinal secretion [5, 6], we examined the possibility that flavonols might act on the intestinal epithelial cell to stimulate ion transport.

In this study, the colonic epithelial cell line T_{84} was used as a model to study the effects of flavonols on the enterocyte. These cells grow as well-differentiated monolayers which exhibit vectorial chloride secretion when mounted in Ussing chambers and exposed to a variety of neurohormonal stimuli [7]. Chloride secretion, monitored by a change in the short-circuit current (Isc) necessary to nullify the potential difference across the cell monolayer, is stimulated by agents which increase cAMP, such as vasoactive intestinal peptide (VIP) or prostaglandin E_1 [8, 9], and also by agents which act through Ca^{2+}-mediated pathways, such as carbachol, histamine and calcium ionophores [10, 11]. Chloride secretion occurs through Cl^- channels located on the apical membrane of confluent monolayers [12], and is the result of the Cl^- electrochemical gradient generated by the concerted action of three transporters: the basolateral Na^+,K^+,Cl^- co-transporter, the Na^+,K^+-ATPase pump, and K^+ channels [9]. The Na^+ and K^+ imported into the cell by the co-transporter are recycled to the extracellular compartment, respectively, by the Na^+,K^+-ATPase pump and by at least two distinct K^+ efflux channels (activated separately by VIP and by carbachol). Because the secretory response in T_{84} cells is well characterized and reflects the direct action of secretagogues on the enterocyte, we chose this model to characterize the effects of dietary flavonols on colonic secretion.

MATERIALS AND METHODS

Chemicals. Quercetin, kaempferol, myricetin, rutin, barium chloride, superoxide dismutase (SOD) (from bovine erythrocytes), catalase (from bovine liver), desferroxamine B, carbachol, and Fe(III)-EDTA were obtained from Sigma, St. Louis, MO. Bumetanide was provided by Biomol Research Laboratories, Plymouth Landing, PA. VIP was from Peninsula, Belmont, CA. ^{32}P as inorganic phosphate

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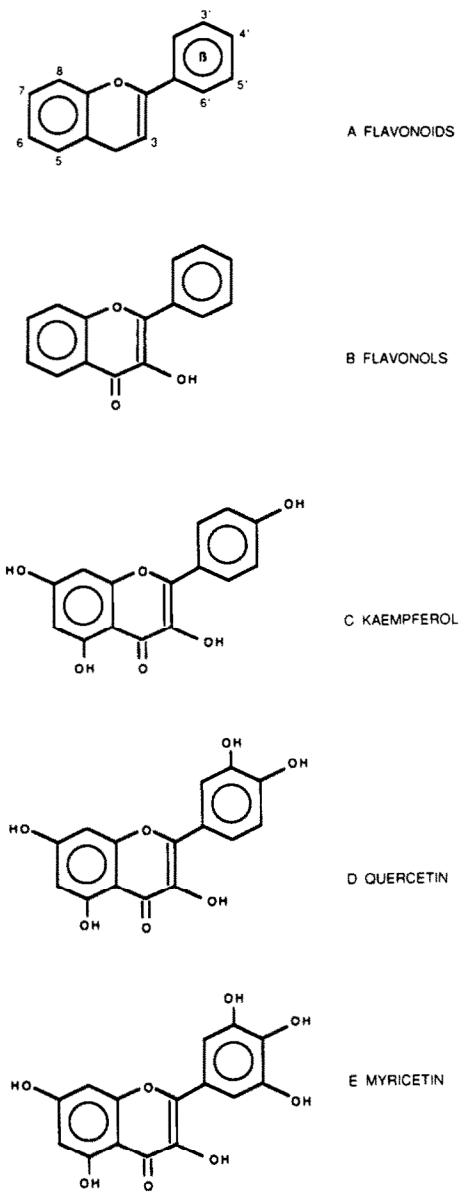

Fig. 1. Chemical structures of flavonoids (A), flavonols (B), kaempferol (C), quercetin (D), and myricetin (E).

was obtained from ICN, Irvine, CA. Culture medium was obtained from the Tissue Culture Facility of the University of North Carolina, Chapel Hill, NC, or from Gibco, Grand Island, NY.

Growth and maintenance of T_{84} cells. T_{84} cells were provided by Dr. K. Dharmsathaphorn (University of California, San Diego). These cells were cultured at 5% CO_2 and 37° in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 5% (v/v) newborn calf serum. Cells were seeded onto collagen-coated Nuclepore filters previously glued onto plastic rings (surface area: 2.9 cm^2, approximately 10^6 cells/filter). These filters were then set on glass beads to allow medium (supplemented with 5000 units/L penicillin, and 5000 μg/L streptomycin sulfate) to bathe both sides of the cell monolayer. The confluent monolayers used for secretory studies were maintained for at least 7 days after the filters were seeded.

Secretory studies. The cell monolayers on the filter/ring units were mounted in a modified Ussing chamber as previously described [7], and both sides of the monolayer were bathed with a Ringer's solution containing 115 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.4 mM KH_2PO_4, 2.5 mM K_2HPO_4, 25 mM $NaHCO_3$, and 10 mM glucose. Quercetin, myricetin, and kaempferol were dissolved in ethanol and added to the Ringer's solution at a 1:100 dilution (final concentrations of ethanol 1%). The medium was warmed to 37° with a circulating water jacket and gently mixed and oxygenated with a constant inflow of 95%O_2/5%CO_2. During secretory studies, spontaneous tissue potential differences were short-circuited by an automatic voltage clamp (model DVC-1000, World Precision Instruments, New Haven, CT) with Ag-$AgCl_2$ electrodes, and the current necessary to maintain this short circuit (Isc) recorded at 1-min intervals. Instrument calibration was performed prior to each experiment using a filter/ring unit without cells. All comparative studies used matched pairs of monolayers seeded at the same time and studied concurrently. Spot checks of the T_{84} monolayers after completion of the experiments showed that cells exposed to 100 μM quercetin for > 1 hr could still exclude trypan blue.

Cellular protein phosphorylation. T_{84} cell protein phosphorylation responses were studied using methods previously described [13]. Briefly, cells were labeled with $^{32}P_i$ in a phosphate-free buffer, exposed to 100 μM quercetin for 5 min, scraped from the filters, and homogenized with a glass–Teflon homogenizer. Phosphoproteins contained in the supernatant fraction after centrifugation at 436,000 *g* for 15 min were precipitated with 10% trichloroacetic acid and resolved by two-dimensional polyacrylamide gel electrophoresis. Phosphoproteins were detected by autoradiography using X-OMAT AR5 film exposed at room temperature.

cAMP Assay. T_{84} cells grown to confluence on filters were washed twice with Ringer's solution and immersed in 15 mL of Ringer's solution supplemented with 0.2 mM 3-isobutyl-1-methylxanthine and 10 mM glucose, warmed to 37°, and equilibrated with 95% O_2, 5% CO_2. Quercetin (final concentration 100 μM) or VIP (final concentration 1 nM) was added to the solution, and after different time periods, the filters containing the cells were transferred into 12 mm × 75 mm plastic tubes and placed in liquid nitrogen. The cAMP was then extracted by boiling the cells for 7 min in 1 mL of 5 mM KH_2PO_4, 5 mM K_2HPO_4, 1 mM EDTA, and 0.1 mM 3-isobutyl-1-methylxanthine. The supernatant resulting from centrifugation at 15,000 *g* for 7.5 min was assayed for cAMP according to the method described by Gettys *et al.* [14].

RESULTS

Stimulation of secretion with flavonols. As shown in Fig. 2, addition of 100 μM quercetin to the mucosal

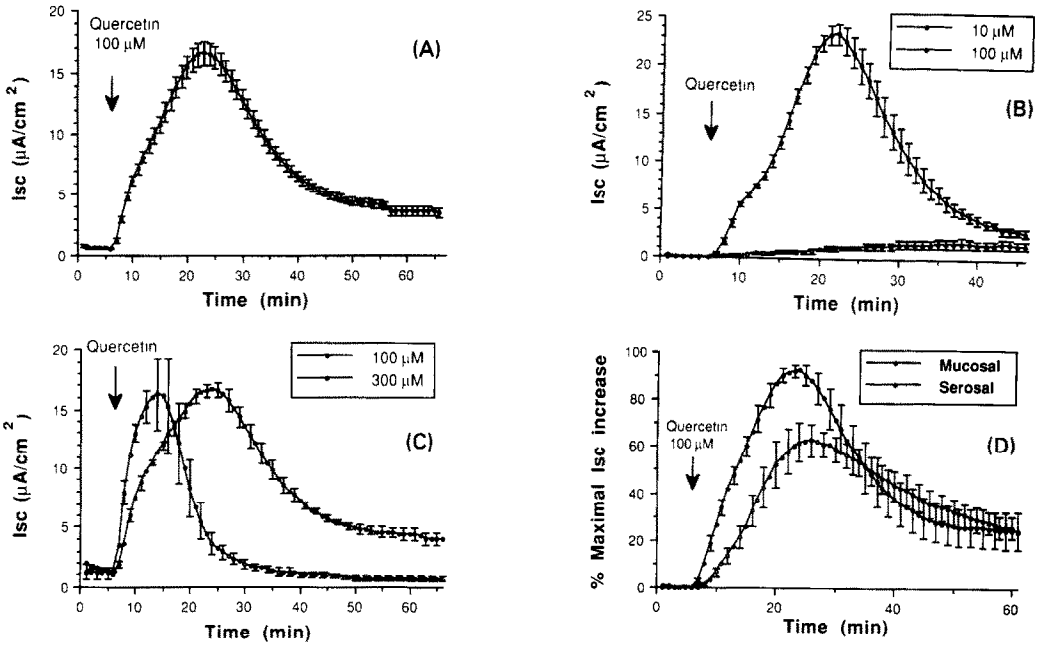

Fig. 2. Secretory effect of quercetin. T_{84} monolayers were grown to confluence on semipermeable membranes, mounted on modified Ussing chambers, and stimulated with quercetin as described in Materials and Methods. The resulting Isc's were recorded every minute and the resulting means and SEM (alternated for clarity in panels C and D) shown. Panel A (top left): Incubation with 100 μM quercetin added to the mucosal compartment (1–36 min: N = 47; 37–66 min: N = 20–46). Panel B (top right): Incubation with either 10 or 100 μM quercetin added to the mucosal compartment (three matched pairs). Panel C (bottom left): Incubation with either 100 or 300 μM quercetin added to the mucosal compartment (three matched pairs). Panel D (bottom right): Incubation with 100 μM quercetin added to either the serosal or the mucosal side of the T_{84} cell monolayer. Within each of the seven matched pairs, the Isc increases were normalized using the maximal Isc increase; the resulting means and SEM are shown [mean maximal Isc response: 53 ± 8.7 μA (18 μA/cm^2)].

side of the cell monolayer produced an increase in Isc which peaked after 15–20 min to a maximum value above baseline of 16.6 μA/cm^2(SEM = 0.9 μA/cm^2, N = 48, 48.1 μA/filter). The Isc was unaffected in control filters exposed to ethanol alone at a final concentration of 1%.

A threshold response to quercetin was obtained at 10 μM (Fig. 2B). The maximal Isc increase produced by 300 μM quercetin was the same as that produced by 100 μM, but the response was more rapid in onset and shorter in duration (Fig. 2C). As shown in Fig. 2D, quercetin stimulated a similar secretory response whether added to the serosal or the mucosal side of the monolayer. However, the maximal response elicited from the serosal side was only 68% of the maximal response obtained from the mucosal side (P = 0.01 for a smaller serosal response, paired two-tailed *t*-test with 6 df, mean maximal mucosal Isc response: 11.9 ± 1.9 μA/cm^2).

The effects of flavonols structurally related to quercetin were also investigated. The maximal Isc increase observed with 100 μM kaempferol was 26 ± 2% of the maximal increase obtained with 100 μM quercetin (three paired experiments). For myricetin, secretion was detected only at 300 μM and not at 100 μM. A minimal response was obtained with a 300 μM concentration of the glycosylated quercetin-rutinoside (rutin) (data not shown).

Effects of modulators of the metabolism of reactive oxygen species on quercetin-stimulated chloride secretion. It is possible that the reactive oxygen species (superoxide, hydrogen peroxide, or hydroxyl radical) produced by quercetin upon auto-oxidation and/or redox cycling may mediate its secretory effect. In this case, the Isc response should be altered by compounds or enzymes which modulate the production or degradation of these species. Quercetin-induced secretion was therefore studied after cells were preincubated with superoxide dismutase (SOD) (to enhance the conversion of superoxide to hydrogen peroxide), catalase (to enhance the conversion of hydrogen peroxide to water and oxygen), iron (to facilitate the Haber–Weiss reaction in which hydrogen peroxide is converted to hydroxyl radical), or desferroxamine B (to chelate iron and prevent the Haber–Weiss reaction). If quercetin-induced secretion is mediated by hydrogen peroxide, then catalase should inhibit the secretory response to quercetin. Similarly, if secretion is mediated by hydroxyl radical, then the Isc response should be inhibited by desferroxamine and enhanced by Fe^{3+}. Finally, if extracellular superoxide is the key mediator, then secretion should be blocked by the combination of SOD and catalase. As shown in Table 1, little effect on quercetin-induced secretion was observed after preincubation with 50 μM

Table 1. Effects of modulators of the metabolism of reactive oxygen species on quercetin-induced secretion in T_{84} cells

Agent	Maximal Isc (% control)	N	P (≠ 100)
SOD (470 units/mL) + catalase (450 units/mL)	104 ± 12.6	6	0.78
Catalase (450 units/mL)	117 ± 7.8	6	0.08
Desferroxamine B (50 μM)	97 ± 5.6	6	0.68
Fe^{3+} (50 μM)	75 ± 9.2	6	0.04

T_{84} cells mounted in modified Ussing chambers were incubated with the different modulators of reactive oxygen species metabolism for 10–15 min, and quercetin was added to a final concentration of 100 μM. In each matched pair, the maximal short-circuit current (Isc) change induced by quercetin in the presence of the modulator is expressed as the percentage of the maximal Isc change induced by quercetin in the absence of the modulator. Values are means ± SEM. The mean maximal Isc changes in the control monolayers were, respectively, 21.9 ± 3.3, 15 ± 2.5, 15.8 ± 1.66, and 18.3 ± 2.7 μA/cm² for the experiments studying the effects of SOD plus catalase, catalase, desferroxamine B, and Fe^{3+}. P values were calculated using two-tailed *t*-tests with 5 df.

desferroxamine B or a combination of 470 units/mL SOD and 450 units/mL catalase. A slight enhancement in secretion, which did not reach statistical significance ($0.1 > P > 0.05$), was noted with 450 units/mL catalase, while 50 μM Fe(III)-EDTA produced a significant ($P < 0.05$) inhibition of quercetin-induced secretion. However, as discussed previously, these last two effects were opposite of what was expected should either hydrogen peroxide or the hydroxyl radical mediate secretion. In the aggregate, these findings do not support a role for reactive oxygen species in the secretory response of quercetin.

Intracellular mechanism of secretion. T_{84} cell apical chloride secretion is dependent on the chloride gradient across the mucosal membrane of the cell. This gradient is generated by the basolateral Na^+,K^+,Cl^- co-transporter with the imported Na^+ and K^+ recycled outside the cell by the Na^+,K^+-ATPase pump and K^+ efflux channels [15]. The role of these transport systems in quercetin-induced chloride secretion was studied using bumetanide, which inhibits the Na^+,K^+,Cl^- co-transporter, and barium chloride, which inhibits a VIP-responsive K^+ channel [10, 16]. Table 2 demonstrates the inhibitory effects of 0.3 mM bumetanide and 6 mM $BaCl_2$ on the secretory response elicited by 100 μM quercetin. Compared with matched controls, the Isc response obtained 20 min after addition of quercetin was only 19 ± 1% and 31 ± 5% of the expected response for bumetanide and barium chloride, respectively. Thus, quercetin-induced secretion appears to require active Na^+,K^+,Cl^- co-transport and K^+ efflux mechanisms.

One approach to determining which intracellular signalling pathway(s) mediates the quercetin secretory response is to evaluate whether quercetin exhibits synergism when administered with other secretagogues. The interactions between carbachol, VIP, and quercetin were therefore studied. In Fig. 3A, cells were exposed either to carbachol (final concentration 10 μM) or to quercetin (final concentration 50 μM). After 15 min, quercetin was added to the cells previously exposed to carbachol and vice versa. In these matched pairs, the effect of

Table 2. Effects of ion transport inhibitors on quercetin-induced secretion in T_{84} cells

Agent	Isc (% control)	N	P (≠ 100)
Bumetanide (0.3 mM)	19 ± 1	4	0.0001
Barium chloride (6 mM)	31 ± 5	4	0.0007

T_{84} cells were incubated for 15 min with either bumetanide or barium chloride, and quercetin was added to a final concentration of 100 μM. In each matched pair, the Isc change induced by quercetin after 20 min in the presence of the inhibitor is expressed as the percentage of the Isc change induced by querecetin alone. Bumetanide was dissolved in 0.1 M NaOH and added to the cells at a 1:100 dilution; the corresponding control also contained 1:100 dilution of 0.1 M NaOH. Values are means ± SEM. The maximal Isc responses in the control monolayers were 12.1 ± 0.59 and 13.4 ± 1.2 μA/cm², respectively, for the experiments studying the effects of bumetanide and barium chloride. P values were calculated using two-tailed *t*-tests with 3 df.

quercetin followed by carbachol (or carbachol followed by quercetin) in one monolayer can be compared to the initial effect of quercetin (or carbachol) alone in the other. Analyzed in this fashion, quercetin alone produced an Isc increase of 9.4 ± 0.4 μA/cm² (27 ± 1.2 μA, N = 3) after 15 min, while carbachol produced an Isc increase of 2.3 ± 1 μA/cm² (7 ± 3.5 μA, N = 3) after 3 min. Carbachol added to cells responding maximally to quercetin produced an additional Isc increase of 34.9 ± 5 μA/cm² (101 ± 14.4 μA) resulting in a combined total Isc increase of 44.4 ± 5 μA/cm² (129 ± 14.4 μA). Quercetin added to cells previously exposed to carbachol produced an Isc increase of 19.1 ± 1.7 μA/cm² (55 ± 4.8 μA). However, in the latter case, quercetin was added after completion of the carbachol response and the interaction between quercetin and carbachol may be suboptimal. In additional experiments, the secretory response produced by a simultaneous dose of carbachol and

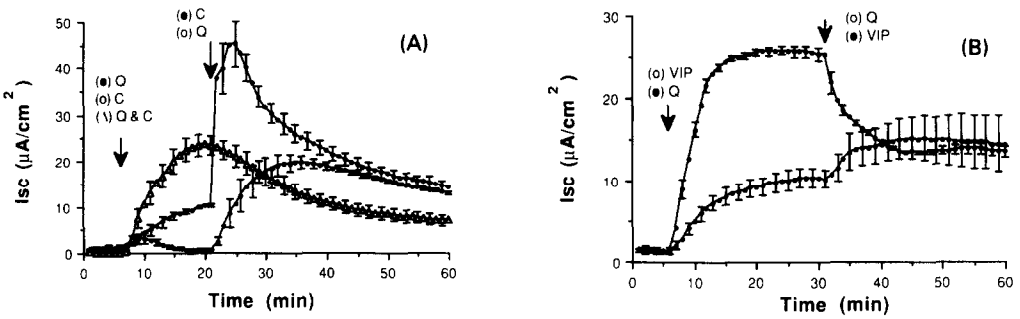

Fig. 3. Interactions between quercetin, carbachol and VIP. Pairs of confluent T_{84} monolayers were mounted in modified Ussing chambers and their secretory responses (mean Isc and SEM) compared. Panel A (left panel): In each pair, one monolayer was exposed to 10 μM carbachol [C] (serosal surface) or to 50 μM quercetin [Q] (mucosal surface); after 15 min, 50 μM quercetin was added to the monolayer previously exposed to carbachol and vice versa. Additional monolayers were also exposed to a combined concentration of 10 μM carbachol and 50 μM quercetin [Q&C]. The following symbols are used: (●) quercetin followed by carbachol (N = 3), (○) carbachol followed by quercetin (N = 3), (△) quercetin and carbachol added simultaneously (N = 5). Panel B (right panel): In each pair, one monolayer was exposed to 1 nM VIP (serosal surface) and the other to 50 μM quercetin [Q] (mucosal surface). After 15 min, 50 μM quercetin was added to the monolayer previously exposed to VIP and vice versa. The following symbols are used: (●) quercetin followed by VIP (N = 3), (○) VIP followed by quercetin (N = 3).

quercetin was also evaluated. A peak Isc with an intermediate value of $23.6 \pm 2.2\ \mu A/cm^2$ ($69 \pm 6.5\ \mu A$, N = 5) was obtained 14 min after addition of the combined secretagogues. In all the different sequences, the Isc increases produced by cells exposed to the combination of quercetin and carbachol were greater than the sum of the individual Isc changes produced by quercetin and carbachol ($9.4 \pm 2.3 = 11.7\ \mu A/cm^2$). The difference in the degrees of synergism probably reflects the different timing of the maximal effect of quercetin and carbachol. In contrast, as shown in Fig. 3B, when the interaction between quercetin and VIP was analyzed in the same fashion, no such synergism, but a possible inhibitory effect was demonstrated. The synergism between carbachol and quercetin, but not between VIP and quercetin, suggests that quercetin may induce secretion through pathways related to the ones activated by VIP, but not by carbachol. This possibility was explored further in the following phosphorylation studies.

Phosphorylation and intracellular cAMP studies. Previous studies have shown that T_{84} cells exhibit distinct phosphorylation responses to stimuli acting via cAMP or via Ca^{2+} [13, 17]. Phosphoproteins p83, p29, and p23 are examples of proteins exhibiting increased phosphorylation in cells stimulated by agents which act via Ca^{2+}, such as carbachol, histamine, and ionomycin. By contrast, phosphoproteins p37, p18, and p23 exhibit increased phosphorylation in cells exposed to agents which act via cAMP, such as VIP and forskolin. Each of these five phosphoproteins showed increased labeling in monolayers stimulated with forskolin plus carbachol (Fig. 4, comparing panels A and B). When monolayers were stimulated with 100 μM quercetin, only three of these five phosphorylation responses were observed: quercetin stimulated the phosphorylation of p37, p18, and p23, but not p29 or p83 (Fig. 4, comparing panels C and D). These results suggest that quercetin activates intracellular signaling mechanisms mediated by cAMP, but not by Ca^{2+}.

In an attempt to study whether quercetin induces the generation of cAMP, this second messenger was measured in cells exposed to 100 μM quercetin for different time periods ranging from 1 to 20 min. Surprisingly, as shown in Table 3, only a modest increase in cAMP levels was detected. These increases were minimal when compared with the mean 85-fold increase in cAMP demonstrated with control monolayers exposed to 1 nM VIP for the same time periods (data not shown).

DISCUSSION

We have demonstrated that quercetin is a potent stimulator of ion transport in T_{84} cells. When added to either the mucosal or serosal side of the cell monolayer, quercetin produced a concentration-dependent increase in Isc, which peaked 15–20 min after the addition of 100 μM quercetin. The observed Isc response is consistent with the possibility that quercetin can act directly on the enterocyte to stimulate electrogenic Cl^- secretion. Of the related hydroxylated flavonols, kaempferol was less potent than quercetin, while myricetin and the glycosylated quercetin-rutinoside (rutin) produced minimal responses. Considering the effective mucosal–serosal barrier, the observation that quercetin can act from either side of the monolayer suggests that its effect is not initially mediated by cellular surface components selectively present on either side of the cell (e.g. receptors). It is also possible that quercetin, being a small hydrophobic molecule, penetrated the cell and produced its effect intracellularly. The similar time courses of the secretory responses produced by the addition of quercetin to either side of the cell do not support the possibility that the effect of quercetin is localized to one side of the cell

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