Modulation of the Glucagon-Like Peptide-1 Receptor Signaling by Naturally Occurring and Synthetic Flavonoids

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ABSTRACT
The glucagon-like peptide-1 receptor (GLP-1R) is a promising target for the treatment of type II diabetes mellitus because of its role in metabolic homeostasis. In recent years, difficulties with peptide therapies have driven the search for small-molecule compounds to modulate the activity of this receptor. We recently identified quercetin, a naturally occurring flavonoid, as a probe-dependent, pathway-selective allosteric modulator of GLP-1R-mediated signaling. Using Chinese hamster ovary cells expressing the human GLP-1R, we have now extended this work to identify the structural requirements of flavonoids to modify GLP-1R binding and signaling (cAMP formation and intracellular Ca²⁺ mobilization) of each of the GLP-1R endogenous agonists, as well as the clinically used exogenous peptide mimetic exendin-4. This study identified a chemical series of hydroxyl flavonols with the ability to selectively augment calcium (Ca²⁺) signaling in a peptide agonist-specific manner, with effects only on truncated GLP-1 peptides [GLP-1(7–36)NH₂ and GLP-1(7–37)] and exendin-4, but not on oxyntomodulin or full-length GLP-1 peptides [GLP-1(1–36)NH₂ and GLP-1(1–37)]. In addition, the 3-hydroxyl group on the flavone backbone (i.e., a flavonol) was essential for this activity, however insufficient on its own, to produce the allosteric effects. In contrast to hydroxyl flavonols, catechin had no effect on peptide-mediated Ca²⁺ signaling but negatively modulated peptide-mediated cAMP formation in a probe-dependent manner. These data represent a detailed examination of the action of different flavonoids on peptide agonists at the GLP-1R and may aid in the development of future small molecule compounds targeted at this receptor.

Introduction
Glucagon-like peptide-1 (GLP-1) is an endogenous incretin hormone released by L cells of the intestine in response to nutrient intake that stimulates insulin secretion (Drucker and Nauck, 2006). GLP-1 has attracted interest from the pharmaceutical industry in recent years as a treatment for type II diabetes and its accompanying obesity, because it has multiple antidiabetic actions. For example, GLP-1 stimulates insulin and suppresses glucagon secretion, inhibits gastric emptying, and reduces appetite and food intake, therefore aiding weight loss (Drucker and Nauck, 2006). In addition, GLP-1 increases pancreatic β-cell mass by inducing neogenesis, proliferation, and antiapoptosis of the β-cells (Vahl and D’Alessio, 2004). However, the therapeutic potential of GLP-1 is limited because of its rapid degradation in the plasma by dipeptidyl peptidase-IV and its relatively narrow therapeutic window associated with nausea at high doses (Larsen et al., 2001; Vilsboll et al., 2003). Clinical studies revealed that replacement therapies with metabolically stable GLP-1 mimetics, or treatment with dipeptidyl peptidase-IV inhibitors, can improve the management of hyperglycemia for some patients. Both exenatide (Byetta; Amylin Pharmaceuticals, Inc., San Diego, CA) and liraglutide (Victoza; Novo Nordisk A/S, Bagsvaerd, Denmark) have been approved by the U.S. Food and Drug Administration for the treatment of type II diabetes (Kolterman et al., 2003; Drucker et al., 2010). However, as peptide analogs of GLP-1, these require administration by intravenous or subcutaneous injection and have been associated with significant adverse side effects, including pan-
creatitis (Olansky, 2010). Therefore, orally active small-molecule agonists or modulators that augment signaling via the GLP-1 receptor are highly desirable.

GLP-1 is a member of the glucagon peptide superfamily and exerts its effects by binding to the GLP-1R, a family B G protein-coupled receptor (GPCR). The GLP-1R is primarily expressed in pancreatic β-cells and is principally coupled to Gαs, thereby mediating its effects through generation of cAMP, causing cell depolarization and increased cytosolic calcium (Ca²⁺) concentration, ultimately resulting in augmentation of insulin secretion (Drucker et al., 1987; Holz et al., 1993; Wheeler et al., 1993). Although insulin release downstream of GLP-1R activation is known to be critically dependent upon the formation of cAMP, there is also a role for mobilization of intracellular Ca²⁺ and activation of mitogen-activated protein kinases such as extracellular signal-regulated kinase 1 and 2 (ERK1/2) in augmentation of insulin response (Baggio and Drucker, 2007). Furthermore, for many therapeutically relevant effects of GLP-1R activation, including the modulation of appetite, the underlying GLP-1R-mediated signaling is not fully understood, but physiological responses are known to be a composite of multiple signaling pathways.

The GLP-1R system is further complicated because the receptor is activated by multiple endogenous ligands, including four variants of GLP-1 and the related peptide oxyntomodulin, and can also be activated by exogenous mimetics such as exendin-4. The four active secreted forms of GLP-1 include a full-length peptide, GLP-1(1–37), and a truncated form, GLP-1(7–37), each of which has an amidated counterpart: GLP-1(1–36)NH₂ and GLP-1(7–36)NH₂. In the past, the search for nonpeptide small-molecule agonists for any family B GPCR has been largely unsuccessful. Recently, however, a number of small-molecule compounds have been identified that are able to activate the GLP-1R. These include a series of substituted quinoxalines, the most potent of which is compound 2 (Knudsen et al., 2007; Teng et al., 2007), a series of pyrimidines (Sloop et al., 2010), and a cyclobutane derivative (Chen et al., 2007).

We have also reported that quercetin can modulate GLP-1R activation in both a pathway and ligand-specific manner (Koole et al., 2010). Quercetin selectively modulated Ca²⁺ signaling for the high-affinity agonists GLP-1(7–37), GLP-1(7–36)NH₂, and exendin-4 but not oxyntomodulin; however, it had no effect on any of the peptide ligands in cAMP accumulation or ERK1/2 phosphorylation assays. In separate studies, experiments in streptozotocin-induced diabetic rats showed significantly lower plasma glucose levels, urine output, and urine glucose content when these animals are treated with an appropriate amount of quercetin compared with control diabetic rats (Vessal et al., 2003; Shetty et al., 2004), suggesting that quercetin may have potential in the management of type II diabetes, effects that may be mediated in part, via modulation of the GLP-1R. Quercetin is a flavonol, a subgroup of the flavonoids that are found in a wide variety of plants and plant derivatives and are widely consumed in the human diet (Hertog and Hollman, 1996). In this study, we investigated the structure/activity relationship of various flavonoids (flavones, flavonols, isoflavones, and catechins; Fig. 1) in the allosteric modulation of GLP-1R signaling and identify a potential structural scaffold that may be useful in development of small-molecule modulators for future drug discovery programs.

![Fig. 1. Structures of flavonoids used in this study. The numbers on the ring positions of the 2-phenylchrome-4-one scaffold are labeled on the flavone structure.](image-url)
Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and Flu-o-4 acetoxyethyl ester were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia). AlphaScreen reagents, 125I-exendin(9–39), 96-well microtiter plates, GPC filters, plates, and Microscint 4 scintillant were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). The bicinchoninic acid protein assay kit was purchased from Thermo Fisher Scientific (Rockford, IL). GLP-1 and GLP-1 peptide analogs were purchased from American Peptide (Sunnyvale, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or BDH Merck (Melbourne, VIC, Australia) and were of an analytical grade.

Cell Culture. FlpIn Chinese hamster ovary cells stably transfected with human GLP-1R (FlpInCHO-huGLP-1R) and FlpIn 3T3 cells stably expressing the calcitonin receptor Leu polymorphism were generated using Gateway technology (Invitrogen) as described previously (May et al., 2007a). Cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and incubated in a humidified environment at 37°C in 5% CO2.

Radioligand Binding Assays. Membrane preparations of FlpInCHO-huGLP-1R were prepared as described previously (Avlani et al., 2004). Protein concentration was determined using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific) following the manufacturer's instructions, with bovine serum albumin (BSA) as standard. Competition binding assays were performed in 96-well plates using 20 μg of membrane expressing GLP-1R. Membranes were incubated in HEPES buffer [1 mM HEPES, 10 mM MgCl2, 100 mM NaCl, and 1% (w/v) BSA, pH 7.4] containing 0.5 nM 125I-exendin(9–39) and increasing concentrations of unlabeled ligand for 1 h. For interaction studies, competition of 125I-exendin(9–39) binding by each orthosteric agonist was performed in the presence of increasing concentrations of each flavonoid. For all experiments, nonspecific binding was defined by 1 μM exendin(9–39). Incubation was terminated by rapid filtration through Whatman GF/C filters [presoaked in 0.03% (v/v) polyethylenimine for a min of 2 h] using a 96-well harvester (Tomtec, Hamden, CT). Filters were washed three times with 0.9% (w/v) NaCl and 0.3% (w/v) BSA and were allowed to dry before addition of 30 μl of scintillant and determination of radioactivity by scintillation counting.

cAMP Accumulation Assay. FlpInCHO-huGLP-1R cells were seeded at a density of 5 × 104 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO2. Growth media were replaced with stimulation buffer [phenol-free DMEM containing 0.1% (w/v) BSA and 1 mM 3-isobutyl-1-methylxanthine] and incubated for a further 1 h at 37°C in 5% CO2. Cells were stimulated with peptide ligand and/or flavonoid and incubated for 30 min at 37°C in 5% CO2. The reaction was terminated by rapid removal of the buffer and addition of 50 μl of ice-cold 100% ethanol. After ethanol evaporation, 75 μl of lysis buffer [0.1% (w/v) BSA, 0.3% (v/v) Tween 20 and 5 mM HEPES, pH 7.4] was added and 10 μl of lystate was transferred to a 384-well ProxiPlate. Five microliters of acceptor bead mix (1.0% AlphaScreen cAMP acceptor beads diluted in lysis buffer) and 15 μl of donor bead mix [0.3% AlphaScreen cAMP donor beads and 0.025% AlphaScreen cAMP biotinylated cAMP (133 units/μl)] diluted in lysis buffer, and preincubated for a minimum of 30 min) were added in reduced lighting conditions. Plates were incubated at room temperature overnight before measurement of the fluorescence using a Fusion-α plate reader (PerkinElmer Life and Analytical Sciences) with standard AlphaScreen settings. All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel and normalized to maximal peptide response.

Intracellular Ca2+ Mobilization Assay. FlpInCHO-huGLP-1R cells were seeded at a density of 5 × 104 cells/well into 96-well culture plates, and receptor-mediated intracellular Ca2+ mobilization was determined as described previously (Werry et al., 2005). Because of their autofluorescence at high concentrations, each flavonoid was added 30 min before addition of peptide agonist in the FlexStation (Molecular Devices, Sunnyvale, CA). Fluorescence was determined immediately after peptide addition, with an excitation wavelength set to 485 nm and an emission wavelength set to 520 nm, and readings taken every 1.36 s for 120 s. Peak magnitude was calculated using five-point smoothing, followed by correction against basal fluorescence. The peak value was used to create concentration–response curves. Data were normalized to the maximal response elicited by the peptide.

Data Analysis. All data obtained were analyzed in Prism (ver. 5.0.2; GraphPad Software Inc., San Diego, CA). Concentration response binding and signaling data were fitted with a three-parameter logistic equation as described previously (May et al., 2007a), assuming a slope of 1:

$$E = \frac{(E_{\text{max}} - \text{basal})}{1 + 10^{\frac{\log EC_{50} - \log (X)}{10}}}$$

where E is response, Emax and basal are the top and bottom asymptotes of the curve, respectively, Log[A] is the logarithm of the agonist concentration, and LogEC50 is the logarithm of the agonist concentration that gives a response halfway between Emax and basal. Likewise, this equation was used in inhibition binding, instead replacing EC50 with IC50. In this case, basal defines the specific binding of the radioligand that is equivalent to nonspecific ligand binding, whereas Emax defines radioligand binding in the absence of a competing ligand. In a similar manner, the IC50 value represents the molar concentration of A required to generate a response halfway between Emax and basal.

In addition to fitting the three-parameter logistic equation, Ca2+ interaction data were fitted to the following to an operational model of allosterism and agonism (Leach et al., 2007; Aurelio et al., 2009) to derive functional estimates of modulator affinity and cooperativity:

$$E = \text{Basal} + \frac{(E_{\text{max}} - \text{Basal})}{1 + 10^{\frac{\log EC_{50} - \log (X)}{10}}}$$

where Emax is the maximum attainable system response for the pathway under investigation, [A] and [B] are the concentrations of orthosteric agonist and allosteric modulator/agonist, respectively, Kd is the dissociation constant of the allosteric modulator, n is a transducer slope factor linking occupancy to response, α is the cooperativity factor governing allosteric effects of the modulator on orthosteric agonist binding affinity, β is the factor governing allosteric effects on signaling efficacy, and γA and γB are operational measure of the ligands’ respective signaling efficiencies that incorporate receptor expression levels and efficiency of stimulus-response coupling (Leach et al., 2007; Aurelio et al., 2009). In all cases, the value of α was fixed to 1 because binding studies revealed neutral cooperativity between the orthosteric peptides and each of the flavonoids. All analytical data are expressed in logarithms as mean ± S.E.M. and, where relevant, statistical analysis was performed by one-way analysis of variance and Dunnett’s post test using Prism 5.0.2, and statistical significance accepted at p < 0.05.

Results

Hydroxyl Flavonoids Selectively Augment Intracellular Ca2+ Signaling via the GLP-1R. The GLP-1R is promiscuous, in that it has been shown to couple to multiple G proteins, activating multiple signaling pathways (Montrose-Rafizadeh et al., 1999; Bavec et al., 2003; Koole et al., 2010). Although the GLP-1R is known to preferentially couple to Gαs, it can also couple to Gαq proteins, resulting in mobili-
zation of intracellular Ca\(^{2+}\). In a previous study, we showed that quercetin could impart stimulus bias with respect to Ca\(^{2+}\) signaling but in a peptide-specific manner. Here we investigated the ability of a range of flavonoid compounds (Fig. 1) to modulate GLP-1R-mediated Ca\(^{2+}\) signaling. All of the flavonoid compounds in this study lacked intrinsic efficacy because no change was observed in the basal Ca\(^{2+}\) signaling in the presence of these compounds alone (data not shown). However, concentration response curves, established for the peptide ligands in the absence and presence of 30 \(\mu\)M concentrations of each flavonoid suggested a structure-activity relationship (SAR) for allosteric effects of selective flavonoids on peptide-mediated Ca\(^{2+}\) signaling (Fig. 2). In agreement with our previous study (Koole et al., 2010), augmentation of GLP-1(7–36)NH\(_2\), GLP1(7–37), or exendin-4 peptide efficacy and potency was observed at this concentration of quercetin; however, no significant change was observed for oxyntomodulin (Figs. 2 and 3, Table 1). In addition, 3',4'-dihydroxyflavonol displayed positive modulation of Ca\(^{2+}\) responses (to a lesser, but significant degree) induced by GLP-1(7–36)NH\(_2\), GLP-1(7–37), or exendin-4, with an observed increase in both maximal effect and potency of the agonist responses (Figs. 2 and 3, Table 1). This flavonol also displayed probe dependence, with no observed changes in oxyntomodulin-induced Ca\(^{2+}\) signaling. A general trend for modulation of Ca\(^{2+}\) responses can also be observed by hydroxyl flavonols, in that 4'-hydroxyflavonol increased the \(E_{\text{max}}\) and potency of the high-affinity peptide agonists but not oxyntomodulin; however, only the change in potency of GLP-1(7–36)NH\(_2\) reached statistical significance (\(p < 0.05\%\)) (Figs. 2 and 3, Table 1). In contrast, compounds that do not belong to the hydroxyl flavonol class of flavonoids did not alter the responses of any peptide agonist in this signaling pathway (Figs. 2 and 3, Table 1).
In agreement with previous findings (Koole et al., 2010), neither GLP-1(1–36)NH₂ nor GLP-1(1–37) displayed any agonism in Ca\(^{2+}\) signaling pathway in either the absence or presence of any of the tested flavonoids (data not shown). Control experiments showed that all ATP responses in the presence of 30 \(\mu\)M any flavonoid tested remained robust and constant. In addition, Ca\(^{2+}\) signaling mediated by calcitonin in calcitonin receptor Leu polymorphism (another family B GPCR)-expressing FlpIn 3T3 cells was unaltered by these flavonoids (data not shown). This, in addition to the probe dependence displayed by the hydroxyl flavonol compounds, is consistent with these compounds’ mediating their effects specifically at the GLP-1R.

To further investigate the interaction between the hydroxyl flavonols (4′-hydroxyflavonol, 3′,4′-dihydroxyflavonol, and quercetin) and the high-affinity peptide agonists, interaction studies between a range of concentrations (1–50 \(\mu\)M) of these modulators and each of the peptides were performed (Fig. 4). Analysis of the data using an allosteric operational model revealed pKᵦ estimates of the flavonols, in addition to cooperativity (β) estimates, the rank order of cooperativity of these modulators and each of the peptides were performed in duplicate. +, data statistically different from peptide alone (\(p < 0.05\)) as measured by one-way analysis of variance followed by a Dunnett’s post test.

**Table 1**

Differential effects of flavonoids on peptide agonists of the human GLP-1R in calcium mobilization in FlpInCHO cells stably expressing the human GLP-1R

Data were analyzed using eq. 1. pEC\(_{50}\) value represent the negative logarithm of the concentration of agonist that produces half the maximal response. E\(_{\text{max}}\) represents the maximal response normalized to that of peptide alone. All values are mean ± S.E.M. of four independent experiments. Data were analyzed with one-way analysis of variance and Dunnett’s post test.

**Orthosteric Peptide Ligand**

<table>
<thead>
<tr>
<th>30 (\mu)M Flavonoid Compound</th>
<th>GLP-1(7–36)NH₂</th>
<th>GLP-1(7–37)</th>
<th>Exendin-4</th>
<th>Oxyntomodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC(_{50})</td>
<td>pEC(_{50})</td>
<td>pEC(_{50})</td>
<td>pEC(_{50})</td>
</tr>
<tr>
<td>Peptide alone (no flavonoid)</td>
<td>8.19 ± 0.06</td>
<td>8.07 ± 0.06</td>
<td>8.19 ± 0.05</td>
<td>6.89 ± 0.07</td>
</tr>
<tr>
<td>Flavonol</td>
<td>8.15 ± 0.06</td>
<td>7.99 ± 0.06</td>
<td>8.03 ± 0.08</td>
<td>6.90 ± 0.13</td>
</tr>
<tr>
<td>Flavonol</td>
<td>8.02 ± 0.08</td>
<td>8.04 ± 0.05</td>
<td>8.09 ± 0.13</td>
<td>6.81 ± 0.11</td>
</tr>
<tr>
<td>3′,4′-Dihydroxyflavonol</td>
<td>8.47 ± 0.08*</td>
<td>8.37 ± 0.11</td>
<td>8.41 ± 0.12</td>
<td>6.88 ± 0.10</td>
</tr>
<tr>
<td>3′,4′-Dihydroxyflavonol</td>
<td>8.68 ± 0.07*</td>
<td>8.55 ± 0.06*</td>
<td>8.57 ± 0.10*</td>
<td>6.95 ± 0.14</td>
</tr>
<tr>
<td>Quercetin</td>
<td>8.72 ± 0.07*</td>
<td>8.65 ± 0.09*</td>
<td>8.81 ± 0.09*</td>
<td>6.84 ± 0.08</td>
</tr>
<tr>
<td>Quercetin</td>
<td>8.18 ± 0.06</td>
<td>7.97 ± 0.05</td>
<td>7.96 ± 0.08</td>
<td>6.90 ± 0.09</td>
</tr>
<tr>
<td>Luteolin</td>
<td>8.27 ± 0.09</td>
<td>8.06 ± 0.10</td>
<td>8.08 ± 0.06</td>
<td>6.95 ± 0.12</td>
</tr>
<tr>
<td>Genistein</td>
<td>8.06 ± 0.05</td>
<td>8.01 ± 0.06</td>
<td>8.16 ± 0.09</td>
<td>6.84 ± 0.09</td>
</tr>
<tr>
<td>Catechin</td>
<td>8.20 ± 0.07</td>
<td>8.11 ± 0.09</td>
<td>7.97 ± 0.08</td>
<td>6.84 ± 0.10</td>
</tr>
</tbody>
</table>

* \(P < 0.05\), statistically significant from peptide alone.
reductions in the observed maximal response (Fig. 5, Table 3). Furthermore, the potency of the cAMP response produced by GLP-1(7–37) was also significantly reduced (8-fold) in the presence of catechin, whereas cAMP responses to GLP-1(1–37), exendin-4, and oxyntomodulin were unaltered (Fig. 5, Table 3). All other flavonoids studied showed no modulation.

### Table 2

<table>
<thead>
<tr>
<th>Flavonol</th>
<th>(pK_b)</th>
<th>(\log) Estimated Log(\beta)</th>
<th>GLP-1(7–36)NH(_2)</th>
<th>GLP-1(7–37)</th>
<th>Exendin-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4’-Hydroxyflavonol</td>
<td>4.7 ± 0.2</td>
<td>0.40 ± 0.10 (2.5)</td>
<td>0.39 ± 0.09 (2.5)</td>
<td>0.49 ± 0.16 (3.1)</td>
<td></td>
</tr>
<tr>
<td>3’,4’-Dihydroxyflavonol</td>
<td>4.5 ± 0.1</td>
<td>0.79 ± 0.12 (6.2)</td>
<td>0.75 ± 0.09 (5.6)</td>
<td>0.80 ± 0.10 (6.3)</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>4.4 ± 0.2</td>
<td>0.99 ± 0.13 (9.8)</td>
<td>0.98 ± 0.09 (9.6)</td>
<td>1.04 ± 0.10 (10.9)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Characterization of the interaction between a range of concentrations (1–50 \(\mu\)M) of flavonols [4’-hydroxyflavonol (a, d, g), 3’,4’-hydroxyflavonol (b, e, h), and quercetin (c, f, i)] and GLP-1(7–36)NH\(_2\) (a–c), GLP-1(7–37) (d–f), and exendin-4 (g–i) in an intracellular \(\text{Ca}^{2+}\) mobilization assay using FlpInCHO cells stably expressing the human GLP-1R. Data are normalized to the maximal response elicited by peptide alone and analyzed with an allosteric operational model as defined in eq. 2. All data are mean ± S.E.M. of three independent experiments performed in duplicate.

All values are mean ± S.E.M. of three independent experiments analyzed according to eq. 2. \(pK_b\) is the negative logarithm of the estimated affinity of the flavonol compounds. This value was derived from the interaction data of GLP-1(7–36)NH\(_2\). Consistent values were obtained from GLP-1(7–37) and exendin-4 analyses. Estimated Log\(\beta\) is the logarithm of the factor governing the allosteric effects on activation between flavonols and the peptide agonists. Antilogarithms are shown in parentheses (\(\beta\)).
of GLP-1R peptide ligands in cAMP accumulation assays (Fig. 5, Table 3).

**Flavonoids Selectively Modulate the Binding Affinity of Antagonists and Agonists of the GLP-1R.** Equilibrium binding studies were performed between each of the flavonoids and a radiolabeled orthosteric GLP-1R antagonist \([1^{25}I]-exendin(9–39)\) to establish the ability of flavonoids to modify peptide binding affinity. All the flavonoids revealed weak negative cooperativity with the antagonist, the greatest degree of inhibition being observed in hydroxyl flavonols (4'-hydroxyflavonol, 3',4'-dihydroxyflavonol, and quercetin) (Fig. 6).

To establish the ability of the different flavonoids to modulate orthosteric agonist affinity, competition binding studies were performed with GLP-1(7–36)NH₂, GLP1(7–37), GLP-1(1–36)NH₂, GLP-1(1–37), oxyntomodulin, or exendin-4 in
Table 3

Differential effects of flavonoids on peptide agonists of the human GLP-1R in cAMP accumulation assays in FlpInCHO cells stably expressing the human GLP-1R.

<table>
<thead>
<tr>
<th>FLAVONOID COMPOUND</th>
<th>Molar concentration (M)</th>
<th>GLP-1(7–36)NH2</th>
<th>Exendin-4</th>
<th>Oxyntomodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide alone (no flavonoid)</td>
<td>10−5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Flavone</td>
<td></td>
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<tr>
<td>Flavonol</td>
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<td></td>
<td></td>
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<tr>
<td>-Hydroxyflavonol</td>
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<tr>
<td>-Dihydroxyflavonol</td>
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<tr>
<td>Quercetin</td>
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<tr>
<td>Luteolin</td>
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<tr>
<td>Daidzein</td>
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<td>Genistein</td>
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<tr>
<td>Catechin</td>
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</tbody>
</table>

Data were analyzed using eq. 1. pEC50 values represent the negative logarithm of the concentration of agonist that produces half the maximal response. Max represents the maximal response normalized to that of peptide alone. All values are mean ± S.E.M. of four independent experiments. Data were analyzed with one-way analysis of variance and Dunnett’s post test.

The absence or presence of 30 μM concentrations of each flavonoid (Fig. 6). No significant modulation in agonist affinity of any peptide was observed in interaction binding studies with any of the flavonoids studied (Fig. 6). This suggests the effects of the hydroxyl flavonols on peptide-mediated Ca2+ signaling and catechin on cAMP signaling are principally driven by changes in orthosteric ligand efficacy.

Discussion

The GLP-1R is a major target for the treatment and management of type II diabetes but peptides, despite the approval of several drugs (exenatide and liraglutide), do not provide ideal therapeutics because their use is complicated by the route of administration. This has driven the search for low molecular weight, orally active compounds that activate or augment GLP-1R signaling as the idealized therapeutic drug. Recent drug discovery efforts for the GLP-1R have focused on targeting sites for allosteric modulation. Allosteric interactions are often complex because ligands can alter the biological properties of the endogenous ligand by modulating the affinity and/or efficacy as well as having the potential to exhibit their own agonism. This can be complicated if there are multiple endogenous ligands (as is the case for the GLP-1R), because the allosteric interaction can vary with the nature of the orthosteric ligand, a property termed “probe dependence” (May et al., 2007b). These allosteric effects can also be manifested in a pathway-dependent manner, which adds an additional level of complexity. An example of this is observed for the GLP-1R with the actions of Novo Nordisk compound 2, a small-molecule allosteric agonist that modulates oxyntomodulin preferentially over GLP-1 peptides in cAMP accumulation assays yet displays no modulation in Ca2+ or ERK1/2 phosphorylation assays (Koole et al., 2010).

We recently found that the flavonol quercetin lacked intrinsic efficacy but selectively modulated intracellular Ca2+ responses in a probe-dependent manner. At concentrations above 1 μM, quercetin augmented signaling of the high-affinity agonists GLP-1(7–36)NH2, GLP-1(7–37), and exendin-4 but had no effect on oxyntomodulin signaling (Koole et al., 2010). Quercetin also engenders “stimulus bias” at the level of the GLP-1R, in that no modulation of cAMP signaling was observed. To systematically explore the SAR of quercetin as a modulator of GLP-1R signaling, a selection of flavonoid compounds were analyzed based on the flavone (2-phenylchromen-4-one) backbone (Fig. 1). In addition, two isoflavones containing a 3-phenylchromen-4-one backbone were examined. Important structural features of flavonoids were identified in this study that distinguished those that have the ability to modulate GLP-1R-mediated signaling from those that are inactive. A series of hydroxyl flavonols displayed no intrinsic efficacy, but all modulated Ca2+ signaling in a probe-dependent manner. The greater the number of hydroxyl substituents contained in these flavonol compounds, the more pronounced the observed modulator ability. A structure-activity series can therefore be assigned to these compounds; quercetin, which contains hydroxyl groups at positions 3, 5, 7, 3’, and 4’ (see Fig. 1), displayed the largest degree of modulation, which is evident by its larger cooperativity factor (β = 9.6–10.9 depending on the orthosteric peptide). 3’,4’-Dihydroxyflavonol with hydroxyl groups at positions 3, 3’, and 4’ showed a smaller degree of modulation.
than quercetin ($\beta = 5.6–6.3$) but greater than that by 4'-hydroxyflavonol ($\beta = 2.5–3.1$) with hydroxyl groups at only positions 3 and 3'. It is noteworthy that replacement of the hydroxyl groups at positions 3’ and 4’ by the more bulky methoxy groups (3’,4’-dimethoxyflavonol) removed the ability of the compound to modulate Ca$^{2+}$ signaling. The pres-

Fig. 6. Characterization of the inhibition of $^{125}$I-exendin(9–39) binding by peptide agonists and/or flavonoids using membranes prepared from FlpInCHO cells stably expressing the human GLP-1R: GLP-1(7–36)NH$_2$ (a), GLP-1(7–37) (b), exendin-4 (c), oxyntomodulin (d), GLP-1(1–36)NH$_2$ (e), and GLP-1(1–37) (f) in the presence of 30 $\mu$M flavonoid compound. g, inhibition of flavonoid compounds alone. The peptide-alone curve represents inhibition binding by GLP-1(7–36)NH$_2$. Data are presented as percentage specific binding, with nonspecific binding measured in the presence of 1 $\mu$M exendin(9–39). All values are mean ± S.E.M. of four independent experiments performed in duplicate.
ence of just the flavonol backbone (hydroxyl at position 3) was also inactive. However, the hydroxyl group at position 3 of the 2-phenylchromen-4-one scaffold was essential but not sufficient on its own for modulation of Ca$^{2+}$ responses by the high-affinity peptide ligands. The flavone luteolin is structurally very similar to quercetin, lacking only the hydroxyl group at position 3. Consequently, luteolin did not modulate Ca$^{2+}$ signaling of any of the peptide ligands (Figs. 2 and 3) indicating that this hydroxyl group is essential for the allosteric actions of hydroxyl flavonoids. This is also supported by the lack of activity observed for the isoflavones daidzein and genistein, which lack the 3-hydroxy group because of the presence of the bulky hydroxyphenyl group at this position.

The hydroxyl flavonols tested all imparted stimulus bias; no modulation in peptide-mediated cAMP signaling was observed. The flavonoid catechin also displayed pathway-selective modulation. However, in contrast to hydroxyl flavonols, analysis of the actions of catechin revealed no modulation in Ca$^{2+}$ signaling but interesting differences in the modulation of peptide agonist function for cAMP accumulation. Catechin contains a slightly different scaffold (a chromene backbone) compared with all the other tested compounds, lacking the carbonyl group at position 4 (Fig. 1). This introduces flexibility into the phenolic structure; however, it contains all the same hydroxyl groups as quercetin. The introduction of flexibility into the backbone removes the ability of the flavonoid to modulate peptide-mediated Ca$^{2+}$ signaling, but imparts an inhibition in the ability of some, but not all, orthosteric peptides to generate cAMP; the efficacy was significantly reduced for the endogenous ligands GLP-1(1–36)NH$_2$ and GLP-1(7–36)NH$_2$, whereas the potency (but not the efficacy) was reduced for GLP-1(1–37), with no significant changes in GLP-1(1–37), oxyntomodulin or exendin-4 signaling. Exendin-4 and the truncated forms of GLP-1 are thought to be functionally equivalent because they have similar pharmacology; however, negative cooperativity between catechin and the truncated GLP-1 peptides versus neutral cooperativity with exendin-4 implies that the conformational states adopted by the truncated GLP-1 peptides and exendin-4 are different. Likewise, using the Novo Nordisk allosteric modulator compound 2, positive binding cooperativity was observed between compound 2 and truncated forms of GLP-1 but neutral cooperativity for exendin-4 (Koole et al., 2010). The subtle differences in the inhibition of cAMP signaling of GLP-1(7–36)NH$_2$ and GLP-1(7–37) in the presence of catechin also suggest that these two truncated versions of GLP-1 may produce different receptor conformational states.

Taken together, the results described above suggest that the difference in the backbone between hydroxyl flavonols and catechin is sufficient to engender unique receptor conformations that can either augment Ca$^{2+}$ signaling or inhibit cAMP signaling of endogenous/exogenous ligands. In binding assays, hydroxyl flavonols and catechin both displayed weak inhibition of the radioligand but displayed neutral cooperativity with the peptide agonists. This implies that the observed allosteric pathway-selective cooperativity (positive for hydroxyl flavonols, negative for catechin) is purely driven by changes in orthosteric efficacy. The lack of effects of the flavonoids in competition binding assays suggests that they are acting at an allosteric site to produce these effects.

The GLP-1R is pleiotropically coupled, and it is clear that the physiological response leading to insulin secretion is a composite of activation of multiple signaling pathways by multiple ligands (Baggio and Drucker, 2007). Because the underlying GLP-1R-mediated signaling to target many of the therapeutically important effects is not fully understood, further work is required to identify the optimal combination of signaling outputs and pathway bias to target for treatment of type II diabetes. Thus, it is becoming increasingly important for screening efforts to focus on multiple signaling outputs, regardless of coupling strength, rather than focusing on a single endpoint, because the ultimate clinical efficacy may be determined by an optimal combination of collateral efficacies. In addition, for receptors such as the GLP-1R that involve the interplay between multiple endogenous ligands, probe dependence of allosteric drugs is a major consideration. The truncated forms of GLP-1 are considered the predominant ligands for the GLP-1R, oxyntomodulin and the full-length GLP-1 forms playing a minor role in comparison. However, when studying allosteric interactions, it is important to study the effects on all endogenous ligands because there is the potential for allosteric effects on weak endogenous agonists that may potentiate the activity of these ligands to physiologically relevant levels, effects that may be beneficial or contribute to unwanted or adverse side effects.

Flavonoid compounds are naturally occurring, and the fact that their structural backbone can engender receptor conformations that either augment Ca$^{2+}$ signaling or inhibit cAMP signaling may aid in developing compounds with favorable pharmacological properties for the treatment of disease conditions. The lack of effect observed on the calcitonin receptor Leu polymorphism and ATP responses in Ca$^{2+}$ signaling assays, in addition to the probe dependence observed for GLP-1R agonists, are consistent with the idea that these compounds act at the GLP-1R. However, at the high concentrations used in this study, flavonoids are reported to exert multiple pharmacological effects, in addition to those that may be beneficial to treatment type II diabetes. Some of these are discussed extensively in Middleton et al., 2000. As a consequence of this lack of specificity, flavonoids are unlikely to provide a good starting structure for development of selective allosteric ligands; however, the flavonol scaffold may provide a useful tool for studying GLP-1R modulation, in addition to providing proof of concept for the use of SAR to probe allosteric interactions at this receptor.

In conclusion, we have shown that naturally occurring and synthetic flavonoids can differentially traffic stimuli imparted to the GLP-1R by multiple but not all endogenous agonists. An SAR approach revealed crucial roles of the 3-hydroxyl group and multiple substituent hydroxyl groups of the phenyl ring for positive allosteric modulator activity. Additional phenyl groups at positions 5 and 7 of the chromone-4-one ring further enhance the positive modulator activity. These data represent a detailed examination of the role of naturally occurring compounds on peptide agonists at the GLP1-R, and may aid drug discovery efforts in the development of viable therapeutics for the management of type II diabetes.

Authorship Contributions

Participated in research design: Wootten, Simms, Woodman, and Sexton.

Conducted experiments: Wootten.

Contributed new reagents or analytical tools: Woodman.
References
Larsen J, Hyleboeck B, Ng K, and Damsbo P (2001) Glucagon-like peptide-1 infusion must be maintained for 24 h/day to obtain acceptable glycemia in type 2 diabetic patients who are poorly controlled on sulphonylurea treatment. Diabetes Care 24:1416–1421.
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