Citrus Flavonoids Stimulate Secretion by Human Colonic T_{84} Cells[1,2]

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ABSTRACT Flavonoids, compounds containing a 2-phenylbenzo(γ)pyrane nucleus, are universally distributed among vascular plants. Even though flavonoids are ingested in a normal diet in average quantities of 1 g daily, their effects on the digestive system have only been recently investigated. This study used an in vitro model of colonic secretion, monolayers of T_{84} colonic adenocarcinoma cells mounted in Ussing chambers, to demonstrate that 100 μmol/L of either tangeritin or nobiletin, polymethoxylated flavonoids contained in citrus fruits, stimulated sustained electrogenic chloride secretion with a maximal short-circuit current of 3.3 μA/cm^2. In contrast, naringin and hesperidin, glycosylated citrus flavonoids, stimulated minimal secretion, suggesting that carbohydrate substitutions inhibited their secretory potential. The secretion stimulated by tangeritin and nobiletin was synergistic with carbachol but not with vasoactive intestinal peptide and was inhibited by barium chloride, bumetanide, H-89, and Cl^- depletion. These properties suggest that tangeritin and nobiletin stimulated Cl^- secretion via the cAMP pathway; however, these flavonoids did not stimulate cAMP production to the extent seen with vasoactive intestinal peptide. These flavonoids did not autooxidize, suggesting that reactive oxygen species did not mediate this secretion. These observations suggest that dietary citrus flavonoids may modulate colonic secretion, possibly through direct interaction with intracellular secretory pathways. J. Nutr. 123: 259–268, 1993.

INDEXING KEY WORDS:

- *human T_{84} cells* • *flavonoids*
- *secretion* • *tangeritin*
- *nobiletin*

Flavonoids, a class of compounds that have the basic structural feature of a 2-phenylbenzo(γ)pyrone nucleus (**Fig. 1***A*), are universally distributed among vascular plants, where they may serve as natural transport regulators for the plant growth substance auxin (Jacobs and Rubery 1988). Plant flavonoids are ingested in the normal human diet in quantities of 1 g or more a day (Brown 1980). Although these compounds may not be vitamins as previously postulated, they may constitute a class of semi-essential food components on the basis of their ubiquity, their essential role in insects, and their multiple biologic actions (Kuhnau 1976). Flavonoids have antioxidant and chelating properties and have been shown to modulate various cellular enzymes including hydrolases, transferases, hydroxylases, oxidoreductases and kinases (reviewed in Havsteen 1983, Middleton, Jr. 1984). Their potential use as antiinflammatory, antiviral, antitumor or antiallergenic agents has also been suggested (Bors et al. 1990, Middleton and Kandaswami 1992, Salup et al. 1992).

The effects of the dietary flavonoids on the function of the digestive system have only recently been investigated. We have previously observed that the 3,5,7,4′-quadrahydroxyflavone, kaempferol, the 3,5,7,3′,4′-pentahydroxyflavone, quercetin (Fig. 1*B*) and the 3,5,7,3′,4′,5′-hexahydroxyflavone, myricetin, stimulated electrogenic chloride secretion by colonic T_{84} cell monolayers in Ussing chambers. We also observed that this secretory potential was sharply dependent on the individual flavonoid structure because quercetin, with five hydroxyl substitutions, was more potent than kaempferol, which contains four hydroxyl substitutions, while myricetin, with six hydroxyl substitutions, was the least potent (Nguyen et al. 1991). Thus, the secretory potential of various classes of flavonoids must be evaluated separately.

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Flavonoids contained in citrus fruits include the polymethoxylated flavones concentrated in fruit peels such as the 5,6,7,8,4′-pentamethoxyflavone in tangerine, tangeritin (Fig. 1*C*) (Nelson 1934), and the 5,6,7,8,3′,4′-hexamethoxyflavone in orange, nobiletin (Fig. 1*D*) (Swift 1960). Tangeritin and nobiletin may be the main natural resistance factor against pathogenic fungi in citrus fruits (Ben-Aziz 1967). Naringin (Fig. 1*E*, 4′,5,7-trihydroxyflavanone-7-rhamnoglucoside) and hesperidin (Fig. 1*F*, 3′,5,7-trihydroxy-4′-methoxyflavanone-7-rhamnoglucoside) are examples of glycosylated flavanones also contained in citrus fruits (Pritchett and Merchant 1946). Because these flavonoids are natural constituents of a regular diet, especially those rich in citrus fruit flavoring or extracts, we studied their secretory potential in this report.

Secretory potential was evaluated using the T_{84} colonic adenocarcinoma cell line. T_{84} cells grow as well-differentiated and polarized monolayers that exhibit serosal-to-mucosal chloride secretion when studied in Ussing chambers after exposure to a variety of neurohormonal stimuli (Dharmsathaphorn et al. 1984). This secretion, monitored by a change in the short-circuit current (Isc)[3] necessary to nullify the difference in potential across the cell monolayer, is stimulated by agents that increase cAMP [e.g., vasoactive intestinal peptide (VIP) or prostaglandin E_1 (Dharmsathaphorn et al. 1985, Weymer et al. 1985)], cGMP [e.g., *E. coli* heat-stable enterotoxin (Huott et al. 1988)], or intracellular Ca^{2+} [e.g., carbachol, histamine and calcium ionophores (Dharmsathaphorn and Pandol 1986, Dharmsathaphorn et al. 1989)]. Chloride secretion occurs through activated chloride channels located on the apical membrane of the T_{84} cell. This secretion is dependent on the chloride gradient between the intracellular and extracellular spaces generated by the concerted actions of three types of transporters, the basolateral $Na^+,K^+,2Cl^-$ cotransporter, the Na^+,K^+-ATPase pump, and K^+ efflux channels. The Na^+ and K^+ cations imported into the cell by the $Na^+,K^+,2Cl^-$ cotransporter are respectively recycled to the extracellular compartment by the Na^+,K^+-ATPase pump and at least two different K^+ efflux channels (activated separately by VIP and by carbachol) (Fondacaro 1986). Because the secretory response in T_{84} cells is well characterized and reflects the direct action of different secretagogues on a well-defined and uniform population of cells, we chose this model to characterize the effects of the dietary citrus flavonoids on colonic secretion.

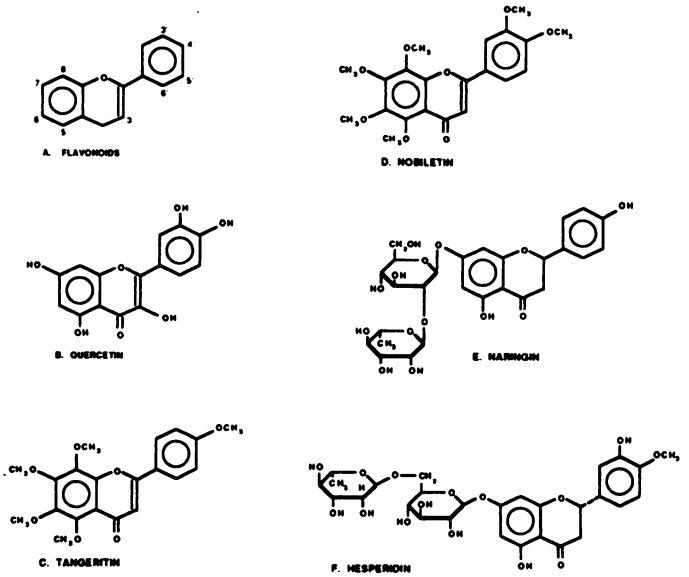

FIGURE 1 Chemical structure of the different flavonoids studied. *A*) 2-phenylbenzo(γ)pyrone nucleus of flavonoids, *B*) polyhydroxylated flavonol quercetin (3,5,7,3′,4′-pentahydroxyflavone), *C*) polymethoxylated citrus flavone, tangeritin (5,6,7,8,4′ pentamethoxyflavone), *D*) polymethoxylated citrus flavone, nobiletin (5,6,7,8,3′,4′ hexamethoxyflavone), *E*) glycosylated citrus flavone, naringin (4′,5,7-trihydroxyflavanone-7-rhamnoglucoside), *F*) glycosylated citrus flavone, hesperidin (3′,5,7-trihydroxy-4′-methoxyflavanone-7-rhamnoglucoside).

MATERIALS AND METHODS

Chemicals. Quercetin, carbachol, barium chloride and bumetanide were obtained from Sigma Chemical (St. Louis, MO). Vasoactive intestinal peptide was obtained from Peninsula (Belmont, CA) and the isoquinolinesulfonamide H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) was from Calbiochem (San Diego, CA). The citrus flavonoids nobiletin, tangeritin, naringin and hesperidin were the gift of John A. Attaway, Florida Department of Citrus, Lake Alfred, FL. 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 the T_{84} cells. T_{84} cells (passes 30–60) were provided by Jonathan A. Cohn (Division of Gastroenterology, Duke University). These cells were cultured at 5% CO_2 and 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 supplemented with 5% (v/v) newborn calf serum. Cells were seeded on collagen-coated Nucleopore filters previously glued onto plastic rings (surface area 2.9 cm^2, ~10^6 cells per filter). These filters were then set on glass beads to allow medium (supplemented with 5000 U/L penicillin and 5000 μg/L streptomycin sulfate) to bathe both sides of the cell

[3]Abbreviations used: H-89, (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide); Isc, short-circuit current; VIP, vasoactive intestinal peptide.

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monolayer. The confluent monolayers used for secretory studies were maintained for at least 7 d after seeding.

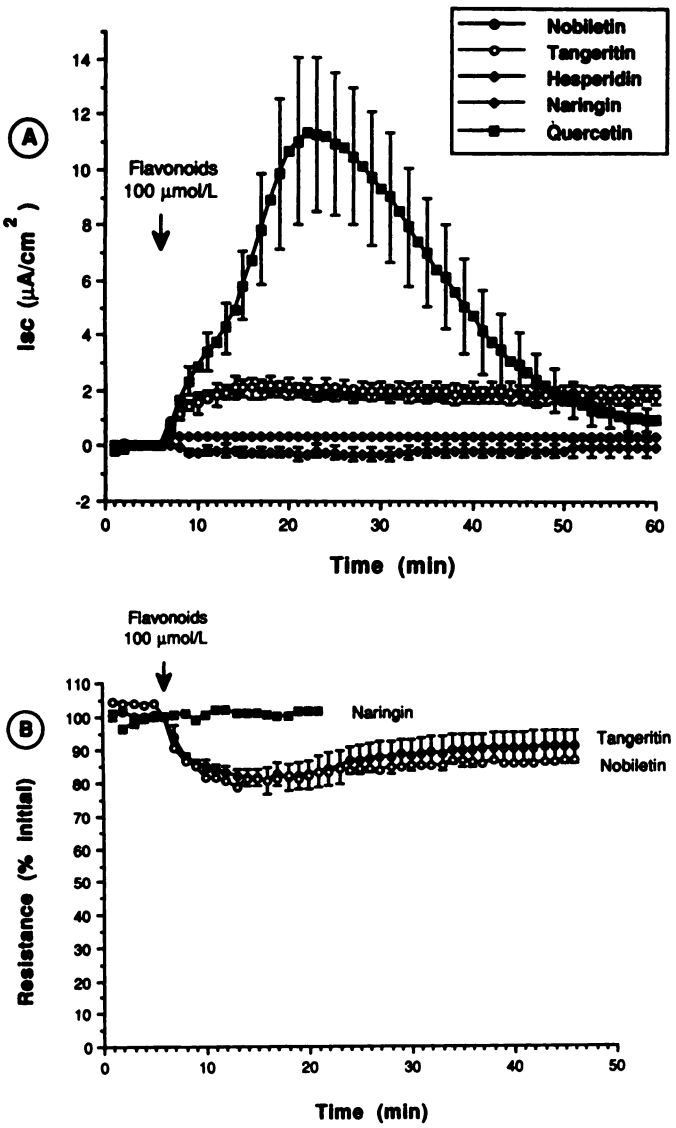

FIGURE 2 Panel A: secretory response of T_{84} cells stimulated by different flavonoids. After 5 min of baseline recording, 100 µmol/L of nobiletin, tangeritin, hesperidin, naringin or quercetin was added to the mucosal side of T_{84} cell monolayers mounted in Ussing chambers and studied as described in Materials and Methods. The results shown are derived from seven experiments. In each experiment, the monolayers seeded at the same time were studied on the same day, allowing the comparison between tangeritin (n = 7), nobiletin (n = 7), quercetin (n = 7), and either naringin (n = 5) or hesperidin (n = 1). The resulting means and SEM (when appropriate) are shown. *Panel B*: monolayer resistance after addition of different flavonoids. After 5 min of baseline recording, 100 µmol/L of either tangeritin (n = 3), nobiletin (n = 2), or naringin (n = 2) were added to the mucosal side of the T_{84} cell monolayers mounted in Ussing chambers and the resistance determined as detailed in the Methods. For each monolayer, the resistance values were normalized to the initial resistance at the time of flavonoid addition and the resulting means and SEM (when appropriate) are shown. Isc = short-circuit current.

Secretory studies. The secretory potential of the cell monolayer was studied in modified Ussing ch ambers as previously described (Nguyen et al. 1991). Both sides of the monolayer were bathed with Ringer's solution containing 115 mmol/L NaCl, 1.2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 0.4 mmol/L KH_2PO_4, 2.5 mmol/L K_2HPO_4, 25 mmol/L $NaHCO_3$ and 10 mmol/L glucose. The flavonoids were dissolved in 100% ethanol and added to the Ringer's solution at a 1:100 dilution (final concentration of ethanol: 1%; in control experiments, this concentration does not have effect on the Isc). The medium was warmed to 37°C with a circulating water jacket and gently mixed and aerated with a constant inflow of 95% air-5% CO_2. During secretory studies, spontaneous tissue potential differences were short-circuited by an automatic voltage clamp (model DVC-1000, World Precision Instruments, Sarasota, FL) with Ag-$AgCl_2$ electrodes, and the current necessary to maintain the short-circuit current (Isc) recorded at 1-min intervals. For studies of monolayer conductance, a constant current of 100 µA was generated by the voltage clamp and the corresponding voltage recorded at 1-min intervals. The monolayer resistance was calculated using Ohm's law. 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.

Cyclic nucleotide assays. T_{84} cells grown to confluence on six-well plates were washed twice and overlaid with 1 mL of Ringer's solution that had been supplemented with 0.2 mmol/L 3-isobutyl-1-methylxanthine and 10 mmol/L glucose, warmed to 37°C and equilibrated with 95% air-5% CO_2. Flavonoids, dissolved in ethanol (final flavonoid concentration: 100 µmol/L, final ethanol concentration in flavonoid-treated and control cells: 1%), or known secretagogues such as VIP (final concentration: 10 nmol/L) or *E. coli* heat-stable enterotoxin (final concentration: 1 µmol/L) were then added to the cells. After 10 min of incubation at 37°C in 95% air-5% CO_2, the treatment was stopped by removing the solution overlying the cells and adding 500 µL of ice-cold 70% ethanol to each well. The extracts were next transferred to plastic tubes and the wells washed with an additional 500 µL of 70% ethanol. After centrifugation at 2000 × g for 15 min, the resulting supernatant was dried at 60°C under a constant inflow of nitrogen and stored at −10°C. Intracellular levels of either cAMP or cGMP were subsequently assayed by RIA using kits purchased from Amersham (Arlington Heights, IL) (cGMP kit, no. RPA 525; cAMP kit, no. RPA 509).

Oxygen consumption. For the studies of flavonoid autoxidation, 20 µL of 20 mmol/L flavonoid dissolved in 100% ethanol was added to 2 mL of 100 mmol/L K_2HPO_4-KH_2PO_4, pH 8.3, for a final flavonoid con-

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centration of 200 μmol/L. Oxygen consumption was then studied at 25°C in a water-jacketed cell (Gilson Medical Electronics, Middleton, WI) which was equipped with a Clark electrode attached to an oxygen monitor (YSI Model 5300, Yellow Springs Instrument, Yellow Springs, OH).

Statistical analyses. Unless mentioned otherwise, means and SEM are given. For comparisons between matched monolayers, the probabilities obtained using paired two-tailed *t* tests are given.

RESULTS

Stimulation of secretion. In **Figure 2*A***, the secretory responses stimulated by 100 μmol/L of either tangeritin, nobiletin, hesperidin, naringin or quercetin in seven experiments are compared. In each of these experiments the T_{84} monolayers seeded at the same time were studied on the same day, allowing the comparison between tangeritin, nobiletin, quercetin and either naringin or hesperidin. Tangeritin or nobiletin produced a similar increase in Isc, which reached a maximal value after 10–15 min and was sustained for up to 1 h. The secretion stimulated by quercetin was greater but more transient, as previously observed (Nguyen et al. 1991). The glycosylated flavonoids naringin and hesperidin stimulated much smaller secretion. When all the experiments using flavonoids during a similar period were reviewed, the average maximal Isc changes produced by the different flavonoids were 3.0 ± 0.2 $\mu A/cm^2$ for nobiletin (n = 28), 3.4 ± 0.3 $\mu A/cm^2$ for tangeritin (n = 36), 0.2 ± 0.4 $\mu A/cm^2$ for hesperidin (n = 5), 0.3 ± 0.1 $\mu A/cm^2$ for naringin (n = 11) and 14.1 ± 0.3 $\mu A/cm^2$ for quercetin (n = 14). In separate experiments, the monolayer resistance was monitored after addition of 100 μmol/L of tangeritin, nobiletin or naringin. As shown in Figure 2*B*, compared with the initial resistance at the time of flavonoid addition, the resistances of the tangeritin- and nobiletin-treated monolayers decreased by 19 ± 4% (n = 3) and 21% (n = 2), respectively. In contrast, the resistance of the naringin-treated monolayers was not affected. These changes of resistance paralleled the Isc changes and most likely reflected the electrogenic secretion stimulated by the different flavonoids.

As shown in **Figure 3**, similar secretory responses were obtained whether 100 μmol/L tangeritin or nobiletin was added to either the mucosal or the serosal side of the T_{84} cell monolayer. Similarly, the same small secretory response to naringin was noted whether it was added to the serosal or mucosal side of the T_{84} monolayer (data not shown). In separate experiments, we observed that a threshold response was obtained with 10 μmol/L of either tangeritin or nobiletin and that 100 μmol/L tangeritin and nobiletin still stimulated secretion when added to

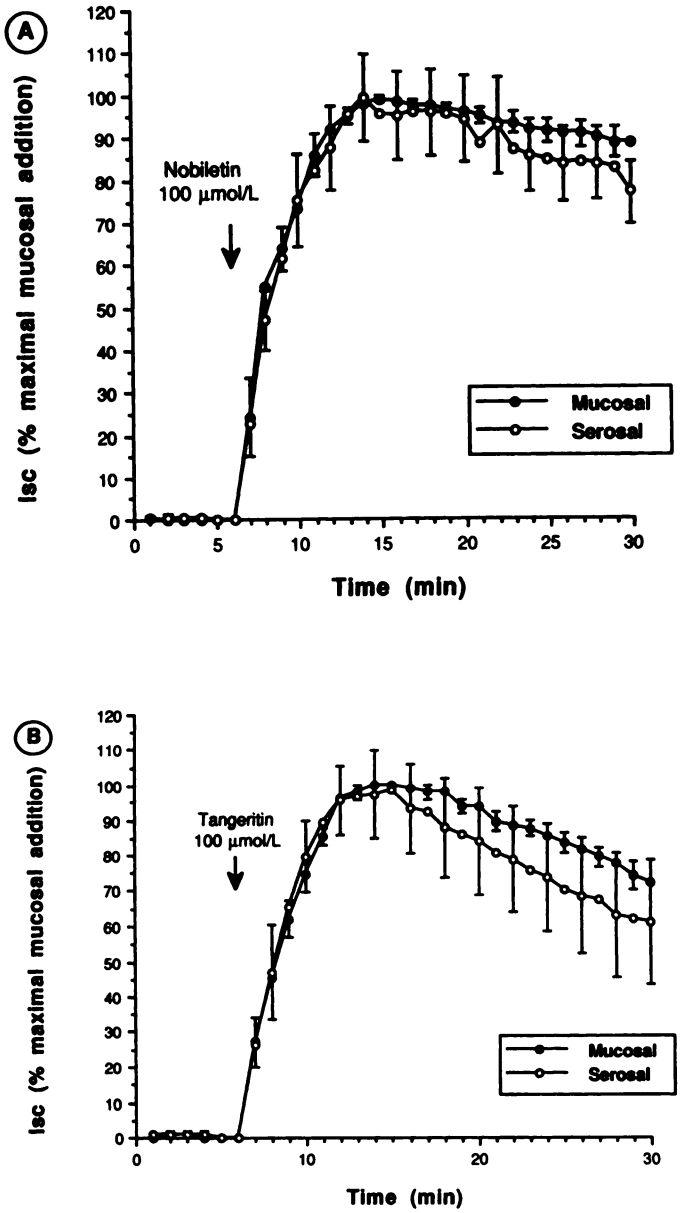

FIGURE 3 Secretory response of T_{84} cells stimulated by nobiletin (*panel A*) or tangeritin (*panel B*) added to the mucosal or serosal side of the monolayer. After 5 min of baseline recording, 100 μmol/L of either nobiletin or tangeritin were added to either the mucosal or the serosal side of T_{84} cell monolayers mounted in Ussing chambers and studied as described in the Methods. With each pair of monolayers (tangeritin: 5 pairs, nobiletin: 3 pairs), the short-circuit current (Isc) values were normalized to the maximal Isc value obtained when flavonoids were added to the mucosal side of the monolayer. The resulting means and SEM are shown.

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cells previously incubated with 100 μmol/L naringin for 15 min (data not shown).

Interaction with other secretagogues. Because it is likely that the in vivo effects of the citrus flavonoids will be exerted in concert with other endogenous secretory agents, the interaction between flavonoids and known secretagogues acting via cAMP (e.g., VIP) or intracellular Ca^{2+} (e.g., carbachol) was investigated.

Addition of 100 μmol/L carbachol alone produced a transient moderate increase in the Isc with a peak value of 3.8 ± 1 μA/cm^2 (n = 3) (**Fig. 4***A*). However, in T_{84} cells previously treated with 100 μmol/L tangeritin for 25 min, the same addition of 100 μmol/L carbachol resulted in a peak Isc increase of 42 μA/cm^2, from 2.8 ± 1 μA/cm^2 to a maximal value of 44.8 ± 7.9 μA/cm^2 (n = 3). The carbachol-stimulated Isc increase in tangeritin-treated cells was significantly greater than the increase stimulated in untreated cells (P = 0.046). Conversely, whereas 100 μmol/L tangeritin alone produced an Isc change of 3.1 ± 1 μA/cm^2, in cells pretreated with 100 μmol/L carbachol for 10 min, addition of 100 μmol/L tangeritin resulted in an additional increase of 9.6 μA/cm^2, from 1.4 ± 0.3 to 10.8 ± 3.4 μA/cm^2. In a similar fashion, whereas 100 μmol/L carbachol alone produced an Isc increase of 4.5 ± 0.7 μA/cm^2 (n = 3), in cells pretreated with 100 μmol/L nobiletin for 25 min the same concentration of carbachol produced an Isc increase of 51.4 μA/cm^2, from 1.7 ± 0.3 to 53.1 ± 15.8 μA/cm^2 (n = 3) (Fig. 4*B*). Again, 100 μmol/L nobiletin produced an Isc increase of 1.7 μA ± 0.3 μA/cm^2 in untreated T_{84} but an increase of 11.0 μA/cm^2 in cells exposed to 100 μmol/L carbachol for 10 min (from 2.4 ± 0.3 to 13.4 ± 0.3 μA/cm^2). Synergism between either nobiletin or tangeritin and carbachol was therefore demonstrated because, in both cases, the combined effect of the flavonoid and carbachol was greater than the sum of the individual effects (Isc 1 min after addition of carbachol to cells previously stimulated with flavonoid vs. sum of the maximal Isc stimulated by flavonoid and the maximal Isc stimulated by carbachol; P = 0.030 for nobiletin, P = 0.026 for tangeritin). In separate experiments, we determined that simultaneous addition of flavonoid and carbachol also demonstrated synergism, with an Isc response intermediate between the one obtained when carbachol followed flavonoids and the one obtained when flavonoids followed carbachol (data not shown). The different degrees of synergism most likely reflected the timing of the maximal effects of flavonoids and carbachol.

In contrast, **Figure 5** shows a different interaction between VIP and the citrus flavonoids. T_{84} cells treated with 10 nmol/L VIP showed a maximal Isc response of 16.2 ± 1.7 μA/cm^2 (n = 4) (Fig. 5*A*). However, when cells were pretreated with 100 μmol/L of tangeritin for 25 min, the additional Isc response to VIP was only 5.5 μA/cm^2, from 4.8 ± 1.0 to 10.3 ±

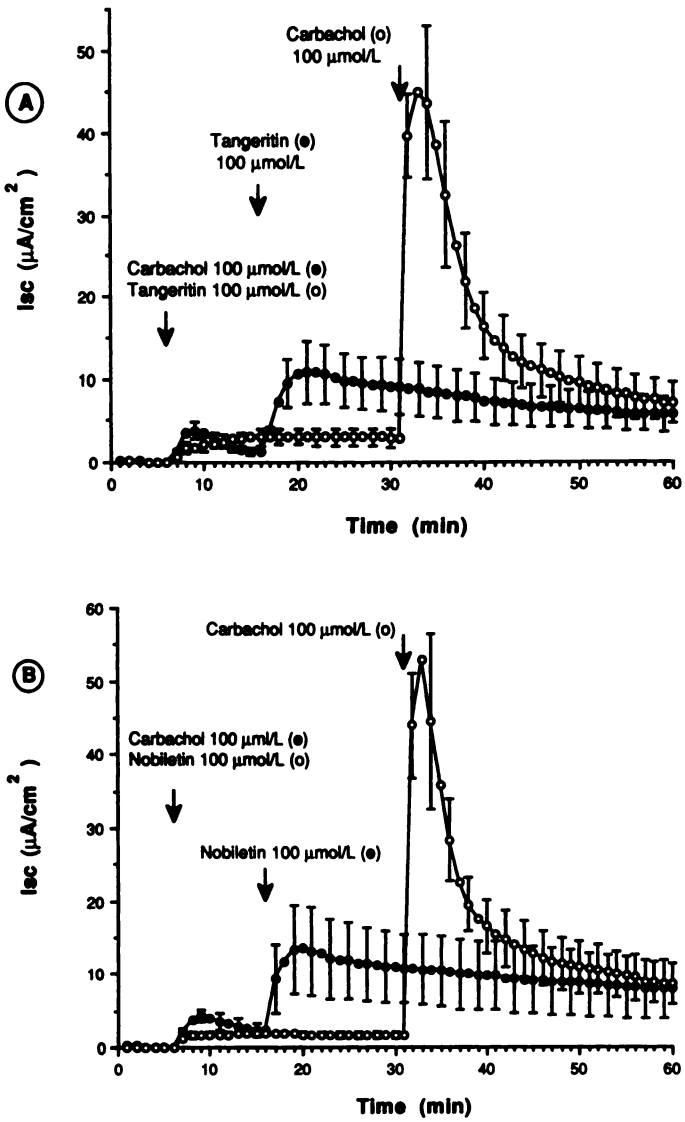

FIGURE 4 Synergism between carbachol and tangeritin (*panel A*) or nobiletin (*panel B*). After 5 min of baseline recording, 100 μmol/L of either carbachol or flavonoid were added to matched T_{84} cell monolayers that were seeded at the same time and studied in Ussing chambers concurrently. Flavonoids (100 μmol/L) were then added to the cells previously treated with carbachol for 10 min while carbachol (100 μmol/L) was added to the cells previously treated with flavonoids for 25 min. Carbachol was added to the serosal side of the cell monolayer and flavonoids to the mucosal side. For each panel, the means and SEM of three experiments are shown. Isc = short-circuit current.

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1.0 $\mu A/cm^2$ (n = 4). The combined effect of VIP and tangeritin was therefore less than additive. In cells maximally stimulated by 10 nmol/L VIP, subsequent addition of 100 μmol/L tangeritin produced an inhibitory effect, with the Isc decreasing from 16.2 to 7.2 $\mu A/cm^2$. Similar results were found when nobiletin was substituted for tangeritin (Fig. 5*B*).

In separate experiments, similar interactions between flavonoids and carbachol or VIP were demonstrated when flavonoids were added to the serosal (instead of the mucosal side) of the cell monolayer (data not shown).

Effect of inhibitors of secretory pathways. We next studied the effects of different inhibitors of secretion on the flavonoid-stimulated response in T_{84} cells. Bumetanide inhibits the basolateral $Na^+,K^+,2Cl^-$ cotransporter. This cotransporter produces the high intracellular Cl^- concentration necessary to drive Cl^- anions down the gradient from the intracellular into the extracellular mucosal compartment. Barium chloride, on the other hand, inhibits the cAMP-sensitive (but not Ca^{2+}-sensitive) K^+ efflux channel. This channel allows potassium cations, accumulated intracellularly by the $Na^+,K^+,2Cl^-$ cotransporter, to flow out of the cell. Thus, whereas 3 mmol/L barium chloride inhibits the secretion produced by VIP and prostaglandin E_1 (Mandel et al. 1986, Weymer et al. 1985), it does not inhibit the secretion stimulated by carbachol (Dharmsathaphorn and Pandol 1986). Finally, H-89 is a recently synthesized isoquinolinesulfonamide that is a potent and selective inhibitor of cAMP-dependent protein kinase (Chijiwa et al. 1990).

As shown in **Figure 6***A*, pretreatment of the T_{84} cells with 0.3 mmol/L bumetanide for 15 min inhibited the secretion produced by 100 μmol/L tangeritin. The maximal Isc stimulated by tangeritin decreased by 47%, from the maximal value of 3.7 ± 0.4 $\mu A/cm^2$ obtained in control cells after 12 min (n = 3) to the maximal value of 2.0 ± 0.4 $\mu A/cm^2$ obtained in treated cells after 5 min (n = 3) (significant decrease, P = 0.038). When the secretion stimulated by tangeritin after 35 min of incubation was considered, it was decreased by 75% in the treated cells (0.6 ± 0.3 $\mu A/cm^2$ in treated cells vs. 2.2 ± 0.1 $\mu A/cm^2$ in controls, n = 3). As shown in Fig. 6*B*, 3 mmol/L barium chloride also inhibited the maximal secretion stimulated by 100 μmol/L tangeritin by 76%, from 4.4 ± 0.4 $\mu A/cm^2$ in matched control cells (n = 4) to 1.0 ± 0.1 $\mu A/cm^2$ in treated cells (n = 4) (significant decrease, P = 0.006). As shown in Fig. 6*C*, the secretion stimulated by 100 μmol/L tangeritin was also completely inhibited by a 30-min preincubation with 30 μmol/L of the cAMP-dependent protein kinase inhibitor, H-89 (n = 2). In contrast, as expected, the secretion associated with the subsequent addition of 100 μmol/L carbachol, which was mediated by intracellular Ca^{2+}, was still demonstrated in the presence of this inhibitor (the decreased response reflects the absent

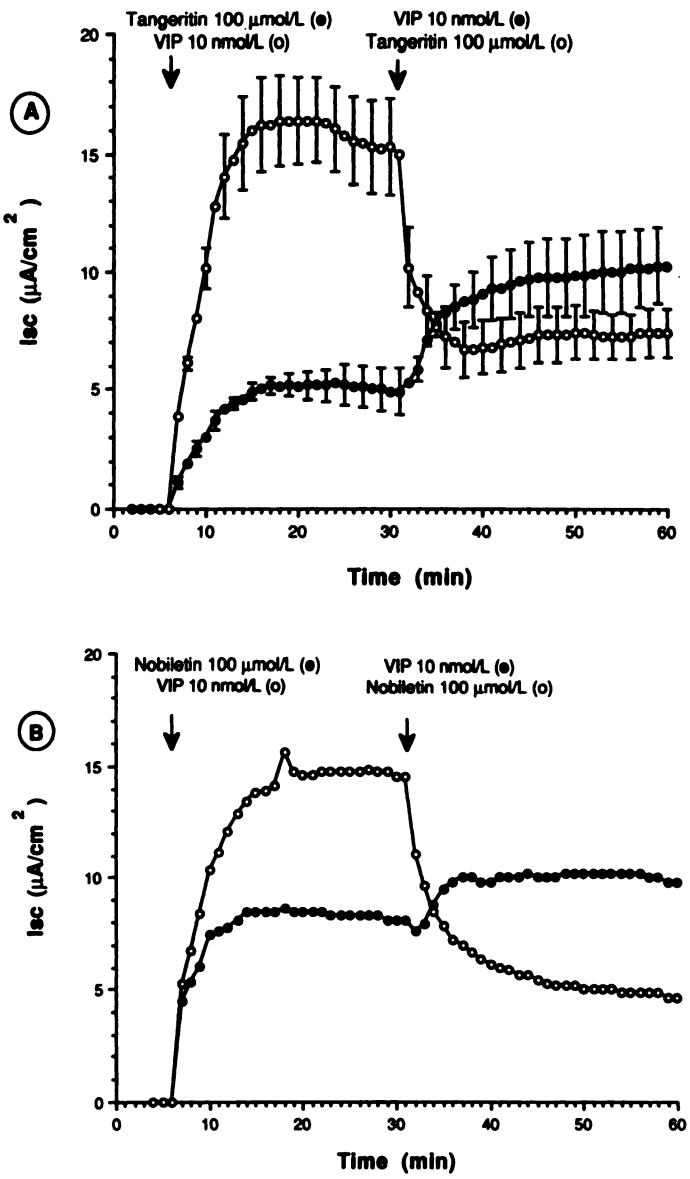

FIGURE 5 Lack of additive effect between vasoactive intestinal peptide (VIP) and tangeritin (*panel A*) and nobiletin (*panel B*). After 5 min of baseline recording, either 10 nmol/L VIP or 100 μmol/L flavonoid were added to matched T_{84} cell monolayers that were seeded at the same time and studied in Ussing chambers concurrently. After an additional 25 min, 10 nmol/L VIP was added to the cells previously treated with flavonoid while 100 μmol/L flavonoid were added to the cells previously treated with VIP. Vasoactive intestinal peptide was added to the serosal side of the monolayer and tangeritin to the mucosal side. The means and SEM of four experiments are shown in *panel A*, and the means of two experiments shown in *panel B*. Isc = short-circuit current.

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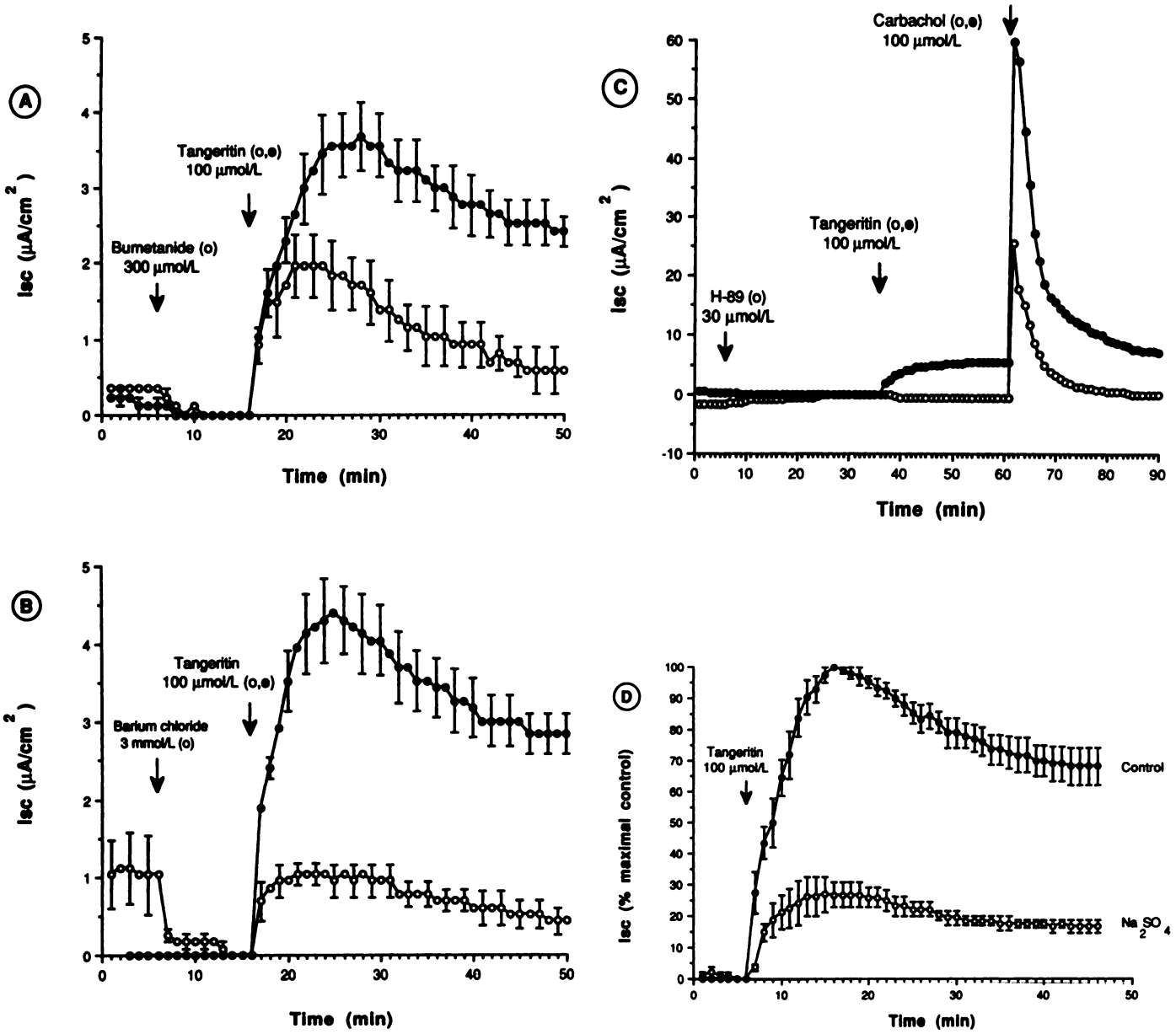

FIGURE 6 Inhibition of tangeritin-stimulated secretion by bumetanide (*panel A*), barium chloride (*panel B*), H-89 (*panel C*) and chloride depletion (*panel D*). In *panels A, B* and *C*, after 5 min of baseline recording, 300 µmol/L bumetanide, 3 mmol/L barium chloride or 30 µmol/L H-89 were added to T_{84} cell monolayers mounted in Ussing chambers as described in Materials and Methods. After an additional 10 min, 100 µmol/L tangeritin was added to both the T_{84} cell monolayer previously incubated with bumetanide, barium chloride or H- 89 and to matched control monolayers that were seeded at the same time and studied concurrently. Bumetanide and barium chloride were added to the serosal side of the cell monolayer, tangeritin to the mucosal side of the cell monolayer and H-89 to both sides of the cell monolayer. Because bumetanide was dissolved in NaOH, the serosal compartments of the bumetanide-treated and control monolayers both had a final concentration of 1 mmol/L NaOH. In *panel D*, Cl^- in the medium was depleted by substituting Na_2SO_4 for NaCl. After 5 min of baseline recording, 100 µmol of tangeritin was added to the mucosal side of the cell monolayer. The resulting short-circuit current (Isc) changes were normalized to the maximal Isc obtained on control experiments using normal Ringer's solution. In *panel A*, the means and SEM of three experiments are shown; in *panel B*, the means and SEM of four experiments are shown; in *panel C*, the means of two experiments are shown; in *panel D*, the means and SEM of three experiments are shown.

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synergism with tangeritin, now blocked by H-89).

Finally, to determine whether the secretory effects demonstrated with the different flavonoids were dependent on the presence of Cl^- in the medium, we substituted sodium chloride in the medium with sodium sulfate. As shown in Fig. 6*D*, in this medium, the Isc changes were only 27 ± 3% (n = 3) of the values obtained with control studies using normal Ringer's solution (significant decrease, P = 0.008).

Effect of intracellular cyclic nucleotides. The synergism between tangeritin or nobiletin and carbachol, but not VIP, and the inhibitory effect of barium chloride and H-89 all suggest that cyclic nucleotides mediate the secretion stimulated by these citrus flavonoids. To further evaluate this possibility, T_{84} cells were stimulated with 100 μmol/L of either tangeritin or nobiletin for 10 min and the resulting intracellular levels of cAMP and cGMP assayed by RIA. Compared with a control incubation with the ethanol carrier alone, the intracellular levels of cAMP in cells treated with tangeritin, nobiletin and 10 nmol/L VIP were 113, 116 and 735%, respectively, of control values (n = 2, with duplicate determinations, data not shown). Similarly, the intracellular levels of cGMP in cells treated with tangeritin, nobiletin and 1 μmol/L *E. coli* heat stable enterotoxin were 97, 107 and 2143%, respectively, of control values (n = 2, with duplicate determination, data not shown).

Absence of autooxidation. We previously demonstrated that certain multihydroxylated flavonoids such as quercetin and kaempferol produced reactive oxygen species through autooxidation (Canada et al. 1990). Because reactive oxygen species may stimulate T_{84} secretion (Tamai et al. 1992), we evaluated whether the multimethoxylated flavonoids tangeritin and nobiletin could autooxidize and generate reactive oxygen species, which might in turn mediate T_{84} secretion. This possibility was evaluated through studies of the oxygen consumption by these citrus flavonoids using air-tight cells fitted with a Clark oxygen electrode. As shown in **Figure** 7, tangeritin did not autooxidize, suggesting that reactive oxygen species did not mediate its secretory effect. Similar observations were also noted for nobiletin (data not shown).

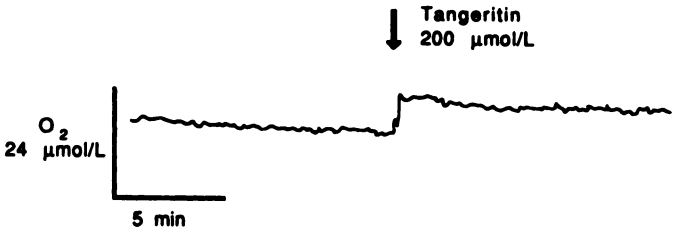

FIGURE 7 Lack of autooxidation of tangeritin (*panel A*) and nobiletin (*panel B*). The oxygen consumption by 200 μmol/L tangeritin in a 2-mL water-jacketed oxygen consumption cell was monitored continuously using a Clark electrode attached to an oxygen monitor. The tracing shown is representative of two experiments. In a different experiment, similar results were obtained when nobiletin was substituted for tangeritin.

DISCUSSION

We have shown that the polymethoxylated citrus flavones nobiletin and tangeritin induced secretion in T_{84} cells mounted in Ussing chambers. These responses were sustained for more than 1 h. Even though nobiletin and tangeritin alone produced only moderate secretion with an average Isc change of 3.0–3.5 $\mu A/cm^2$, their marked synergism with carbachol, resulting in a combined response exceeding 40 $\mu A/cm^2$, suggests that these compounds may have substantial stimulatory effects on intestinal or colonic secretion when combined with endogenous muscarinic agonists. On the other hand, citrus flavonoids can also partially inhibit the secretion produced by VIP; they may therefore exert inhibitory effects on the intestinal secretion stimulated by endogenous secretagogues acting via cyclic nucleotides, such as VIP or prostaglandins.

Neither nobiletin nor tangeritin underwent autooxidation, and, therefore, neither could generate reactive oxygen species through an oxidation-reduction mechanism. Secretory effects resulting directly or indirectly from the production of reactive oxygen species, such as those derived from monochloramine (Tamai 1992), will not be a consideration in this case. The secretion stimulated by the citrus flavonoids was inhibited by bumetanide and Cl^- depletion, suggesting that it was dependent on the chloride gradient between the intracellular and the luminal spaces generated by the $Na^+,K^+,2Cl^-$ cotransporter. Therefore, similar to the secretion produced by other known secretagogues in the T_{84} model, the electrogenic secretion stimulated by the citrus flavonoids reflects Cl^- flow through activated apical chloride channels.

The marked synergism between carbachol and the citrus flavonoids suggests that carbachol and flavonoids do not share the same effector pathway mediated by intracellular Ca^{2+}. On the other hand, the secretory effects of VIP and the citrus flavonoids were less than additive, implying that both agents shared a common cyclic nucleotide-dependent pathway. This possibility is further supported by the finding that 3 mmol/L barium chloride inhibited the secretion produced by both citrus flavonoids, similar to the inhibition produced by agents acting through cAMP, [e.g., VIP and prostaglandin E_1 (Mandel et al. 1986, Weymer et al. 1985)] but not by agents acting through Ca^{2+} [e.g., carbachol (Dharmsathaphorn and Pandol 1986)]. It has been demonstrated that barium chloride acts by inhibiting the K^+ efflux channel that

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is activated by cyclic nucleotides but not intracellular Ca^{2+}. The inhibitory effect of H-89 also confirms that the cAMP pathway is the operative system in citrus flavonoid-stimulated cell secretion. Because H-89, a specific inhibitor of the cAMP-dependent protein kinase, also inhibited the secretion stimulated by the citrus flavonoids, we can infer that this secretion requires the activation of the cAMP-dependent protein kinase, a downstream event in the signal transduction cascade mediated by cAMP.

Direct assays of cAMP and cGMP in cells treated with citrus flavonoids, however, did not show the marked elevations of cyclic nucleotides demonstrated in control treatments with VIP (for cAMP) or *E. coli* heat-stable enterotoxin (for cGMP). It is possible that, analogous to adenosine, there is a discrepancy between the concentrations required for the stimulation of Cl^- secretion and for cAMP production and that high concentrations of flavonoids will be required for a significant increase in cAMP (Barrett et al. 1990). The limited solubility of the flavonoids prevented the direct evaluation of this hypothesis. Alternatively, because flavonoids have been shown to modulate different intracellular enzymatic pathways (Havsteen 1983, Middleton, Jr. 1984), it is possible that the lipophilic citrus flavonoids can also penetrate T_{84} cells and directly activate the enzymes that modulate the secretory action of cAMP. Indeed, we have previously shown that the flavonol quercetin, although it did not markedly increase cAMP, could nonetheless produce the protein phosphorylation response resulting from the activation of the cAMP-dependent protein kinase (Nguyen et al. 1991). The inhibitory effect of H-89 also suggests that the activation of cAMP-dependent protein kinase is required for the secretory effect of flavonoids.

Comparison of the secretions produced by the polymethoxylated citrus flavones and the polyhydroxylated flavonols quercetin, kaempferol and myricetin is also of interest. Even though the citrus flavones differ from the flavonols only by the nature of the substitutions on the flavane nucleus (methoxyl vs. hydroxyl), neither the oxidation of flavones to flavonols nor the reduction of flavonols to flavones seems to occur in plants, suggesting that these flavonoid subclasses are differentiated early in their biosynthesis (Kuhnau 1976). The secretion stimulated by tangeritin or nobiletin is comparable to the one previously demonstrated for kaempferol and is higher than the one previously demonstrated for myricetin (Nguyen et al. 1991). However, the secretion stimulated by the citrus flavones is lower than that stimulated by quercetin. This difference may relate to the ability of quercetin to inhibit protein kinase C and the inability of either tangeritin or nobiletin to do so (Ferriola et al. 1989). Because it has been suggested that protein kinase C inhibits secretion (Warhurst et al. 1991), the additional secretory response demonstrated with quercetin may result from its ability to prevent the inhibitory effect of this kinase. The complex interaction between quercetin and the different pathways that modulate secretion is consistent with the multiphasic secretory response observed with quercetin (compared with the more simple response demonstrated by the citrus flavones).

In this report, the glycosylated citrus flavonoids hesperidin and naringin stimulated minimal secretion. Combined with the previous observation that rutin, a glycosylated form of quercetin, did not stimulate secretion (Nguyen et al. 1991), these findings would suggest that glycosylation impairs the potential of a flavonoid to activate T_{84} cell secretion.

Flavonoids constitute a significant part of the typical Western diet and their presence in the alimentary tract may be prolonged by the enterohepatic circulation previously demonstrated (Kuhnau 1976). In addition, intestinal bacteria can also hydrolyze the naturally occurring inactive glycosylated flavonoids into aglycones, thus activating their secretory potential (Bokkenheuser et al. 1987). The citrus flavones are a significant component of foods flavored with citrus peels or extracts. The observations made in this report suggest that these dietary compounds may play a role in the modulation of intestinal or colonic fluid and electrolyte secretion.

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