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Role of protein phosphatases in the activation of CFTR (ABCC7) by genistein and bromotetramisole

JIEXIN LUO,[1] TANG ZHU,[1] ALEXANDRA EVAGELIDIS,[1] MARY D. PATO,[2] AND JOHN W. HANRAHAN[1]
[1]*Department of Physiology, McGill University, Montréal, Québec H3G 1Y6; and* [2]*Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0*

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Luo, Jiexin, Tang Zhu, Alexandra Evagelidis, Mary D. Pato, and John W. Hanrahan. Role of protein phosphatases in the activation of CFTR (ABCC7) by genistein and bromotetramisole. *Am J Physiol Cell Physiol* 279: C108–C119, 2000.—Genistein and bromotetramisole (Br-t) strongly activate cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7) chloride channels on Chinese hamster ovary cells and human airway epithelial cells. We have examined the possible role of phosphatases in stimulation by these drugs using patch-clamp and biochemical methods. Genistein inhibited the spontaneous rundown of channel activity that occurs after membrane patches are excised from cAMP-stimulated cells but had no effect on purified protein phosphatase type 1 (PP1), PP2A, PP2B, PP2C, or endogenous phosphatases when assayed as [^{32}P]PO_4 release from prelabeled casein, recombinant GST-R domain fusion protein, or immunoprecipitated full-length CFTR. Br-t also slowed rundown of CFTR channels, but, in marked contrast to genistein, it did inhibit all four protein phosphatases tested. Half-maximal inhibition of PP2A and PP2C was observed with 0.5 and 1.5 mM Br-t, respectively. Protein phosphatases were also sensitive to (+)-*p*-Br-t, a stereoisomer of Br-t that does not inhibit alkaline phosphatases. Br-t appeared to act exclusively through phosphatases since it did not affect CFTR channels in patches that had low apparent endogenous phosphatase activity (i.e., those lacking spontaneous rundown). We conclude that genistein and Br-t act through different mechanisms. Genistein stimulates CFTR without inhibiting phosphatases, whereas Br-t acts by inhibiting a membrane-associated protein phosphatase (probably PP2C) that presumably allows basal phosphorylation to accumulate.

cystic fibrosis; phosphorylation; PP2C; (iso)flavonoids; phenylimidazothiazoles; cystic fibrosis transmembrane conductance regulator

THE CYSTIC FIBROSIS transmembrane conductance regulator (CFTR; ABCC7-human gene nomenclature committee) chloride channel is activated by phosphorylation (6, 43) and is gated by hydrolyzable nucleotides (1, 2, 30). Phosphorylation by protein kinase A (PKA) provides the primary stimulus for channel activation (10, 11, 39). Protein kinase C phosphorylation modulates responsiveness of the channel to PKA (26, 31, 34, 43). Less is known regarding the dephosphorylation of CFTR, although it is equally important for controlling CFTR activity under physiological conditions. A membrane-associated phosphatase reduces the open probability in cell-attached membrane patches and causes CFTR channels to run down rapidly when excised from cAMP-stimulated Chinese hamster ovary (CHO), airway epithelial, or human colon cells (4, 16, 32, 43, 44). Protein phosphatases type 2A and especially 2C (PP2A and PP2C, respectively) have been implicated in regulating CFTR (7, 19, 32, 43, 45). CFTR and PP2C are specifically coimmunoprecipitated by antibodies against the other protein, and PP2C can be copurified by nickel chelate chromatography of histidine-tagged CFTR, suggesting that they form a stable regulatory complex (52).

The (iso)flavonoid genistein has attracted interest because of its ability to stimulate CFTR channels, including those with disease-causing mutations (14, 21, 23, 24, 41, 42, 50, 51). In most preparations, genistein stimulation requires elevated PKA activity. Genistein stimulation is associated with increased phosphorylation of tryptic peptides, which also become phosphorylated during forskolin stimulation (41). These data are consistent with phosphatase inhibition, although recent patch-clamp and biochemical studies indicate that activation of genistein is at least partially independent of phosphatase and demonstrate direct interaction of genistein with the channel (12, 14, 36, 40, 47–49).

Wild-type and mutant CFTR channels in transfected cells are also activated by phenylimidazothiazoles such as (−)-*p*-bromotetramisole (Br-t) and levamisole, which are well-known inhibitors of liver, bone, and kidney (LBK) alkaline phosphatases. Although most recent data suggest that CFTR is regulated by protein phosphatases (notably PP2C and PP2A) rather than by an alkaline phosphatase, the sensitivities of protein phosphatases to Br-t have not been reported. Consequently, it remains unclear whether phenylimidazothiazoles could act through inhibition of protein phosphatases. Understanding how genistein and Br-t

Address for reprint requests and other correspondence: J. W. Hanrahan, Dept. of Physiology, McGill Univ., 3655 Drummond St., Montréal, Québec, Canada H3G 1Y6 (E-mail: hanrahan@med.mcgill.ca).

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stimulate CFTR should clarify aspects of its regulation and could be relevant to the design of pharmacotherapies for cystic fibrosis.

In this study, we show that genistein and Br-t activate CFTR channels through distinct mechanisms. Genistein does not inhibit protein phosphatases, whereas both enantiomers of Br-t inhibit protein phosphatases at concentrations found previously to activate CFTR channels, and this probably accounts for their stimulatory effect.

MATERIALS AND METHODS

Cell culture. CHO cells stably expressing wild-type CFTR were plated at low density on glass coverslips 3–5 days before patch-clamp experiments. Baby hamster kidney (BHK) cells stably expressing wild-type CFTR were plated in 10-cm-diameter plates for use in immunoprecipitations. Cells were grown at 37°C with 5% CO_2 in modified minimal essential medium (Life Technologies) containing 5% dialyzed FBS.

Patch-clamp studies. CHO cells were placed in a recording chamber (200 μl) containing (in mM) 150 NaCl, 2 $MgCl_2$, and 10 *N*-[Tris(hydroxymethyl)methyl]-2-aminoethane-sulfonic acid (TES), pH 7.4. In most experiments, this solution also contained 0.5 mM Mg^{2+}-ATP. Single-channel currents were recorded from cell-attached and excised patches as described previously (32) and were analyzed using DRSCAN, a pCLAMP-compatible program for analyzing long records (15).

Phosphatases. Recombinant PP1γ was kindly provided by Dr. P. T. W. Cohen, University of Dundee. PP2A and PP2Cα were prepared from turkey gizzard smooth muscle, as described previously (37, 38). PP2B was from Calbiochem (La Jolla, CA).

GST-R domain. cDNA corresponding to amino acids M645–M837 was amplified using Vent polymerase and PCR primers that contained *Bam*H I sites for subcloning into the pGEX-2T vector. Kozak consensus sequences were also added for future studies involving expression in mammalian cells. The pGEX-2T-R domain plasmid was confirmed by DNA sequencing. HB101 cells were transformed and grown to late log phase at 30°C. GST-R domain fusion protein expression was induced for 2–3 h with 0.1 mM isopropyl β-D-thiogalactopyranoside. GST-R domain was purified on glutathione-Sepharose 4B (Sigma) and eluted by addition of 20 mM glutathione at 4°C according to the manufacturer's instructions.

[^{32}P]PO_4 labeling of casein or GST-R domain. Casein (10 mg; Sigma) or GST-R domain fusion protein (200 μg) was labeled by incubation with PKA (600 units; see Ref. 43 for details) and [γ-^{32}P]ATP (50 μCi; Amersham) in 1 ml reaction buffer containing 50 mM Tris·HCl, 0.1 mM EGTA, 10 mM magnesium acetate, and 0.1% β-mecaptoethanol (pH = 7.0) for 5–12 h at 30°C. The reaction was stopped by adding 100 μl of solution containing 100 mM EDTA and 100 mM sodium pyrophosphate. Free [γ-^{32}P]ATP was removed by passing the reaction mix through Sepharose G-50 (Pharmacia Biotech, Sweden) that had been preequilibrated with 50 mM Tris·HCl, 0.1 mM EGTA, 5% glycerol, and 0.1% β-mercaptoethanol (pH = 7.0). To measure radioactivity of the purified substrates, 100 μl TCA (20% wt/vol) were added to 5- or 10-μl aliquots and centrifuged at 10,000 rpm for 1 min at 4°C, and the supernatant was counted by liquid scintillation. The amount of free [γ-^{32}P]ATP was always <1% of the total radioactivity.

Dephosphorylation of [^{32}P]PO_4-casein and [^{32}P]PO_4-GST-R domain fusion protein. Release of [^{32}P]PO_4 from prelabeled casein by PP1 and PP2A was assayed in duplicate as follows. Purified phosphatase (20 μl) was added to 10 μl Tris buffer containing phosphorylated substrate (prepared as described above). The final assay solution contained 100 mM NaCl, 1.34 mM $MgCl_2$, 33 mM Tris-Cl, pH 7.0, and 0.5–40 nM phosphatase as indicated. The mixture was incubated at 22°C, and the reaction was stopped at timed intervals by the addition of 20% TCA. Samples were centrifuged for 2 min at 4,000 rpm at 4°C, and the supernatant was taken for liquid scintillation counting to determine the amount of free [^{32}P]PO_4 released from casein. Spontaneous release of [^{32}P]PO_4 from phosphocasein was low in the absence of phosphatase (typically <1%) and was subtracted from the total quantity released with phosphatase present. The same assay solution was supplemented with 1 mM Ca^{2+}, 2 mM Mn^{2+}, and 10 μg/ml calmodulin (Boehringer Mannheim) when measuring PP2B activity. For PP2C assays, 10 mM $MgCl_2$ was added to the solution described for PP1 and PP2A. Dephosphorylation of [^{32}P]PO_4-GST-R fusion protein was carried out under similar conditions except the reaction was terminated by adding SDS sample buffer (3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 62.5 mM Tris · HCl, pH 6.8), and samples were subjected to SDS-PAGE (12%) and autoradiography.

Dephosphorylation of immunoprecipitated (full-length) CFTR. After cells were lysed with 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 20 mM Tris · HCl (pH = 8), 0.25 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin (RIPA buffer), CFTR was immunoprecipitated on protein G-Sepharose 4B beads using the monoclonal antibody M3A7 (27). CFTR was phosphorylated while still on the beads by incubation with 180 nM PKA catalytic subunit, 20 μM ATP, 10 μCi [γ-^{32}P]ATP, 10 μg BSA, 140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 mM Tris, pH 7.4, for 1 h at 22°C. The reaction was stopped as before, and the beads were carefully washed with RIPA buffer to remove PKA and then incubated in buffer (140 mM NaCl and 10 mM TES) containing 2 nM PP2A and 2 mM $MgCl_2$ or, alternatively, 10 nM PP2C and 10 mM $MgCl_2$. Immunoprecipitates from ~1.5 plates (10 cm^2) of confluent BHK cells overexpressing wild-type CFTR were used in each experiment. The reaction was stopped by adding sample loading buffer. Samples were subjected to 6% SDS-PAGE and autoradiography to assess the amount of [^{32}P]PO_4 remaining on CFTR. Aliquots were taken from the same immunoprecipitated sample when examining dephosphorylation under various test conditions, and identical volumes were used in each lane during SDS-PAGE. This procedure provided consistent loading, as indicated by several experiments in which Western blots were carried out on the same nitrocellulose membranes used for autoradiography (e.g., see Fig. 4*B*).

Preparation of CHO cell fractions for phosphatase assays. Cells were lysed by sonication in 2× lysis buffer containing 100 mM Tris · HCl, pH 7.4, 4 mM EDTA, 4 mM EGTA, 2% wt/vol Nonidet P-40, and protease inhibitors. The insoluble fraction was removed by centrifugation at 3,000 *g* for 15 min to remove unbroken cells and nuclei. The supernatant was centrifuged at 7,800 *g* for 10 min to pellet the mitochondrial fraction, which was washed with lysis buffer, resuspended in 2× buffer, and mixed with an equal volume of 100% glycerol. The supernatant was centrifuged again at 100,000 *g* for 60 min. The pellet from this spin (crude membrane fraction) was treated as described for the mitochondrial fraction. The supernatant was taken as the cytosolic fraction and was mixed with an equal volume of 100% glycerol.

Statistics. Values are presented as means ± SE. Significance was assessed at the 95% confidence level using a Student's *t*-test.

RESULTS

Effect of genistein on CFTR channel rundown. Rapid deactivation of CFTR channels in excised patches can be reversed by exposure to PKA and therefore reflects dephosphorylation of PKA sites by a robust, membrane-associated phosphatase activity (43). If genistein stimulates CFTR at the level of phosphatases, it would be expected to slow the rundown of channel activity in excised patches (e.g., see Ref. 4). To test this prediction, patches were excised from CHO cells that had been prestimulated using 10 μM forskolin. Under control conditions, channel activity declined by >90% within 100 s after excision in bath solution containing 1 mM Mg^{2+}-ATP (Fig. 1*A*), as described previously (4, 43). However, if genistein (50 μM) was added to the bath once forskolin-stimulated channel activity had increased to a stable level, then CFTR channels remained active for >5 min after excision (Fig. 1*B*). Genistein is a well-known tyrosine kinase inhibitor; however, tyrosine kinases were probably not involved in prolonging channel activity in isolated patches. Another broad-range tyrosine kinase antagonist, erbstatin (IC_{50} = 780 nM), had no effect on rundown when

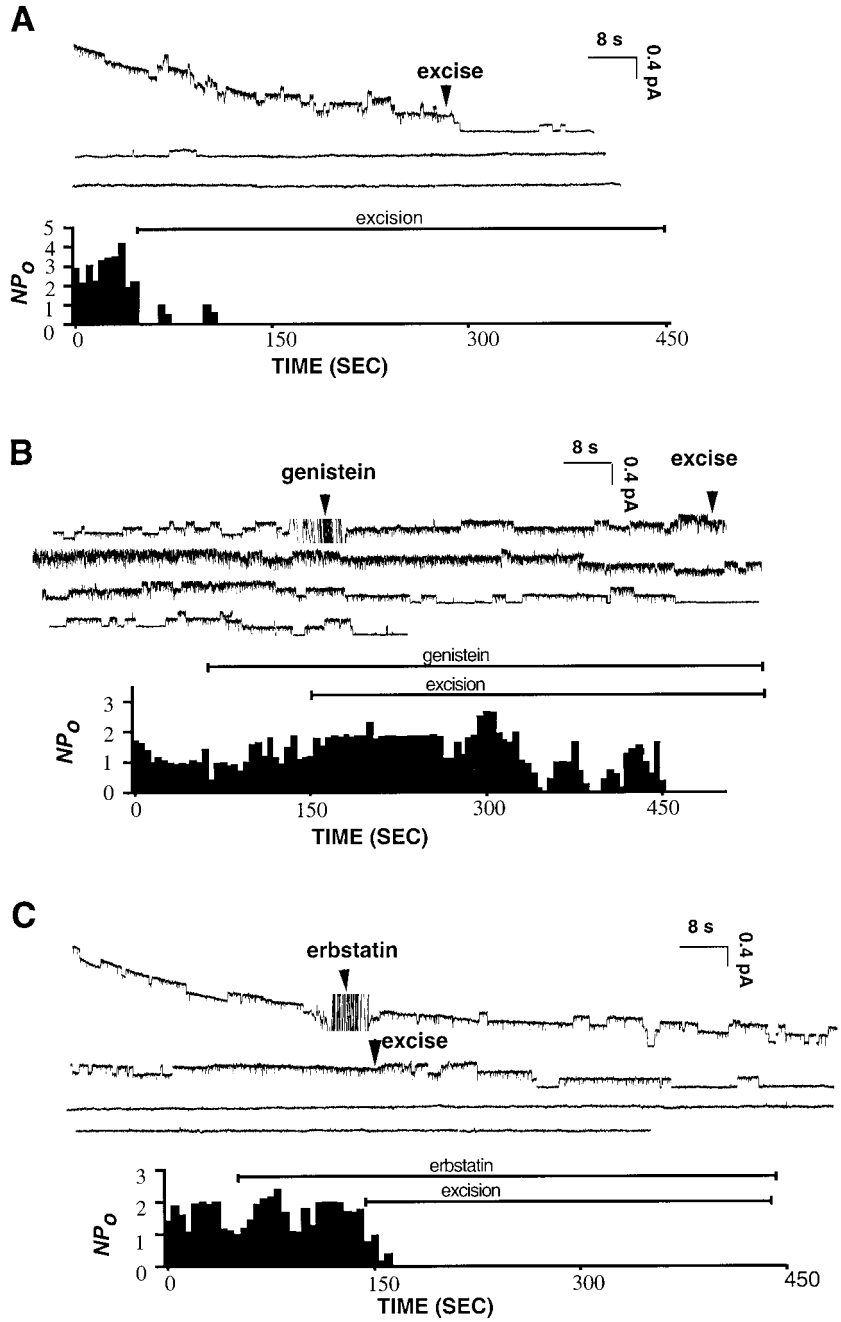

Fig. 1. Inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) channel rundown by genistein (50 μM). Channels were recorded in the cell-attached configuration with forskolin (10 μM) in the bath and then excised in protein kinase A (PKA)-free buffer containing 1 mM Mg^{2+}-ATP. The traces in *A*-*C* are continuous recordings. Mean number of channels open (NP_o) is shown under the traces. *A*: rapid deactivation of channels immediately after isolation of the patch under control conditions. *B*: prolonged activity when genistein (50 μM) was present in the bath. *C*: addition of erbstatin (50 μM), another tyrosine kinase inhibitor, had no effect on rundown. Data are representative of 5 out of 5 experiments.

used at the same concentration (Fig. 1*C*), nor did two other inhibitors (tyrphostin A47 and lavendustin A; data not shown).

Effect of genistein on activities of purified PP2A and PP2C. To determine if genistein directly inhibits protein phosphatases implicated in CFTR regulation, we assayed the effect of genistein on PP2A and PP2C. The amount of each phosphatase used in this and subsequent experiments was chosen based on their activities in preliminary experiments. Under control conditions, purified PP2A (0.5 nM) and PP2C (2 nM) both caused significant $[^{32}P]PO_4$ release from radiolabeled casein within 10 min (7%-17%, Fig. 2, *A* and *B*). Genistein (50 μM) had no effect on PP2A-catalyzed release, which was inhibited ~90% by calyculin A (10 nM) as expected, nor on the residual 10% phosphatase activity remaining in the presence of calyculin A. PP2C-catalyzed $[^{32}P]PO_4$ release from phosphocasein was also genistein insensitive (Fig. 2*B*), although it was inhibited by 90% when free Mg^{2+} concentration ($[Mg^{2+}]$) was reduced (i.e., in nominally Mg^{2+}-free solution containing 10 mM EGTA). Finally, to determine if genistein alters the time course of dephosphorylation, we compared $[^{32}P]PO_4$ release by PP2C at eight different time points. Genistein (50 μM) did not affect PP2C-catalyzed dephosphorylation at any time during the 30-min period (Fig. 2*C*).

Effect of genistein on protein phosphatase activity in cell fractions. Although most studies suggest that CFTR is regulated by PP2C- and PP2A-like phosphatases, particular isoforms of these enzymes or unidentified endogenous phosphatases could potentially be sensitive to genistein and could mediate its stimulatory action in intact cells. We therefore examined the effect of genistein on phosphatase activities in different CHO cell fractions (crude membrane extract, cytosol, and mitochondria). Under control conditions, all three cell fractions caused significant $[^{32}P]PO_4$ release from phosphocasein after 10 min of exposure at 22°C, but none was affected by genistein (50 μM; Fig. 3). Thus we could find no evidence for genistein-sensitive protein phosphatases in CHO cells.

Effect of genistein on dephosphorylation of GST-R domain fusion protein and immunoprecipitated CFTR. The experiments described above indicate that genistein slows channel rundown in excised patches without directly inhibiting protein phosphatases. We considered another possibility; i.e., that genistein in-

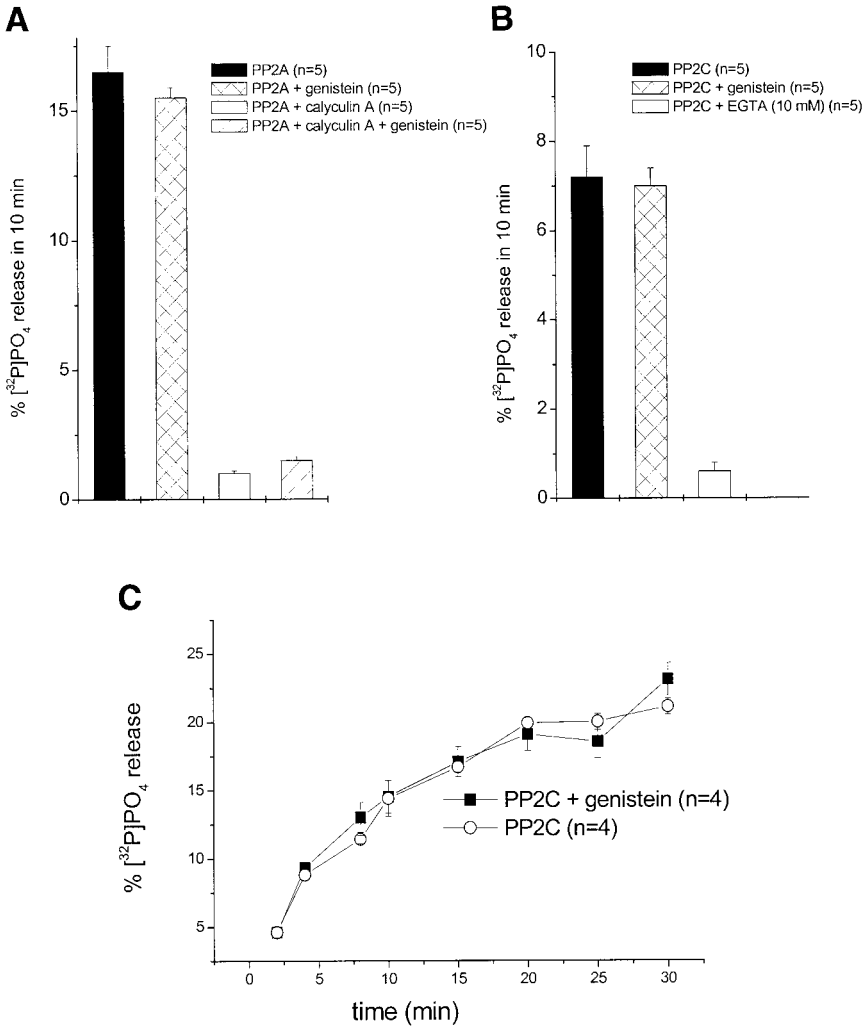

Fig. 2. Lack of inhibitory effect of genistein (50 μM) on protein phosphatase type 2A (PP2A) and PP2C. Casein was prelabeled using [γ-^{32}P]ATP and PKA as described in MATERIALS AND METHODS and was exposed to PP2A (0.5 nM) or PP2C (2 nM) for 10 min. Free $[^{32}P]PO_4$ release was assayed by liquid scintillation, and the percentage was calculated as 100 × (free $[^{32}P]PO_4$/total radioactivity of the reaction mix). *A*: genistein did not affect the activity of PP2A, which was inhibited ~90% by calyculin A (10 nM). *B*: genistein (50 μM) had no effect on PP2C activity, which was inhibited by low Mg^{2+} (nominally Mg^{2+} free + EGTA). *C*: the time course of PP2C-dependent $[^{32}P]PO_4$ release was not affected by genistein. Data are means ± SE; n = 4 or 5 independent assays as indicated.

hibits dephosphorylation at the level of CFTR by making it a less effective substrate for phosphatases. Because most phosphorylation of CFTR is on the R domain, we first examined dephosphorylation of the GST-R domain fusion protein.

GST-R was radiolabeled, and its dephosphorylation was assessed by autoradiography as described in MATERIALS AND METHODS. The entire fusion protein was used since the R domain portion contains >97% of the radioactive phosphate after incubation with PKA and [γ-^{32}P]ATP (52). Significant dephosphorylation of GST-R was evident under control conditions after 10 min of exposure to PP2A or PP2C (compare *lanes 1* vs. *2* and *1* vs. *4*, respectively, Fig. 4*A*). Dephosphorylation by PP2A was not inhibited by genistein (*lane 4*) but was virtually abolished by 10 nM calyculin A, as expected (*lane 3*). Dephosphorylation by PP2C (*lane 5*) was not affected by genistein (*lane 6*) but was strongly inhibited by reducing [Mg^{2+}], as expected (*lane 7*). Genistein also had no detectable effect on the dephosphorylation of GST-R by PP1, PP2B, or alkaline phosphatase (*lanes 8–14*). To test if genistein might protect PKA sites from PP2A or PP2C only in the full-length polypeptide, immunoprecipitated CFTR was phosphorylated in vitro and incubated with phosphatases for 15 min in the presence or absence of genistein. As shown in Fig. 4*B*, genistein (50 μM) had no effect on dephosphorylation of full-length CFTR by PP2A or PP2C. Western blot analysis confirmed that similar amounts of CFTR had been loaded in each lane. These results indicate that genistein does not affect CFTR dephosphorylation, at least under experimental conditions in which the protein is immunoprecipitated (see below).

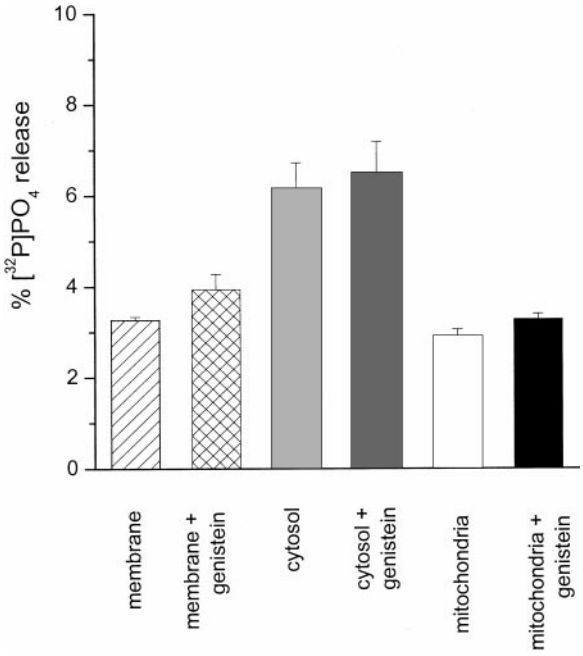

Fig. 3. Effect of genistein (50 μM) on protein phosphatase activities of crude cell fractions. Phosphatase activities of the membrane, cytosolic, and mitochondrial fractions were determined by measuring [^{32}P]PO_4 release from phosphocasein. Note that genistein had no effect on the endogenous phosphatase activities present in membrane, cytosolic, or mitochondrial fractions. Data are means ± SE; n = 4 experiments.

Effect of Br-t on CFTR channel rundown. The alkaline phosphatase inhibitor (−)-*p*-Br-t (2 mM) also slowed deactivation of CFTR channels in excised patches (Fig. 5). Channel activity was sustained for >5 min in seven out of seven patches, although it eventually declined to zero, consistent with previous results (4). However, the stereoisomer (+)-*p*-Br-t, which is often used as a negative control when assaying alkaline phosphatases, also slowed rundown. These data are consistent with recent patch-clamp studies (28) and provide further evidence that channel deactivation is not mediated by one of the "LBK" alkaline phosphatases, which are specifically inhibited by (−)-Br-t.

Effect of Br-t on purified PP2A and PP2C. Because PP2C and PP2A are the most likely candidates to be CFTR phosphatases, we investigated their sensitivities to Br-t using the protocol described above for genistein. As shown in Fig. 6*A*, both enantiomers reduced PP2A (0.5 nM) activity by >90%. Similar results were obtained in PP2C (2 nM) assays, although inhibition by (+)-Br-t was somewhat stronger. Some dephosphorylation occurred even at the highest Br-t concentrations used, which probably explains why Br-t slows deactivation approximately fourfold in patch-clamp experiments but does not completely abolish rundown (4).

We determined the concentration dependence of Br-t inhibition (Fig. 6*B*). (−)-*p*-Br-t was slightly more effective in inhibiting PP2C than PP2A (IC_{50} values of 1 and 2 mM, respectively). Surprisingly, inhibition of PP2C by the "inactive" (+)-Br-t isomer was threefold stronger than by (−)-Br-t (Fig. 6*C*). Least-squares fitting yielded IC_{50} = 0.3 mM for (+)-*p*-Br-t and 1 mM for (−)-*p*-Br-t (Fig. 6*C*). Nevertheless, the concentration of (−)-Br-t required to inhibit PP2C is ~500-fold higher than for comparable inhibition of LBK alkaline phosphatases.

Cytosol had the strongest endogenous phosphatase activity among the cell fractions assayed during genistein experiments (see above); therefore, we examined the Br-t sensitivity of crude cytosolic extracts. Assay conditions were designed to support activity of all known protein phosphatases, i.e., Ca^{2+} (1 mM), calmodulin (10 μg/ml), and Mg^{2+} (12 mM) were all present. Both enantiomers of Br-t inhibited release of [^{32}P]PO_4 from phosphocasein, with (+)-*p*-Br-t being the most potent (Fig. 7). Cytosolic phosphatase activity was nearly abolished when exposed to a mixture of known protein phosphatase inhibitors, i.e., calyculin A to inhibit PP1 and PP2A, EGTA to inhibit PP2B, and low Mg^{2+} to inhibit PP2C.

Effect of Br-t on dephosphorylation of CFTR and GST-R domain fusion protein. The ability of Br-t to inhibit dephosphorylation could depend on the particular substrate used in the assay; therefore, we examined its effect on dephosphorylation of both GST-R domain fusion protein and full-length CFTR. GST-R and immunoprecipitated CFTR were incubated with PKA and [γ-^{32}P]ATP and then were exposed to purified

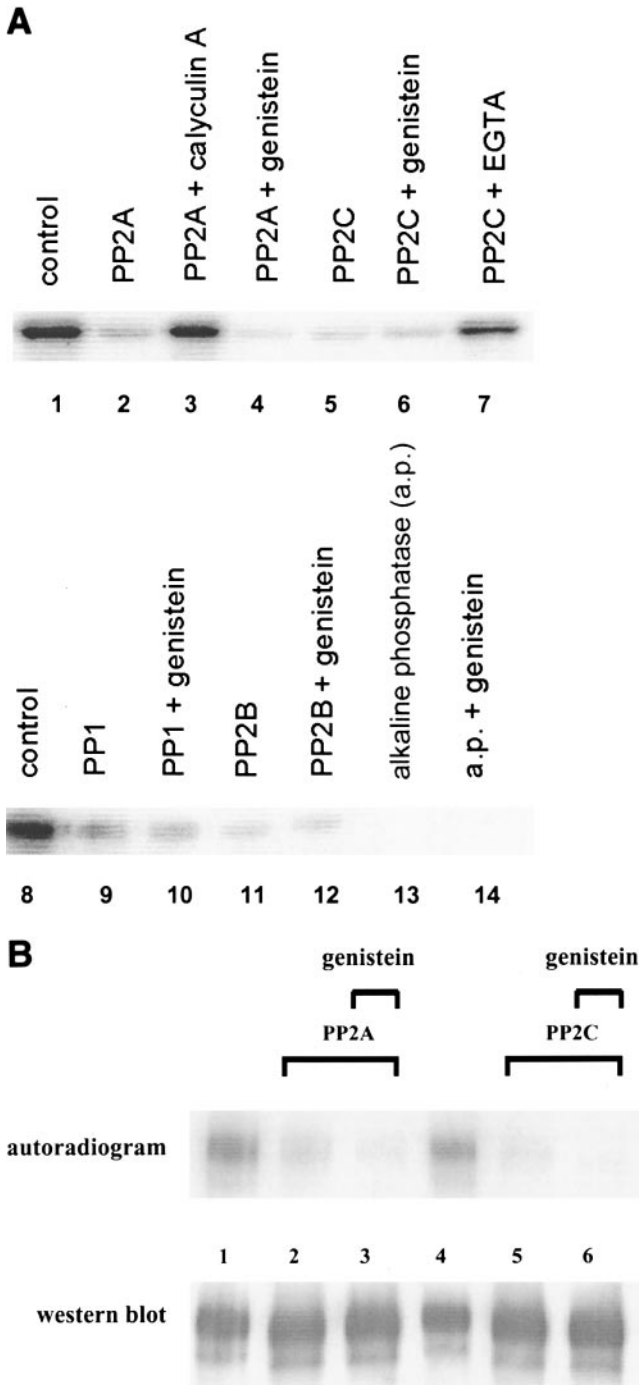

Fig. 4. Effect of genistein (50 μM) on dephosphorylation of GST-R domain fusion protein by purified phosphatases. *A*: GST-R was radiolabeled by preincubation with [γ-^{32}P]ATP and PKA and then was exposed to control buffer or phosphatases for 10 min, run on (12%) SDS-PAGE, and analyzed by autoradiography (see MATERIALS AND METHODS). Comparison of *lanes 2* and *5* with *lane 1* reveals strong dephosphorylation by PP2A (2 nM) and PP2C (10 nM), which was not inhibited by genistein (*lanes 4* and *6*, respectively). PP2A and PP2C activities were inhibited by 10 nM calyculin A (*lane 3*) and by low Mg^{2+} (*lane 7*), respectively. Comparison of *lanes 9* and *11* with *lane 8* shows that PP1 (10 nM) and PP2B (40 nM) also dephosphorylated GST-R, and their activities were unaffected by genistein (*lanes 10* and *12*, respectively). Finally, GST-R was dephosphorylated by alkaline phosphatase (ap) in the absence (*lane 13*) or presence (*lane 14*) of genistein. *B*: effect of genistein on dephosphorylation of full-length CFTR by purified phosphatases. Aliquots of immunoprecipitated CFTR were incubated in appropriate control buffers (*lanes 1* and *4*), with PP2A in the absence (*lane 2*) or presence of genistein (*lane 3*), or with PP2C in the absence (*lane 5*) or presence of genistein (*lane 6*). Western blot of the same membrane shown on *bottom* confirms similar protein loading in each lane; representative of 2 independent experiments.

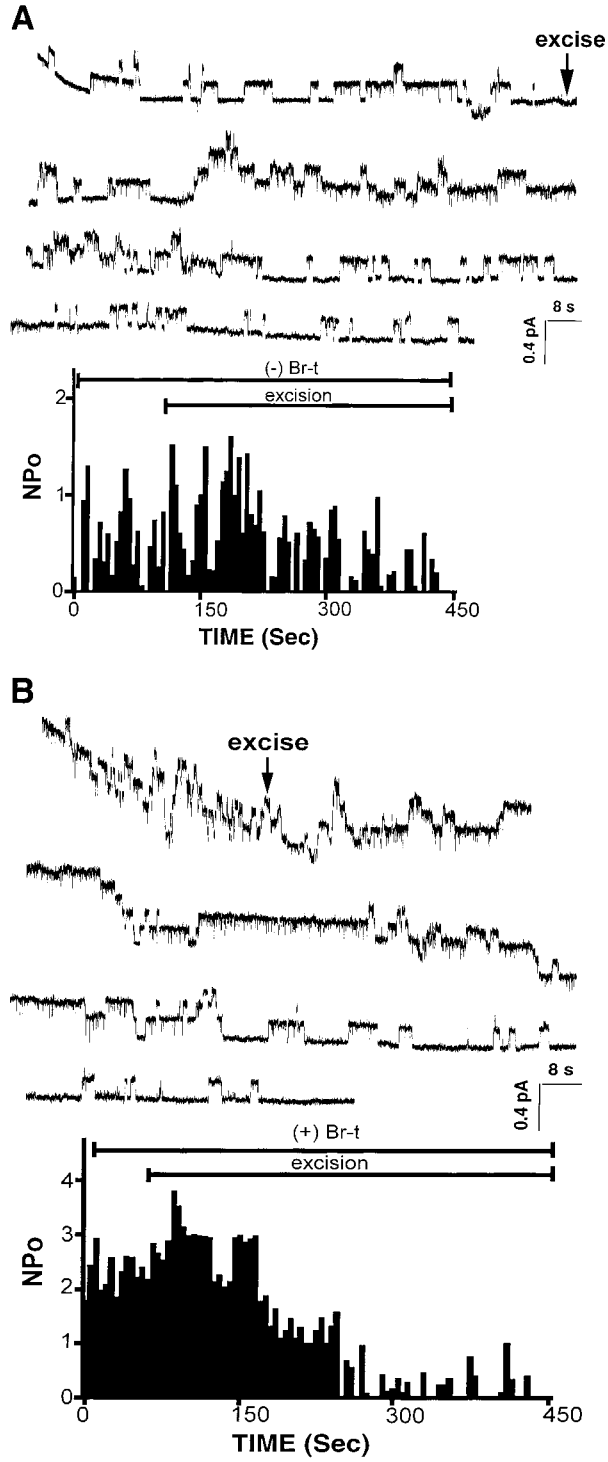

Fig. 5. Effect of bromotetramisole (Br-t) enantiomers (2 mM) on deactivation of CFTR channels in excised patches. Br-t was added at the beginning of the recordings. *A*: rundown was inhibited by (−)-Br-t in 5 of 12 patches (compare Fig. 1, *A* and *C*). *B*: similar inhibition was observed with (+)-Br-t in 5 of 12 patches.

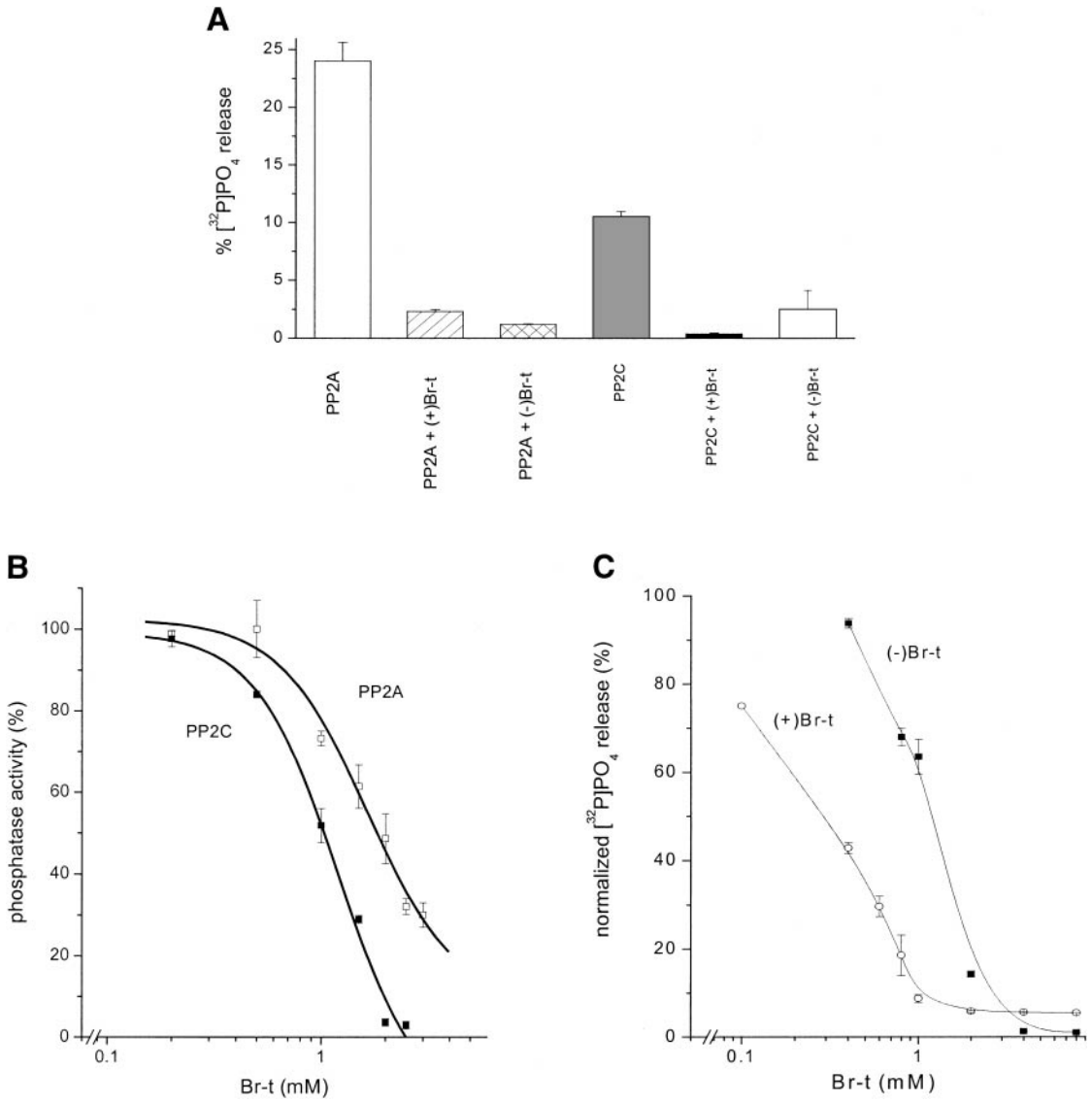

Fig. 6. Effects of (−)-Br-t and (+)-Br-t on activities of PP2A and PP2C. *A*: fraction of $[^{32}P]PO_4$ released from radiolabeled phosphocasein by phosphatases under control conditions and in the presence of Br-t enantiomers. Note that (+)-Br-t was almost as potent as (−)-Br-t in inhibiting dephosphorylation by PP2A (0.5 nM) and was a more potent inhibitor of PP2C (2 nM) than PP2A. *B*: concentration dependence of PP2C (■) and PP2A (□) inhibition by (−)-Br-t. $[^{32}P]PO_4$ release was normalized as the ratio (amount released with PP2C + Br-t)/(amount released with PP2C alone). Note that PP2C was more sensitive than PP2A. *C*: comparison of the inhibition of PP2C by − and + enantiomers of Br-t. Data are means ± SE; $n = 4$ experiments.

PP2A (2 nM) or PP2C (10 nM) in the absence or presence of Br-t, as described in MATERIALS AND METHODS. Br-t strongly inhibited dephosphorylation of GST-R by all four types of protein phosphatase under these conditions (Fig. 8). Dephosphorylation of full-length CFTR by PP2A (2 nM) and PP2C (10 nM) was also inhibited by both enantiomers (Fig. 9). To compare the concentration dependence of Br-t inhibition more quantitatively, the IC_{50} for inhibition of PP2C by each enantiomer was assessed using radiolabeled GST-R as the substrate. As shown in Fig. 10*A*, elevating (−)-Br-t concentration caused progressively stronger inhibition of PP2C, yielding an IC_{50} ~0.5 mM and maximal inhibition at 2 mM. Similar results were obtained with (+)-*p*-Br-t, although the IC_{50} was somewhat lower (0.2 mM; Fig. 10*B*).

DISCUSSION

Only a few potent activators of the CFTR channel have been described to date, and their mechanisms of action remain obscure. In this paper, we have examined the role of protein phosphatases in stimulation by two of these, genistein and Br-t. In addition to activating wild-type CFTR in epithelial cells, both drugs have been shown to stimulate channels with disease-associated mutations. Genistein potentiates forskolin activation of CFTR that have the most common CF mutation,

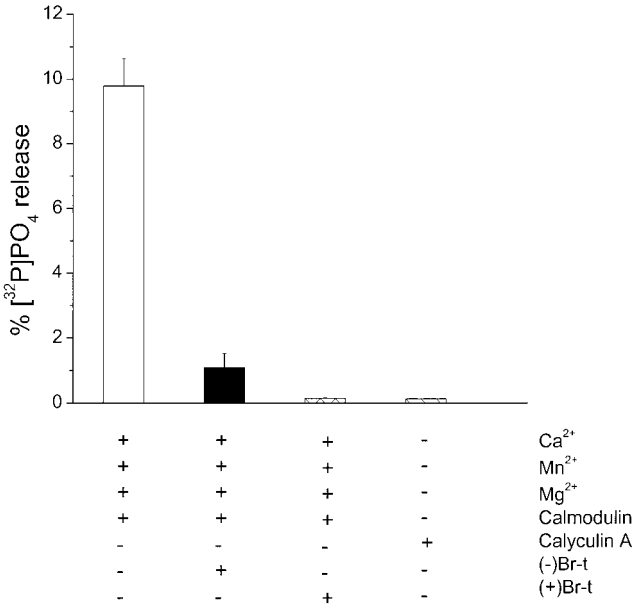

Fig. 7. Effect of Br-t (2 mM) on protein phosphatase activity of cytosolic fraction from Chinese hamster ovary (CHO) cells. Both enantiomers of Br-t (+ and −) inhibited dephosphorylation of phosphocasein when assayed in the presence of 1 mM Ca^{2+}, 1 mM Mn^{2+}, 12 mM Mg^{2+}, and calmodulin. Note that inhibition by (+)-Br-t was significantly stronger than by (−)-Br-t, consistent with the effects on purified PP2A in Fig. 6 and the abundance of PP2A in the cytosol. Data are means ± SE; $n = 5$.

a deletion of phenylalanine 508 (21), whereas Br-t activates several mutants, including G551D, when cells have only basal PKA activity (4).

Mechanisms of CFTR activation by genistein. Early evidence for genistein stimulation of CFTR came from studies of NIH/3T3 cells transfected with CFTR (24). Addition of genistein alone stimulated ^{125}I efflux and CFTR channel activity in cell-attached patches, and these responses were blocked by the tyrosine phosphatase inhibitor orthovanadate. Genistein did not elevate cAMP but rather decreased the concentration of forskolin required for half-maximal stimulation. Similar results were obtained with shark rectal glands (SRG) and primary cell cultures derived from SRG (29). The T84 cell line also responded to tyrphostin-47, another

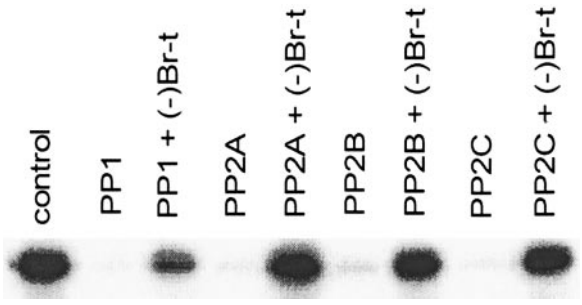

Fig. 8. Effect of Br-t (2 mM) on dephosphorylation of GST-R domain fusion protein. GST-R was phosphorylated as in Fig. 4*A*, incubated with PP1 (10 nM), PP2A (2 nM), PP2B (40 nM), or PP2C (10 nM) for 10 min at 20°C in the absence or presence of (−)-Br-t, run on 12% SDS-PAGE, and exposed to X-ray film for 12 h. Both enantiomers inhibited dephosphorylation of the fusion protein.

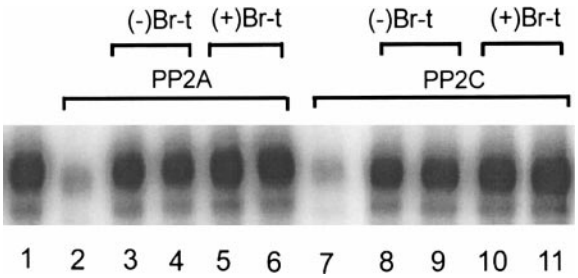

Fig. 9. Effect of Br-t enantiomers (2 mM) on dephosphorylation of full-length CFTR. After immunoprecipitation and radiolabeling as in Fig. 4*B*, CFTR was incubated in control buffer (*lane 1*) or in solution containing PP2A (2 nM; *lanes 2–6*) or PP2C (10 nM; *lanes 7–11*). Each incubation was carried out in duplicate with − and + enantiomers of Br-t. Dephosphorylation by PP2A and PP2C was strongly inhibited by both (−)-Br-t (*lanes 3* and *4* and *lanes 8* and *9*, respectively) and by (+)-Br-t (*lanes 5* and *6* and *lanes 10* and *11*, respectively).

tyrosine kinase inhibitor, strengthening the idea that CFTR channels are tonically inhibited by tyrosine kinases (42); however, subsequent studies indicated that genistein has some other mode of action. The tyrosine

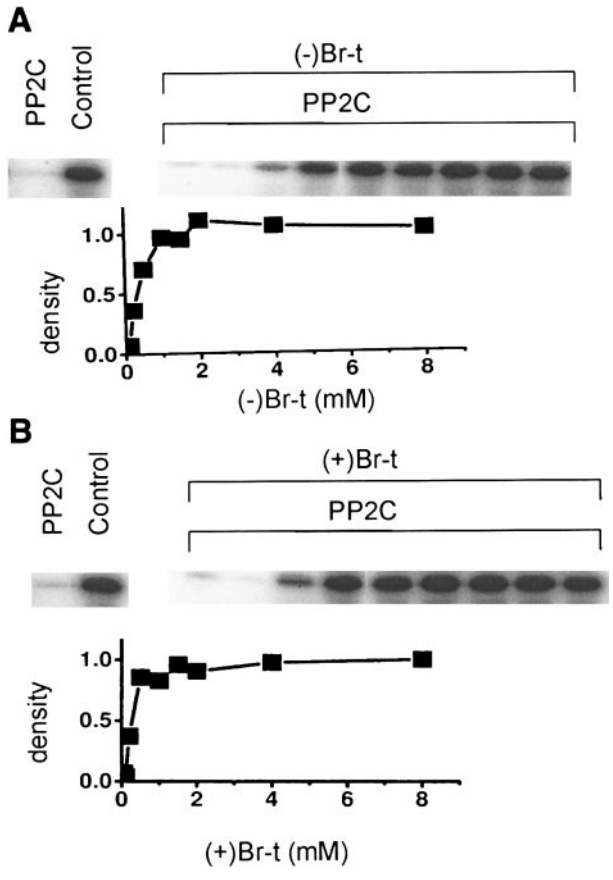

Fig. 10. Concentration dependence of PP2C inhibition by Br-t enantiomers. GST-R domain protein was phosphorylated as before and then was exposed to PP2C (10 nM) for 10 min in the presence of different concentrations of Br-t. Dephosphorylation was quantified by densitometry of autoradiograms after normalizing to the control lane (no phosphatase present during 10 min incubation under same conditions). *A*: dephosphorylation of GST-R domain by PP2C was strongly inhibited at concentrations of (−)-Br-t >1 mM. *B*: (+)-Br-t inhibited PP2C at similar concentrations.

kinase inhibitors erbastatin, herbimycin A, tyrphostin-51, and tyrphostin-47 did not mimick genistein stimulation of CFTR channels in other cell types (12, 23, 49). Moreover, subsequent studies with the tyrosine kinase src revealed that tyrosine phosphorylation stimulates rather than inhibits channel activity. Exposing CFTR to the tyrosine kinase $p60^{c\text{-}src}$ potentiated stimulation by PKA (13) or strongly activated CFTR channels when added alone (25). The latter stimulation was associated with phosphorylation of tyrosines on CFTR.

Genistein has been proposed to stimulate CFTR by inhibiting protein phosphatases that normally counteract its activation by PKA. This hypothesis was supported by the finding that genistein's effect on CFTR channels in NIH/3T3 and Hi-5 insect cells was greatly enhanced by cAMP stimulation (41, 50). Genistein exposure increased CFTR phosphorylation in vivo, as assessed by metabolic labeling of cells with $[^{32}P]PO_4$. Subsequent digestion by trypsin, SDS-PAGE, and TLC yielded phosphopeptide maps during genistein treatment that resembled those during forskolin stimulation (41). Because genistein does not stimulate PKA, these results were most easily explained by inhibition of a CFTR phosphatase. Sustained CFTR-mediated currents across permeabilized T84 and HT-29/B6 cell monolayers after cAMP washout provided further support for the phosphatase hypothesis (23).

Most recently, genistein has been shown to increase the mean open time of single CFTR channels in patches with little phosphatase activity (22). Moreover, repeated genistein stimulations were obtained in the absence of PKA, which argues against a mechanism that requires phosphorylation/dephosphorylation (49). Genistein further increased the activity of channels that had been stimulated by PKA and Mg^{2+}-adenosine 5′-*O*-(3-thiotriphosphate), although the thiophosphoryl moiety would be expected to resist phosphatases (14). On the basis of these findings, it was proposed that genistein alters CFTR gating by directly interacting with the channel, perhaps at a nucleotide binding fold (48). Indeed, direct interaction of genistein with a fusion protein composed of the second nucleotide binding fold (NBF2) and maltose binding protein, and ATPase inhibition, have recently been demonstrated (40). This is compatible with long open times, since inhibitors of hydrolysis at NBF2 are thought to stabilize the open state of the channel (20). Genistein stimulation of CFTR by such a mechanism contrasts with that proposed for multidrug resistance-associated protein (MRP1), another ATP-binding cassette transport protein (18). Genistein is thought to stimulate ATPase activity of MRP1 by interacting at the substrate binding site for drug transport rather than a NBF. There is also evidence that genistein acts on CFTR preferentially from the extracellular side (51). Regardless of the site of interaction, direct binding of genistein to CFTR would not preclude other modes of action such as phosphatase inhibition (17, 48).

Genistein does not inhibit protein phosphatases or dephosphorylation of immunoprecipitated CFTR. The present results argue strongly against a role of phosphatases in the activation of CFTR by genistein. Genistein slowed channel rundown in excised patches but did not inhibit any of the purified protein phosphatases tested (PP1, PP2A, PP2B, and PP2C), nor did it affect the endogenous phosphatase activities of cell fractions. We could find no evidence that the ability of CFTR to serve as a phosphatase substrate was affected by genistein; however, extrapolating from immunoprecipitated CFTR protein to native membrane is problematic because genistein could inhibit dephosphorylation through some affect on channel gating that is disrupted by solubilization. In preliminary experiments, we found that genistein alters the digestion pattern of CFTR produced by limited proteolysis with chymotrypsin (data not shown), further evidence for genistein inducing a conformational change (40). Although a secondary (gating-dependent) effect on phosphatase sensitivity cannot be excluded, the present data are most consistent with stimulation via direct binding of genistein to CFTR and stabilization of the open state.

Mechanism of CFTR activation by Br-t. Br-t has long been known as a potent and specific inhibitor of particular types of alkaline phosphatases (8). LBK alkaline phosphatase activities are abolished by 0.1 mM (−)-*p*-Br-t, whereas intestinal and placental alkaline phosphatases are not inhibited significantly (46). The stereoisomer (+)-*p*-Br-t does not inhibit alkaline phosphatases and is widely used as an inactive analog. In previous work, we found that (−)-Br-t inhibits CFTR rundown in patches from CHO and human airway epithelial cells [NP34 (4, 5)] and also activates quiescent G551D mutant channels that are normally unresponsive to forskolin. Dephosphorylation of CFTR in crude CHO cell membrane preparations was also sensitive to millimolar concentrations of (−)-Br-t. Inhibition of rundown by 1 mM (+)-Br-t was not detected; however, the effects of this enantiomer on dephosphorylation of CFTR protein were not assessed.

In the present study, channel rundown and CFTR dephosphorylation were sensitive to both "active" (−) and "inactive" (+)-Br-t enantiomers, suggesting LBK alkaline phosphatases are not involved. Inhibition of rundown by (+)-Br-t was apparent in two-thirds of the patches and may have been more consistent than in previous experiments because of the higher concentration used (2 vs. 1 mM in Ref. 4). In this study, we found that both enantiomers inhibit PP1, PP2A, PP2B, and PP2C and reduce activity of endogenous phosphatases in crude cell fractions by ~90%. Rundown in excised patches was slowed but not abolished by Br-t; therefore, the residual 10% must be sufficient to eventually cause channels to deactivate. Strong inhibition of protein phosphatases by Br-t was apparent regardless of the substrate used (phosphocasein, full-length phospho-CFTR immunoprecipitates, or phospho-GST-R domain fusion protein).

Direct evidence that Br-t stimulates CFTR by inhibiting phosphatases. Phenylimidazothiazoles (0.1–2 mM) stimulate iodide efflux and activate single CFTR channels in CFTR-expressing CHO and airway cells

without elevating cAMP (4, 5). (−)-Br-t also stimulates CFTR conductance in other preparations (28, 35), although the response seems somewhat weaker [e.g., 15% of the forskolin response in permeabilized T84 cell monolayers (35)]. Such variations would be expected if Br-t stimulation is indirect and mediated by inhibition of phosphatase(s) as originally proposed (4), since the responsiveness of different preparations would depend on their basal PKA activity, which in turn would reflect adenylyl cyclase and phosphodiesterase activities and perhaps other factors. The present results support the proposal that Br-t stimulates channel activity by inhibiting CFTR dephosphorylation, since it inhibited all four major types of protein phosphatases examined.

Higher concentrations of phenylimidazothiazoles are needed for activation of CFTR channels on-cell (EC_{50} = 450 μM) than to inhibit alkaline phosphatase in biochemical assays ($IC_{50} \leq$ 11 μM; see Refs. 5 and 33). We tentatively attributed this difference to a requirement for permeation through the plasma membrane during patch-clamp experiments; however, the present work shows that Br-t inhibits protein phosphatases in the test tube at similar concentrations. Thus the high IC_{50} for CFTR channel activation reflects the lower sensitivity of protein phosphatases to Br-t compared with alkaline phosphatase. PP2C, which is strongly implicated in CFTR channel regulation (32, 45, 52), was inhibited at concentrations that activate CFTR channels in cell-attached patches (5).

Biochemical assays in the present study also revealed that Br-t inhibition of protein phosphatases is not stereospecific. This is consistent with reports that both Br-t enantiomers stimulate chloride conductance in cells expressing endogenous (35) or heterologous CFTR (9). Thus, although the CFTR phosphatase has some functional similarities to alkaline phosphatases [negligible sensitivity to okadaic acid and calyculin A, Ca^{2+} and calmodulin independence, Mg^{2+} dependence, and sensitivity to a polyclonal antibody against alkaline phosphatase (3, 32, 43)], the results of this study are more consistent with regulation by a protein phosphatase. The ability of phosphatase inhibitors to activate CFTR in unstimulated cells, at least in some cell types, implies significant turnover of phosphorylation on CFTR under resting conditions.

When added alone, Br-t does not stimulate the short-circuit current across intact T84 cell monolayers, although partial stimulation is observed when the basolateral membrane is permeabilized using nystatin (35). We also found this negative result in early studies (Becq F, Boucher A, and Hanrahan JW, unpublished observations) and attributed it to the known insensitivity of intestinal alkaline phosphatases to Br-t. Because the present work suggests that CFTR is regulated by a Br-t-sensitive protein phosphatase, the inability of Br-t to stimulate secretion across T84 monolayers now requires some other explanation. One possibility is that Br-t also inhibits other transporters or channels, e.g., basolateral membrane potassium conductance, as recently demonstrated by Mun et al. (35). Such inhibition by Br-t may be direct or mediated by a phosphatase, or it may involve inhibition of some enzyme critical for cellular metabolism. Regardless, inhibition of basolateral potassium channels would depolarize both basolateral and apical membranes and would diminish the apical driving force for chloride. Such depolarization could block secretion and obscure activation of CFTR channels in assays that do not involve measurement of single channel activity. The ability of Br-t to induce a current across T84 monolayers after the basolateral membrane had been nystatin permeabilized and after a transepithelial chloride gradient had been imposed supports this explanation (35). Although patch-clamp studies suggest that phosphatase inhibitors could provide therapeutic benefit (4), the high concentrations of Br-t needed (5), offsetting effects on other channels (35), and the sensitivity of multiple protein phosphatases (present study) indicate that Br-t itself has limited potential as a drug. Establishing the precise isoform of PP2C associated with CFTR may help in the identification of more potent and specific phosphatase inhibitors.

In summary, genistein and Br-t activate CFTR channels through different mechanisms. Genistein appears to act directly, since it did not affect phosphatases, whereas Br-t activation clearly does involve phosphatase inhibition. Br-t inhibited all protein phosphatases when tested at concentrations that stimulate CFTR channels and did not stimulate channels in patches with low endogenous phosphatase activity.

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REFERENCES

1. **Anderson MP, Berger HA, Rich DP, Gregory RJ, Smith AE, and Welsh MJ.** Nucleoside triphosphates are required to open the CFTR chloride channel. *Cell* 67: 775–784, 1991.
2. **Baukrowitz T, Hwang T-C, Nairn AC, and Gadsby DC.** Coupling of CFTR Cl^- channel gating to an ATP hydrolysis cycle. *Neuron* 12: 473–482, 1994.
3. **Becq F, Fanjul M, Merten M, Figarella C, Hollande E, and Gola M.** Possible regulation of CFTR chloride channels by membrane bound phosphatases in pancreatic duct cells. *FEBS Lett* 327: 337–342, 1993.
4. **Becq F, Jensen TJ, Chang X-B, Savoia A, Rommens JM, Tsui L-C, Buchwald M, Riordan JR, and Hanrahan JW.** Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc Natl Acad Sci USA* 91: 9160–9164, 1994.
5. **Becq F, Verrier B, Chang X-B, Riordan JR, and Hanrahan JW.** cAMP- and Ca^{2+}-independent activation of cystic fibrosis transmembrane conductance regulator channels by phenylimidazothiazole drugs. *J Biol Chem* 271: 16171–16179, 1996.
6. **Berger HA, Anderson MP, Gregory RJ, Thompson S, Howard PW, Maurer RA, Mulligan R, Smith AE, and Welsh MJ.** Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel. *J Clin Invest* 88: 1422–1431, 1991.
7. **Berger HA, Travis SM, and Welsh MJ.** Regulation of the cystic fibrosis transmembrane conductance regulator Cl^- chan-

nel by specific protein kinases and phosphatases. *J Biol Chem* 268: 2037–2047, 1993.
8. **Borgers M and Thoné F.** The inhibition of alkaline phosphatase by L-p-bromotetramisole. *Histochemistry* 44: 277–280, 1975.
9. **Cai Z, Lansdell KA, and Sheppard DN.** Inhibition of heterologously expressed cystic fibrosis transmembrane conductance regulator Cl^- channels by non-sulphonylurea hypoglycaemic agents. *Br J Pharmacol* 128: 108–118, 1999.
10. **Chang X-B, Tabcharani JA, Hou Y-X, Jensen TJ, Kartner N, Alon N, Hanrahan JW, and Riordan JR.** Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all ten PKA consensus phosphorylation sites. *J Biol Chem* 268: 11304–11311, 1993.
11. **Cheng SH, Rich DP, Marshall J, Gregory RJ, Welsh MJ, and Smith AE.** Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell* 66: 1027–1036, 1991.
12. **Chiang CE, Chen SA, Chang MS, Lin C-I, and Luk HN.** Genistein directly induces cardiac CFTR chloride current by a tyrosine kinase-independent and protein kinase A-independent pathway in guinea pig ventricular myocytes. *Biochem Biophys Res Commun* 235: 74–78, 1997.
13. **Fischer H and Machen TE.** The tyrosine kinase $p60^{c\text{-}src}$ regulates the fast gate of the cystic fibrosis transmembrane conductance regulator chloride channel. *Biophys J* 71: 3073–3082, 1996.
14. **French PJ, Bijman J, Bot AG, Boomaars WEM, Scholte BJ, and De Jonge HR.** Genistein activates CFTR Cl^- channels via a tyrosine kinase- and protein phosphatase-independent mechanism. *Am J Physiol Cell Physiol* 273: C747–C753, 1997.
15. **Hanrahan JW, Kone Z, Mathews CJ, Luo J, Jia Y, and Linsdell P.** Patch-clamp studies of cystic fibrosis transmembrane conductance regulator chloride channel. *Methods Enzymol* 293: 169–194, 1998.
16. **Haws C, Finkbeiner WE, Widdicombe JH, and Wine JJ.** CFTR in Calu-3 human airway cells: channel properties and role in cAMP-activated Cl^- conductance. *Am J Physiol Lung Cell Mol Physiol* 266: L502–L512, 1994.
17. **He ZP, Raman S, Guo Y, and Reenstra WW.** Cystic fibrosis transmembrane conductance regulator activation by cAMP-independent mechanisms. *Am J Physiol Cell Physiol* 275: C958–C966, 1998.
18. **Hooijberg JH, Broxterman HJ, Heijn M, Fles DLA, Lankelma J, and Pinedo HM.** Modulation by (iso)flavonoids of the ATPase activity of the multidrug resistance protein. *FEBS Lett* 413: 344–348, 1997.
19. **Hwang T-C, Horie M, and Gadsby DC.** Functionally distinct phospho-forms underlie incremental activation of protein kinase-regulated Cl^- conductance in mammalian heart. *J Gen Physiol* 101: 629–650, 1993.
20. **Hwang T-C, Nagel G, Nairn AC, and Gadsby DC.** Regulation of the gating of cystic fibrosis transmembrane conductance regulator Cl channels by phosphorylation and ATP hydrolysis. *Proc Natl Acad Sci USA* 91: 4698–4702, 1994.
21. **Hwang T-C, Wang F, Yang ICH, and Reenstra WW.** Potentiation of ΔF508 channel function by genistein binding to CFTR. *Am J Physiol Cell Physiol* 273: C988–C998, 1997.
22. **Hwang TC, Wang F, Yang IC, and Reenstra WW.** Genistein potentiates wild-type and ΔF508-CFTR channel activity. *Am J Physiol Cell Physiol* 273: C988–C998, 1997.
23. **Illek B, Fischer H, and Machen TE.** Alternate stimulation of apical CFTR by genistein in epithelia. *Am J Physiol Cell Physiol* 270: C265–C275, 1996.
24. **Illek B, Fischer H, Santos GF, Widdicombe JH, Machen TE, and Reenstra WW.** cAMP-independent activation of CFTR Cl channels by the tyrosine kinase inhibitor genistein. *Am J Physiol Cell Physiol* 268: C886–C893, 1995.
25. **Jia F, Seibert F, Chang X-B, Riordan JR, and Hanrahan JW.** Activation of CFTR chloride channels by tyrosine phosphorylation (Abstract). *Pediatr Pulmonol Suppl* 14: 214, 1997.
26. **Jia Y, Mathews CJ, and Hanrahan JW.** Phosphorylation by protein kinase C is required for acute activation of cystic fibrosis transmembrane conductance regulator by protein kinase A. *J Biol Chem* 272: 4978–4984, 1997.
27. **Kartner N and Riordan JR.** Characterization of polyclonal and monoclonal antibodies to cystic fibrosis transmembrane conductance regulator. *Methods Enzymol* 292: 629–652, 1998.
28. **Lansdell KA, Kidd JF, Delaney SJ, Wainwright BJ, and Sheppard DN.** Regulation of murine cystic fibrosis transmembrane conductance regulator Cl^- channels expressed in Chinese hamster ovary cells. *J Physiol (Lond)* 512: 751–764, 1998.
29. **Lehrich RW and Forrest JN Jr.** Tyrosine phosphorylation is a novel pathway for regulation of chloride secretion in shark rectal gland. *Am J Physiol Renal Fluid Electrolyte Physiol* 269: F594–F600, 1995.
30. **Li C, Ramjeesingh M, Wang W, Garami E, Hewryk M, Lee D, Rommens JM, Galley K, and Bear CE.** ATPase activity of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 271: 28463–28468, 1996.
31. **Liedtke CM and Cole TS.** Antisense oligonucleotide to PKC-ε alters cAMP-dependent stimulation of CFTR in Calu-3 cells. *Am J Physiol Cell Physiol* 275: C1357–C1364, 1998.
32. **Luo J, Pato MD, Riordan JR, and Hanrahan JW.** Differential regulation of single CFTR channels by PP2C, PP2A, and other phosphatases. *Am J Physiol Cell Physiol* 274: C1397–C1410, 1998.
33. **Metaye T, Millet C, Kraimps JL, Saunier B, Barbier J, and Begon F.** Effect of bromolevamisole and other imidazo[2,1-b]thiazole derivatives on adenylate cyclase activity. *Biochem Pharmacol* 43: 1507–1511, 1992.
34. **Middleton LM and Harvey RD.** PKC regulation of cardiac CFTR Cl^- channel function in guinea pig ventricular myocytes. *Am J Physiol Cell Physiol* 275: C293–C302, 1998.
35. **Mun EC, Mayol JM, Riegler M, O'Brien TC, Farokhzad OC, Song JC, Pothoulakis C, Hrnjez BJ, and Matthews JB.** Levamisole inhibits intestinal Cl^- secretion via basolateral K^+ blockade. *Gastroenterology* 114: 1257–1267, 1998.
36. **Obayashi K, Horie M, Washizuka T, Mishimoto T, and Sasayama S.** On the mechanism of genistein-induced activation of protein kinase A-dependent Cl^- conductance in cardiac myocytes. *Pflügers Arch* 438: 269–277, 1999.
37. **Pato MD and Adelstein RS.** Dephosphorylation of the 20,000-dalton light chain of myosin by two different phosphatases from smooth muscle. *J Biol Chem* 255: 6535–6538, 1980.
38. **Pato MD and Adelstein RS.** Characterization of a Mg^{2+} dependent phosphatase from turkey gizzard smooth muscle. *J Biol Chem* 258: 7055–7058, 1983.
39. **Picciotto MR, Cohn JA, Bertuzzi G, Greengard P, and Nairn AC.** Phosphorylation of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 267: 12742–12752, 1992.
40. **Randak C, Auerswald EA, Assfalg-Machleidt I, Reenstra WW, and Machleidt W.** Inhibition of ATPase, GTPase and adenylate kinase activities of the second nucleotide binding fold of cystic fibrosis transmembrane conductance regulator by genistein. *Biochem J* 340: 227–235, 1999.
41. **Reenstra WW, Yurko-Mauro K, Dam A, Raman S, and Shorten S.** CFTR chloride channel activation by genistein: the role of serine/threonine protein phosphatases. *Am J Physiol Cell Physiol* 271: C650–C657, 1996.
42. **Sears CL, Firoozmand F, Mellander A, Chambers FG, Eromar IG, Bot AGM, Scholte B, De Jonge HR, and Donowitz M.** Genistein and tyrphostin 47 stimulate CFTR-mediated Cl^- secretion in T84 cell monolayers. *Am J Physiol Gastrointest Liver Physiol* 269: G874–G882, 1995.
43. **Tabcharani JA, Chang X-B, Riordan JR, and Hanrahan JW.** Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature* 352: 628–631, 1991.
44. **Tabcharani JA and Hanrahan JW.** On the activation of outwardly rectifying anion channels in excised patches. *Am J Physiol Gastrointest Liver Physiol* 261: G992–G999, 1991.
45. **Travis SM, Berger HA, and Welsh MJ.** Protein phosphatase 2C dephosphorylates and inactivates cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci USA* 94: 11055–11060, 1997.
46. **Van Belle H, De Broe ME, and Wieme RJ.** Bromotetramisole, a new reagent for use in measuring placental or intestinal

isoenzymes of alkaline phosphatase in human serum. *Clin Chem* 23: 454–459, 1977.
47. **Wang DY, Brihaye P, Smitz J, De Waele M, and Clement P.** Inflammatory cells and mediator concentrations in nasal secretions of patients with cystic fibrosis. *Acta Otolaryngol (Stockh)* 116: 472–476, 1996.
48. **Wang F, Zeltwanger S, Yang IC-H, Nairn AC, and Hwang T-C.** Actions of genistein on cystic fibrosis transmembrane conductance regulator channel gating. *J Gen Physiol* 111: 477–490, 1998.
49. **Weinreich F, Wood PG, Riordan JR, and Nagel G.** Direct action of genistein on CFTR. *Pflügers Arch* 434: 484–491, 1997.
50. **Yang ICH, Cheng T-H, Wang F, Price EM, and Hwang T-C.** Modulation of CFTR chloride channels by calyculin A and genistein. *Am J Physiol Cell Physiol* 272: C142–C155, 1997.
51. **Zhou S-S, Hazama A, and Okada Y.** Tyrosine kinase-independent extracellular action of genistein on the CFTR Cl^- channel in guinea pig ventricular myocytes and CFTR-transfected mouse fibroblasts. *Jpn J Physiol* 48: 389–396, 1998.
52. **Zhu T, Dahan D, Evaglelidis A, Zheng S-X, Luo J, and Hanrahan JW.** Association of cystic fibrosis transmembrane conductance regulator and protein phosphatase 2C. *J Biol Chem* 274: 29102–29107, 1999.