Regulation by ATP and ADP of CFTR Chloride Channels That Contain Mutant Nucleotide-Binding Domains

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Regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is unusual in that phosphorylated channels require cytosolic adenosine triphosphate (ATP) to open. The CFTR contains two regions predicted to be nucleotide-binding domains (NBDs); site-directed mutations in each NBD have now been shown to alter the relation between ATP concentration and channel activity, which indicates that ATP stimulates the channel by direct interaction with both NBDs. The two NBDs are not, however, functionally equivalent: adenosine diphosphate (ADP) competitively inhibited the channel by interacting with NBD2 but not by interacting with NBD1. Four cystic fibrosis–associated mutations in the NBDs reduced absolute chloride channel activity, and one mutation also decreased the potency with which ATP stimulates channel activity. Dysfunction of ATP-dependent stimulation through the NBDs may be the basis for defective CFTR chloride channel activity in some cystic fibrosis patients.

The CFTR Cl^- channel (*1–5*) is predicted to consist of five domains: two membrane-spanning domains, each composed of six putative transmembrane segments, that may contribute to the formation of the channel pore (*4*); a regulatory domain containing several adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) phosphorylation sites that are important for channel opening (6–9); and two NBDs that were defined by their sequence similarity with NBDs in the traffic adenosine triphosphatase (ATPase) or the ATP-binding cassette (ABC) transporter family of proteins (*1, 10, 11*). Some members of this family hydrolyze ATP to actively transport the substrate across the cell membrane (*10–13*).

The function of the NBDs in the CFTR is not clear; they are not shared by any other class of ion channel. The NBDs are also the site of the majority of missense mutations that cause cystic fibrosis (CF) (*14*). Recombinant peptides corresponding to NBD1 (*15, 16*) of the CFTR bind analogs of ATP independently of Mg^{2+}, which suggests that such binding might also occur in the intact protein. However, similar studies have not been performed for NBD2 or the intact CFTR. Moreover, the functional importance of having two NBDs, rather than just one, is not yet clear for any member of the traffic ATPase-ABC transporter family. Moreover, it is not known whether the two NBDs interact or whether they have identical functions. We previously showed that after phosphorylation by PKA, CFTR Cl^- channels require hydrolyzable nucleoside triphosphates and Mg^{2+} on the cytosolic surface to open (*17*). On the basis of these results, we speculated that ATP might regulate the CFTR by means of the NBDs.

We recorded CFTR Cl^- channel activity in excised membrane patches of Cl27i mouse mammary epithelial cells that had been exposed to PKA and MgATP (*17*). After the removal of PKA and MgATP, MgATP was added back to the cytosolic side of the membrane. As the MgATP concentration increased, the probability that the phosphorylated channels were in the open state (P_o) increased (Fig. 1, A and B); the increase in P_o appeared to result from a decrease in the duration of the closed state. An Eadie-Hofstee plot of pooled data (Fig. 1C, solid line) generated a curved line consistent with negative kinetic cooperativity. The kinetic cooperativity cannot be explained by a heterogeneous population of channels because the same curvature was observed with a single channel (Fig. 1C, dotted line). Most models used to explain negative kinetic cooperativity include two or more substrate-effector binding sites (*18*). Therefore, we speculated that MgATP may interact with two different sites in the CFTR: NBD1 and NBD2.

To test this hypothesis, we mutated amino acids in the highly conserved Walker motifs (Fig. 2A) (*19*) of the NBDs and measured the effect of MgATP on the mutant channels. In the Walker A motif, Lys is thought to interact with either the α- or γ-phosphate of ATP (*20*). Mutation of the Walker A Lys in NBD1 to Ala (K464A) (*21*) (Fig. 2B) or that in NBD2 to Met (K1250M) (Fig. 2C) resulted in an altered relation between MgATP concentration and channel activity. Both Walker A Lys mutations decreased the potency with which MgATP stimulates the channel (*22*). The conserved Asp in the Walker B motif is thought to interact with Mg^{2+} during the binding of ATP (*20*). The mutation of Asp in NBD2 to Asn (D1370N) also decreased the potency of MgATP (*23*) (Fig. 2C).

The two hydroxyl amino acids immediately on the 3′ side of the Walker A Lys are present in nearly all members of the traffic ATPase-ABC transporter family. The typical sequence is Lys-Ser-Thr or Lys-Thr-Ser. Substitution of these hydroxyl residues in thymidine kinase altered the catalytic activity of the enzyme (*24*). When we switched the order of the two residues in NBD1 (T465S;S466T) (*21*), the ATP dose-response curve shifted to the left (Fig. 2B), which suggests that MgATP was more potent in stimulating this mutant (*25*). These results indicate that ATP interacts directly with both NBDs to increase channel activity.

We investigated whether ADP also interacts with the CFTR ATP binding sites, thereby possibly inhibiting function; our earlier studies showed that ADP alone does not stimulate channel activity (*17*). In the presence of ATP, increasing concentrations of ADP progressively inhibited current (Fig. 3A). An inverse plot of the data suggested that ADP is a competitive antagonist (*26*). The relative inhibitory potency sequence (mean ± SEM) of 1 mM nucleoside diphosphates in the presence of 0.3 mM ATP was: ADP (84 ± 2% inhibition; $n = 4$) > guanosine diphosphate (51 ± 3%; $n = 5$) ≈ inosine diphosphate (49 ± 3%; $n = 4$) > uridine triphosphate (39 ± 4%; $n = 4$) > cytosine diphosphate (27%; $n = 1$) > adenosine-5′-O-(2-thiodiphosphate) (0%; $n = 2$). This potency sequence is the same as that for nucleoside triphosphate stimulation of channel activity (*17*).

These results suggest that the intracellular ATP-ADP ratio may be more important than the absolute concentration of ATP in regulating the CFTR. Thus, changes in the metabolic state of a cell that alter the ATP-ADP ratio may regulate CFTR Cl^- channel activity in vivo. Their competitive antagonism and similar nucleotide specificity suggested that ATP and ADP interact with the same sites. But does ADP act at both NBDs or only one? Mutation of three different residues in NBD2—K1250M, S1255P, and D1370N (*21*)—abolished or reduced the inhibitory effect of ADP (1 mM) on the current stimulated by 1 mM ATP (Fig. 3B). In contrast, mutations in NBD1—K464A and T465S;S466T—did not alter the inhibitory effect of ADP (*27*). These data suggest that ADP inhibits the CFTR by competing with ATP and that this competition occurs at NBD2.

The two NBDs of the CFTR have diver-

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gent amino acid sequences (*1*). Our data indicate that their function has also diverged: ATP-dependent stimulation of NBD2, but not of NBD1, is competitively inhibited by ADP. Our previous work suggested that hydrolyzable nucleoside triphosphates are required for appreciable channel activity (*17*). Because ADP is a product of ATP hydrolysis, one might predict that ADP would be a competitive antagonist at the site of hydrolysis. Thus, we speculate that if the CFTR hydrolyzes ATP, such hydrolysis may occur at NBD2. In this regard, the CFTR might be similar to some enzymes that contain both ATP catalytic sites and ATP allosteric sites (*28*). Alternatively, ADP may simply compete for binding of ATP to NBD2 but not cause channel opening. It remains to be determined whether the NBDs of other members of the traffic ATPase-ABC transporter family (*29, 30*)—particularly those presumed to contain two identical NBDs—interact with ADP or have divergent functions.

The NBDs are the site of the majority of CF missense mutations (*14*). The regulation of these domains by ATP suggested that some CF mutations in the NBDs may alter ATP-dependent regulation. Many CF-associated mutations, including the most common, deletion of Phe^{508}, generate mutant proteins that are not correctly processed (*31, 32*) and hence may not be delivered to the plasma membrane (*33*). For these mutations, the lack of Cl^- permeability can be explained by the absence of the channel. However, other CFTR variants that contain missense mutations in the NBDs are processed correctly, including G551D in NBD1 and G1349D in NBD2 (*21, 31*). We previously reported that G551D was not functional on the basis of results of a halide efflux assay (*32*). Surprisingly, the G1349D mutant was functional in the halide efflux assay and generated

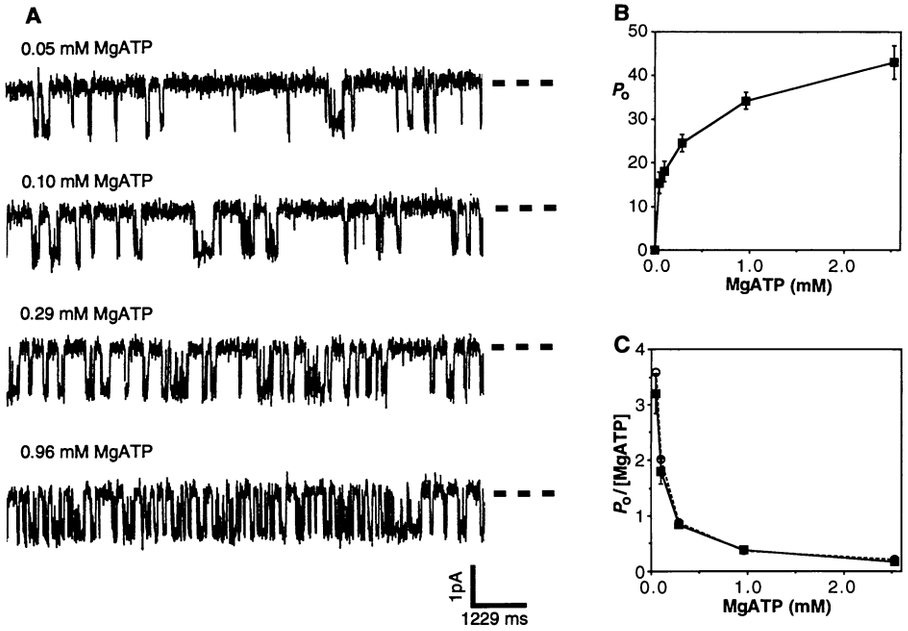

Fig. 1. Effect of MgATP on channel activity in excised, inside-out patches (*40*). (**A**) Effect of MgATP concentration on the activity of a single CFTR Cl^- channel (from a Cl27i cell) that had been phosphorylated with PKA and ATP. Voltage was −100 mV, and the zero current level is indicated by the dashed lines. (**B**) Effect of MgATP concentration on P_o. Data are means ± SEM from six patches. Studies of macroscopic currents in patches that contained large numbers of CFTR Cl^- channels indicate that P_o in the absence of ATP is near 0 (*17*). (**C**) Eadie-Hofstee plot demonstrating negative cooperativity for the effect of ATP on mean P_o (n = 7 excised patches, solid line) and on the P_o of a single CFTR Cl^- channel (broken line). The concentration of MgATP was determined from the concentrations of Mg^{2+} and ATP (the MgATP dissociation constant = 11.48 × 10^3 M^{-1}) (*41*).

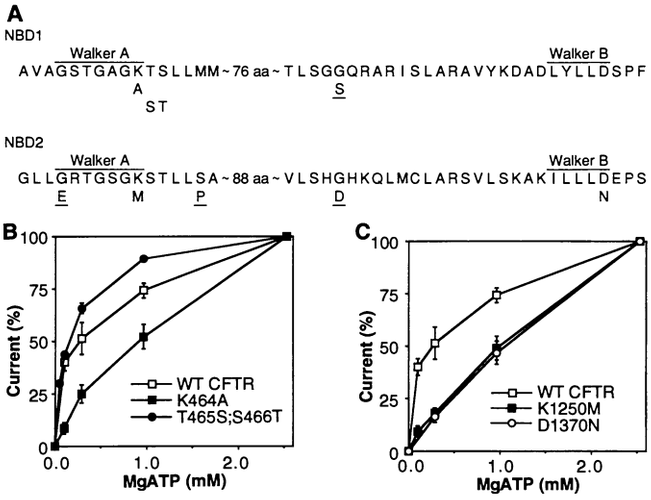

Fig. 2. Effect of site-directed mutations on the function of the CFTR (*40*). (**A**) Amino acid sequences (*21*) of portions of NBD1 and NBD2. Walker A and B motifs (*19*) are indicated by the lines above the sequences. The mutations studied are indicated beneath the sequences; CF-associated mutations are underlined; aa, amino acids. (**B** and **C**) Effect of NBD1 and NBD2 mutations, respectively, on MgATP stimulation of CFTR Cl^- channels. Because of the difficulty in determining the number of channels in a multichannel patch, especially when P_o is very low, the current observed in each excised patch was normalized to the current measured with 2.53 mM MgATP in that patch. All mutants were studied by transient expression in HeLa cells except K1250M, which was studied in NIH 3T3 fibroblasts. The wild-type (WT) CFTR showed the same MgATP dose-response relation in both cell types. In (B) and (C), each data point is the mean ± SEM from four to eight membrane patches.

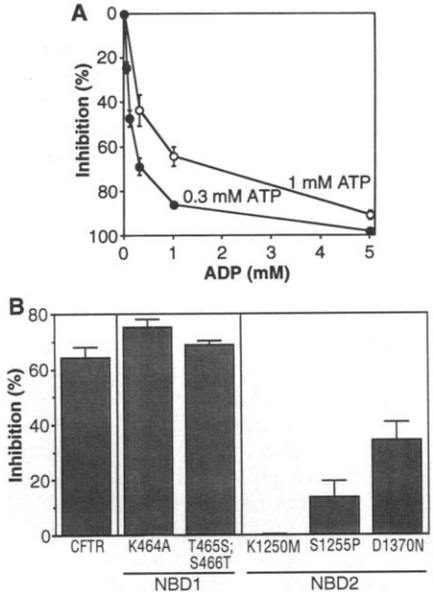

Fig. 3. Competitive inhibition of CFTR Cl^- channels by ADP (*40*). (**A**) Effect of ADP concentration on current in the presence of either 0.3 mM (closed symbols) or 1 mM MgATP (open symbols) in NIH 3T3 fibroblasts that expressed the CFTR. Data are normalized to the current in the absence of ADP and are from four to eight membrane patches for each data point. (**B**) Effect of NBD mutations on ADP-dependent inhibition. MgATP and ADP concentrations were both 1 mM (*27*). Data are from four to eight patches for each mutant.

cAMP-regulated Cl^- channels that had normal biophysical properties when studied with the whole-cell patch-clamp technique (*32*). But because the G1349D mutant causes CF (*34*), we reasoned that there must be some defect in channel function and that perhaps that defect is in its regulation. We examined the effect of ATP on G1349D plus three other CF-associated mutations in the NBDs (Fig. 2A), including G551S (*35*), G1244E (*36*), and S1255P (*37*). The S1255P mutation significantly altered the MgATP dose-response relation; MgATP was less potent at stimulating channel activity (Fig. 4A). The other mutations had minimal effects. However, when we measured the absolute level of channel activity, we found that all the CF mutations markedly reduced P_o (Fig. 4B) (*25*).

How do the G551S, G1244E, and G1349D mutations decrease P_o without dramatically altering the ATP dose-response relation? One possible explanation is that the mutations do not affect the binding affinity of the NBD for MgATP but rather may directly or indirectly alter the transduction mechanism that couples ATP binding to channel opening. Gly^{551} and Gly^{1349} are homologous amino acids in NBD1 and NBD2, respectively (Fig. 2A); both lie within a conserved sequence proposed by Shyamala *et al.* (*38*) to transduce the effect of ATP binding and hydrolysis. Alternatively, if the CFTR hydrolyzes ATP, these mutations might reduce the rate of hydrolysis.

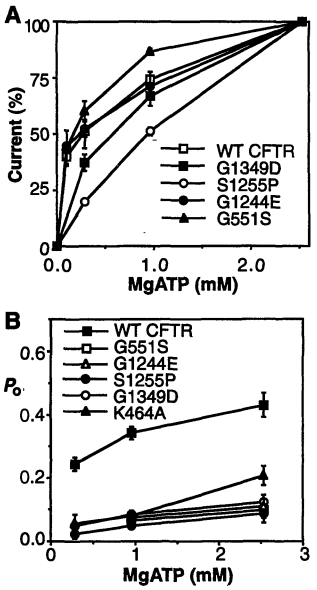

Fig. 4. Effect of CF-associated mutations on the response of CFTR Cl^- channels to ATP (*40*). (**A**) Effect of ATP concentration on current normalized to the response with 2.53 mM MgATP. (**B**) P_o of single channels generated by CF-associated mutations (*40*). The mutant K464A is shown for comparison, and data are from four to nine excised patches for each mutant. All mutants were studied by transient expression in HeLa cells except D1370N, which was studied in C127i cells. Wild-type (WT) CFTR showed the same MgATP dose-response relation when studied in either cell type.

A defining characteristic of CF epithelia is reduced plasma membrane Cl^- permeability (*39*). Our findings suggest two mechanisms by which some CF mutations in the NBDs might reduce Cl^- channel activity: the potency with which ATP stimulates activity by means of the NBDs might be reduced or the absolute level of channel activity might be reduced (irrespective of ATP concentration). Defective regulation of CFTR Cl^- channels through the NBDs may be one mechanism by which CF mutations cause disease.

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21. Mutants are named to include the amino acid residue number preceded by the wild-type amino acid and followed by the amino acid to which the residue was changed, with the use of the single-letter amino acid code. Thus, K464A means that Lys residue 464 was changed to Ala. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
22. We could not determine an absolute Michaelis constant (K_m) because the kinetics are complex and determination of a K_m would require assumptions about a specific model. Moreover, higher MgATP concentrations were not studied because at higher Mg^{2+} concentrations, other channels often appeared in the patches and the rate at which channel activity was lost increased.
23. We previously reported that the CFTR that contained the D1370N mutation was not functional in intact cells using the 6-methoxy-*N*-(3-sulfopropyl) quinolinium halide efflux assay (*32*). The studies described here were performed in excised patches of membrane in the absence of ADP and under conditions in which we could increase the intracellular ATP concentration. We did not study D572N because it was inactive in both the halide efflux assay and in a few recordings with the excised-patch technique. However, because D572N is abnormally processed, it likely never reaches the plasma membrane (*31–33*).
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25. None of the mutations altered single-channel current. At −100 mV, current amplitude (mean ± SEM; in picoamperes) was as follows: CFTR, −1.1 ± 0.0 (n = 7); K464A, −1.2 ± 0.0 (n = 3); T465S;S466T, −1.1 (n = 1); D1370N, −1.2 ± 0.0 (n = 3); K1250M, −1.2 (n = 1); G551S, −1.1 ± 0.1 (n = 4); G1244E, −1.2 ± 0.0 (n = 3); S1255P, −1.2 ± 0.1 (n = 3); and G1349D, −1.1 ± 0.0 (n = 4). Moreover for each mutant, channels were activated by PKA and ATP, which suggests that regulation by phosphorylation was unaltered. Thus, the mutations did not produce gross alterations of the CFTR protein.
26. M. P. Anderson and M. J. Welsh, data not shown.
27. The difference in ADP inhibition is not likely explained by differences in the affinity of these mutants for ATP because K464A shifts the ATP dose-response curve to a degree similar to that caused by K1250M (Fig. 2, B and C). Moreover, when the ATP concentration was reduced to 0.3 mM, 1 mM ADP was still unable to inhibit current in K1250M; at these nucleotide concentrations, ADP inhibited 1.6 ± 3.2% (mean ± SEM) of the current in K1250M (n = 5), compared with 84.4 ± 1.2% in the wild-type CFTR (n = 4).
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40. We used three different cell types and CFTR expression systems. NIH 3T3 fibroblasts infected with a retrovirus-expressing human CFTR or the mutant K1250M were prepared and maintained as described (*4*). C127i cells stably expressing small amounts of the wild-type CFTR and the mutants G1349D and D1370N were generated by calcium phosphate–mediated transfection with a bovine papilloma virus–based vector that contained CFTR cDNA and a neomycin resistance gene (as a selectable marker) under control of the mouse metallothionein MT1 promoter. HeLa cells were used for the transient expression of the CFTR, K464A, T465S;S466T, G551S, D1370N, S1255P, and G1244E. The vaccinia virus–T7 polymerase hybrid expression system was used as described [O. Elroy-Stein, T. R. Fuerst, B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6126 (1989); D. P. Rich *et al.*, *Nature* **347**, 358 (1990)]. The ATP dose-response relation was not affected by the expression system or cell type. We used the excised, inside-out patch-clamp technique as described (*7, 17*). The pipette (external) solution contained 140 mM *N*-methyl-D-glucamine, 2 mM $MgCl_2$, 5 mM $CaCl_2$, 100 mM L-aspartic acid, and 10 mM Hepes-HCl (pH 7.3) (Cl^- concentration = 49 mM). The bath (internal) solution contained 140 mM *N*-methyl-D-glucamine, 3 mM $MgCl_2$, 1 mM EGTA-CSOH, and 10 mM Hepes-HCl (pH 7.3 with HCl) (Cl^- concentration = 147 mM; the estimated free Ca^{2+} concentration <10 nM). Unless otherwise indicated, voltage (refer-

enced to the external surface of the membrane patch) was −40 mV. Studies were performed at 35°C. After excision of a patch from the cell, channels were first phosphorylated with 1 mM ATP and 75 nM catalytic subunit of PKA (Promega), the phosphorylation mixture was then removed, and various concentrations of MgATP were added. P_o was determined from amplitude histograms in patches that contained five channels or less. The number of channels in a patch was determined as the maximum number observed with 2.53 mM MgATP [R. Horn, *Biophys. J.* **60**, 433 (1991)]. Data are presented as the mean ± SEM.

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42. We thank R. Gregory and A. Smith for generously providing some CFTR mutants and stably transfected cells; S. Thompson and R. Mulligan for providing stably transfected cells; M. Keene for constructing a number of the CFTR mutants; B. Plapp for discussions of the kinetics of ATP interactions with the CFTR; L. DeBerg and P. Karp for culturing the cells; T. Mayhew for helping prepare the manuscript; and our colleagues who provided many stimulating discussions. Supported in part by the Howard Hughes Medical Institute, the Cystic Fibrosis Foundation, and the National Heart, Lung, and Blood Institute.

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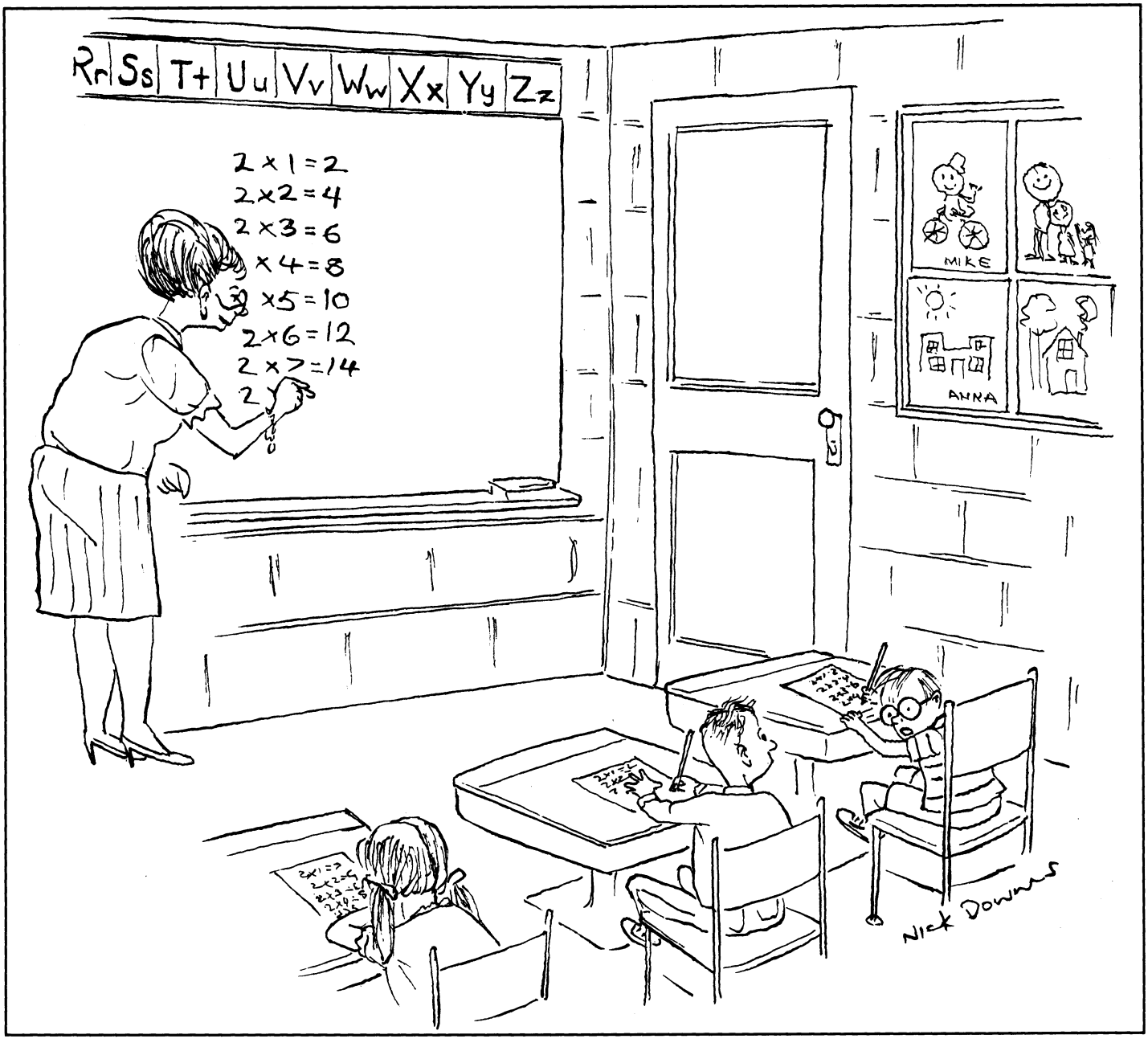

"I worry about the subliminal message."