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Activation of CFTR by UC_{CF}-029 and genistein

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ABSTRACT

The mechanism of action of a novel CFTR activator UC_{CF}-029 on NIH3T3 cells stably expressing ΔF508-CFTR was investigated and its effects compared to those of genistein, a known CFTR activator. This study shows that UC_{CF}-029 and genistein have differing efficacies. The efficacy of UC_{CF}-029 in the presence of forskolin (10 μM) is ~50% that of genistein; however, the EC_{50}'s for both drugs are comparable; 3.5 μM for UC_{CF}-029 and 4.4 μM for genistein. Using NIH3T3 cells stably transfected with K1250A-CFTR we find that CFTR channel open time is unaffected by UC_{CF}-029 or genistein, supporting the hypothesis that these compounds stabilize the open state by inhibiting ATP hydrolysis at NBD2. Our data suggest that the ability of UC_{CF}-029 to augment ΔF508-CFTR channel activity necessitates further interest.

Cystic fibrosis (CF), the most common lethal genetic disease in Caucasians, is caused by mutations in the single gene encoding the cystic fibrosis transmembrane conductance regulator protein, CFTR.[1] CFTR functions as a protein kinase A (PKA)-dependent, cAMP-regulated epithelial chloride channel.[1] Mutations in the CFTR chloride channel result in defective epithelial electrolyte transport. Deletion of the phenylalanine at amino acid position 508 (ΔF508) is the most common disease-associated mutation, having two associated defects; trafficking[2] and function.[3,4] Some ΔF508-CFTR can reach the plasma membrane, although its open probability (Po) is reduced compared to that of wild-type (Wt) channels even in the presence of maximally effective concentrations of cAMP.[3–5]

Recently, attention has been directed towards correcting the defective function associated with ΔF508-CFTR through use of pharmacological means. Several groups of compounds have been shown to improve ΔF508-CFTR function; the isoflavone genistein,[6] the benzimidazolone analogs NS004 and NS1619,[6] curcumin,[7] 6-phenylpyrrolo[2,3-*b*]pyrazines,[8] sulfamoyl-4-oxoquinoline-3carboxamides,[9] and 4′-methyl-4,5′-biothiazole.[10]

High-throughput screening assays for the discovery of novel CFTR activators have generated a novel group of compounds sharing a common structural motif.[11] A comparison of the chemical structures of the CFTR activators used in this study is shown in Figure 1. UC_{CF}-029, a 7,8-benzoflavone (generated based on flavone and benzo[*c*]quinolizinium analogs) has been previously described as an effective CFTR activator,[11,12] and genistein, a widely utilized CFTR activator,[6] is used as our reference compound. Both compounds have been shown to activate CFTR via a cAMP-independent mechanism suggesting a direct interaction with CFTR.[13,11] We investigated the mechanism of action of UC_{CF}-029 on ΔF508-CFTR using both whole-cell and cell-attached patch–clamp techniques. Our data suggest that UC_{CF}-029 activates ΔF508-CFTR at micromolar concentrations. Furthermore, saturating concentrations of genistein and UC_{CF}-029 generate similar whole-cell macroscopic ΔF508-CFTR channel current, suggesting a common mechanism of action.

The cells utilized and the cell-attached and whole-cell patch–clamp electrophysiology were as described previously.[6] UC_{CF}-029, provided by the UC Davis Chemistry group, was prepared as previously described using a modification of the Baker–Venkataraman flavone synthesis.[11] The potency and efficacy of UC_{CF}-029 was quantified on ΔF508-CFTR current using the whole-cell patch–

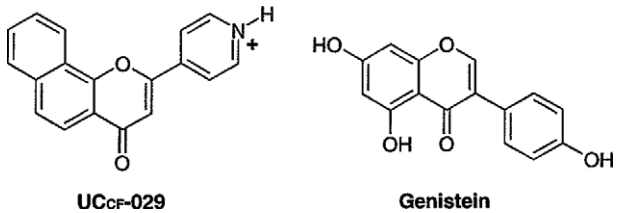

Figure 1. Chemical structures of genistein and UC_{CF}-029. UC_{CF}-029, a 7,8-benzoflavone, contains structural features of both flavones and benzo[*c*]quinoliziniums.

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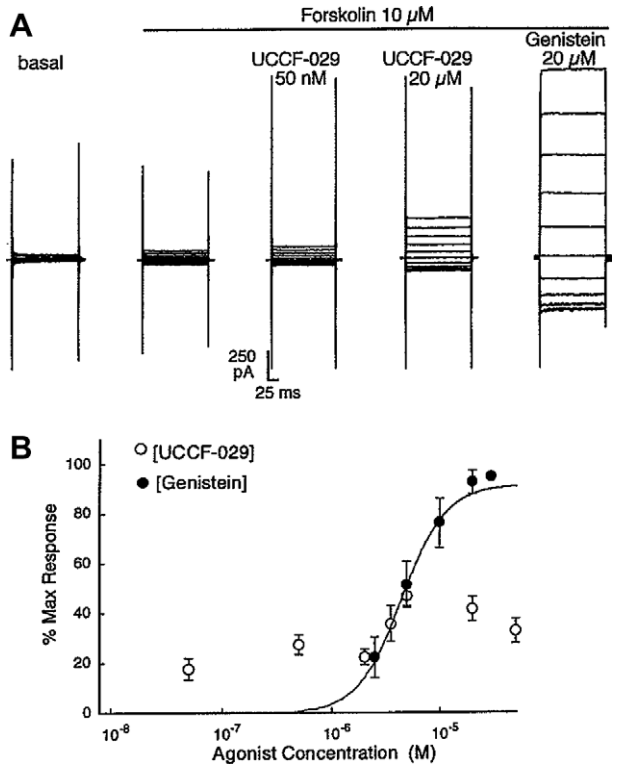

Figure 2. Dose–response relationships of genistein and UC_{CF}-029 in NIH3T3-ΔF508 cells. (A) Concentration-dependent effects of UC_{CF}-029. In the absence of agonists, whole-cell basal current is minimal and addition of 50 nM and 20 μM UC_{CF}-029 incrementally increases current, in the continued presence of 10 μM forskolin. Genistein (20 μM) further potentiates this current. (B) Dose–response relationships of genistein and UC_{CF}-029. Whole-cell cAMP-dependent NIH3T3-ΔF508 CFTR currents at each concentration tested were normalized to that obtained with 20 μM genistein in the same cell. The efficacy of UC_{CF}-029 is ∼50% that of genistein. The EC_{50} for UC_{CF}-029 is 3.5 μM, close to that of genistein (4.4 μM, the genistein dose–response curve was previously published[6]). Genistein (•), n = 5–6 and UC_{CF}-029 (○), n = 4–16.

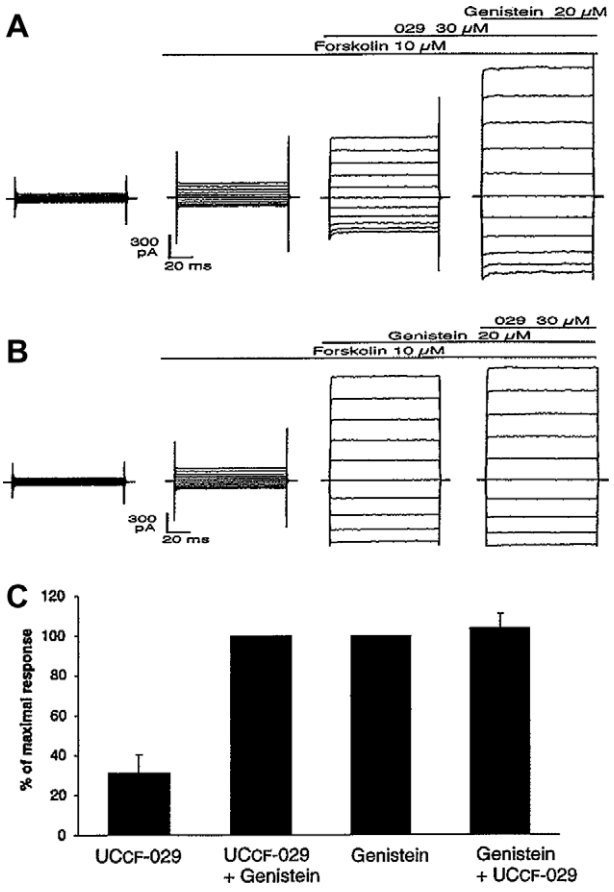

Figure 3. Effect of UC_{CF}-029 and genistein on whole-cell ΔF508-CFTR current. (A) Effect of genistein in the continued presence of UC_{CF}-029. In the continued presence of forskolin (10 μM), UC_{CF}-029 (30 μM) increases the ΔF508-CFTR whole-cell current and genistein (20 μM) further potentiates the current. (B) Effect of UC_{CF}-029 in the continued presence of genistein. In the continued presence of 10 μM forskolin, genistein (20 μM) increases the whole-cell current and there is no additional effect of UC_{CF}-029 (30 μM). (C) Average data of the typical experiments shown in (A and B). Whole-cell current in the presence of genistein is taken as maximal (100%) and the effect of UC_{CF}-029 (30 μM) is compared to that (n = 3).

clamp technique. Genistein was used as a comparison to better appraise the effects of UC_{CF}-029, since the mechanism of action of genistein on CFTR is well understood.[6,14,15] We have previously shown that genistein potentiates forskolin-stimulated ΔF508-CFTR current in a dose-dependent manner, and maximal effect is elicited at 20 μM.[6]

Figure 2A shows a typical whole-cell experiment in which the forskolin-stimulated NIH3T3-ΔF508-CFTR current was augmented with either genistein or UC_{CF}-029. All whole-cell experiments were performed in the presence of a chloride gradient thus yielding the observed rectifying currents. Basal ΔF508-CFTR current is minimal in the absence of agonists, and application of forskolin (10 μM) induced a small increase in current, which incrementally increased with the addition of 50 nM and 20 μM UC_{CF}-029. The addition of 20 μM genistein further potentiates this current. The dose–response relationships of genistein and UC_{CF}-029 summarized from multiple whole-cell patches are shown in Figure 2B. The ΔF508-CFTR current generated at each concentration of agonist tested is normalized to that obtained with 20 μM genistein in the same cell, and similar EC_{50} values for genistein and UC_{CF}-029 were found (4.4 ± 0.5 μM (n = 5–6), 3.5 ± 0.6 μM (n = 4–16), respectively). The maximal whole-cell ΔF508-CFTR current enhanced by genistein is greater than that obtained with a maximal concentration of UC_{CF}-029. These data suggest that the efficacy of UC_{CF}-029 is less (by ∼50%) than that of genistein; however, the potencies are similar (μM range).

In the continued presence of 10 μM forskolin, 30 μM UC_{CF}-029 increased ΔF508-CFTR current by 3.76 ± 1.06-fold (n = 3) and the inclusion of 20 μM genistein in the bath results in a further potentiation of the current. Whole-cell chloride current at +100 mV increased from 8.64 ± 4.67 to 22.31 ± 7.66 pA/pF (n = 3). We assume the whole-cell current generated by genistein plus UC_{CF}-029 is the maximal effect (100%); thus, the current generated by UC_{CF}-029 alone is 31.03 ± 9.57% of the maximum (n = 3), shown in Figure 3A and C. Maximal enhancement of ΔF508-CFTR by genistein prevents further enhancement by UC_{CF}-029, as shown in Figure 3B and C. These data suggest that both genistein and UC_{CF}-029 share a common mechanism of action.

We next examined the effect of UC_{CF}-029 on the forskolin-dependent ΔF508-CFTR channel current in cell-attached patch. Figure 4 shows a typical recording from an NIH3T3 ΔF508 CFTR cell and application of 10 μM forskolin elicits opening of at least 4 channels in that patch (expanded section). In the continued presence of forskolin, addition of 30 μM UC_{CF}-029 increases the ΔF508-CFTR whole-cell current (3.69-fold). Genistein (20 μM) further augments the current (8.29-fold). In similar experiments, the average mean fold increase in current with 20 μM UC_{CF}-029 was 3.76 ± 1.06 (n = 3), compared to the ∼12-fold increase with 20 μM genistein.

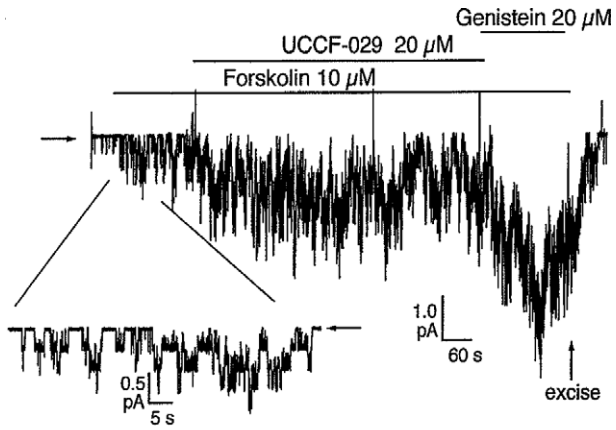

Figure 4. Effect of UC_{CF}-029 on forskolin-dependent ΔF508-CFTR channel current in cell-attached patch. In a recording from an NIH3T3-ΔF508 CFTR cell, 10 μM forskolin generates openings of up to 4 channels. In the continued presence of forskolin, perfusion with 20 μM UC_{CF}-029 generates a 3.69-fold increase in steady-state mean current, which is further potentiated by the addition of 20 μM genistein (8.29-fold). Closed state is denoted by the arrow. Downward deflections are channel openings.

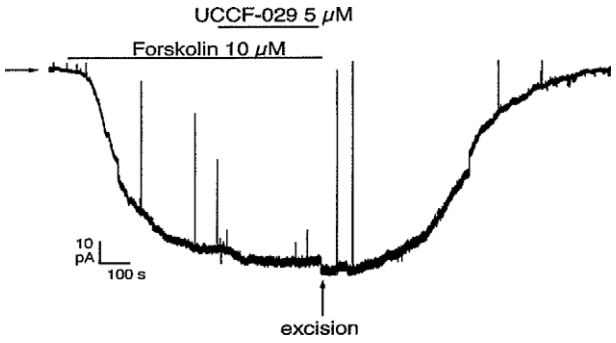

Figure 5. Effect of UC_{CF}-029 and genistein on forskolin-dependent K1250A-CFTR channel current. A continuous cell-attached recording show that 10 μM forskolin elicits a macroscopic K1250A-CFTR current. There is no effect of 5 μM UC_{CF}-029. Excision of the patch results in closure of all K1250A channels upon removal of agonists, and a return to basal activity. Closed state is denoted by the arrow. Downward deflections are channel openings.

To examine whether UC_{CF}-029 acts to stabilize the channel open state, we determined its effect upon the K1250A-CFTR channel (a CFTR channel mutation that can stay open for minutes once opened).[16] A representative recording of K1250A-CFTR in cell-attached patch is shown (Fig. 5), macroscopic current was elicited by maximal concentration of forskolin (10 μM) and subsequent addition of 5 μM UC_{CF}-029 failed to increase the current (fold increase in mean current amplitude was 1.11 ± 0.03, $n = 3$), indicating no effect on Po. Closure of all channels is observed upon excision of the patch and removal of agonists.

UC_{CF}-029 was generated based on two lead compounds, flavones and benzo[*c*]quinolizinium analogs. The isoflavone genistein and the benzo[*c*]quinolizinium MBP-07 have both been shown previously to activate CFTR without increasing intracellular cAMP levels.[17] We compare the effects of UC_{CF}-029 to those of genistein, the most comprehensively studied CFTR activator.[18] Our data suggest that UC_{CF}-029 is not as effective as genistein in activating ΔF508-CFTR channel activity in NIH3T3 fibroblast cells. Whole-cell experiments show that comparative micromolar concentrations of genistein or UC_{CF}-029 yield differing levels of ΔF508-CFTR activity (genistein greater than UC_{CF}-029). Moreover, we find that there is no effect of UC_{CF}-029 on forskolin-stimulated ΔF508-CFTR in the presence of maximally effective concentrations of genistein, indicating that genistein and UC_{CF}-029 likely act on CFTR via a similar mechanism of action.

In addition, we demonstrate that UC_{CF}-029 is unable to further stimulate the K1250A-CFTR chloride current activated by a maximally effective concentration of forskolin. We have previously shown similar results with genistein and benzimidazolone analogs.[6] Our earlier data using relaxation analysis demonstrated that these compounds stabilize open state by inhibiting ATP hydrolysis at NBD2.[6] K1250A-CFTR, a mutation of the Walker A lysine at NBD2 prolongs channel opening by eliminating ATP hydrolysis at NBD2.[19,16]

The original work describing UC_{CF}-029 compared its effects to several other novel compounds generated using combinatorial libraries and with genistein,[11] using the halide-sensitive yellow fluorescent protein on Wt-CFTR transfected Fischer rat thyroid (FRT) cells. Those studies suggested that UC_{CF}-029 was more potent in activating CFTR than genistein. However, the difference in effectiveness of UC_{CF}-029 in our hands versus theirs could be attributed to the following: (1) we used NIH3T3 cells transfected with ΔF508-CFTR, whereas they used FRT cells transfected with Wt-CFTR (interestingly in those same studies, UC_{CF}-029 was not as effective an activator on another CFTR mutation G551D), (2) our experiments used single isolated cells, whereas they used monolayers of cells either grown in 96-well plates for the fluorescent assays or polarized monolayers for Ussing electrophysiological short circuit current measures. Recently, UC_{CF}-029 was shown to be a potent activator of chloride secretion in human bronchial epithelia, although affinity was dependent on basal CFTR activity.[12]

Whilst the results from our study propose that UC_{CF}-029 is less efficacious than genistein in activating ΔF508-CFTR, we suggest that they likely act on CFTR via a similar mechanism of action. Differences found in the potency of UC_{CF}-029 could be assuaged as variances in the cell systems utilized; perhaps one has more basal ΔF508-CFTR channel activity than another, etc. These data enforce the usefulness of using genistein as the benchmark for further development of flavonoids. Further work is required to determine the usefulness of UC_{CF}-029 as a potential CF therapeutic.

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