Articles

Modulation of P-Glycoprotein Phosphorylation and Drug Transport by Sodium Butyrate

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ABSTRACT: P-Glycoprotein (Pgp) expression in cell lines derived from tumors arising from cells which normally express Pgp can be increased by sodium butyrate and other differentiating agents. Although the Pgp level increased 25-fold after sodium butyrate treatment in SW620 human colon carcinoma cells, the intracellular accumulation of vinblastine, adriamycin, and actinomycin D increased rather than decreased. In contrast, colchicine showed the expected decrease in accumulation, as a result of increased efflux. Likewise, treatment of a Pgp-expressing multidrug-resistant SW620 subline with sodium butyrate resulted in active interference with Pgp function. This effect was partially reversed by phorbol esters with a resulting decrease in the accumulation of vinblastine, adriamycin, and actinomycin D. Sodium butyrate, while increasing Pgp levels, inhibited the phosphorylation of Pgp. Time course studies revealed a tight relationship between decreased Pgp phosphorylation and increased vinblastine accumulation after sodium butyrate treatment. Either treatment with phorbol esters or withdrawal of sodium butyrate increased Pgp phosphorylation while decreasing vinblastine accumulation. These studies suggest that the specificity of Pgp transport can be modulated by phosphorylation and that vinblastine, adriamycin, or actinomycin D transport, but not that of colchicine, is diminished after dephosphorylation by sodium butyrate.

The emergence of multidrug resistance as a major obstacle to successful cancer treatment has stimulated studies aimed at identifying putative mechanisms of drug resistance. Beginning with the work of Biedler and Ling (Biedler & Riehm, 1970; Ling & Thompson, 1974), a large body of evidence has been gathered that supports a role for P-glycoprotein (Pgp) in multidrug resistance (van der Bliek & Borst, 1989). Pgp has been found in normal tissues, in unselected cell lines, and in cell lines with acquired multidrug resistance (Fojo et al., 1987). In the latter, this membrane phosphoglycoprotein functions as a drug efflux pump, decreasing intracellular drug concentrations and thus rendering cells resistant to chemotherapy. In normal tissues, the function of Pgp is less well understood. Its presence in tissues such as the colon, kidney, and liver suggests it has a role in detoxification. However, the highest levels of expression are found in the human adrenal gland, where a role in the secretion of adrenal steroids has been postulated, but not proven (Fojo et al., 1987; Yang et al., 1989). No normal substrates for Pgp have been identified to date.

Pgp expression can be increased by differentiating agents in colon cancer cell lines (Mickley et al., 1989). This finding may be linked to the observation that more well-differentiated colon cancers express higher levels of Pgp and that the normal colonic epithelium expresses Pgp (Mickley et al., unpublished observations). In vitro studies have shown that the differentiating agents sodium butyrate, dimethyl sulfoxide, and dimethylformamide increase expression of Pgp in human colon carcinoma cell lines and similarly that sodium butyrate and retinoic acid increase expression of Pgp in neuroblastoma cell lines (Mickley et al., 1989; Bates et al., 1989). Thus, the use of differentiating agents allows the study of Pgp in a system in which Pgp activity may be modulated without drug exposure. Such studies in human colon carcinoma and neuroblastoma cell lines have demonstrated that in some cell lines, but not in all, increased Pgp levels resulting from treatment with a differentiating agent fail to decrease drug accumulation or to increase drug resistance (Mickley et al., 1989; Bates et al., 1989). In SW620 human colon carcinoma cells, a 25-fold increase in Pgp expression after sodium butyrate treatment is accompanied by increased vinblastine accumulation rather than the expected decrease. These observations suggest that the activity of Pgp as a drug efflux pump may depend on factors other than the quantity of Pgp present.

To understand the contradiction of an increase in Pgp concentration and its apparent lack of function in SW620 cells after sodium butyrate treatment and to evaluate possible mechanisms underlying this effect, parental SW620 cells and two multidrug-resistant sublines were examined. We evaluated the effect of sodium butyrate on transport of three other drugs comprising the multidrug resistance phenotype, actinomycin D, adriamycin, and colchicine. We then studied the effect of sodium butyrate on Pgp phosphorylation, postulating a relationship between phosphorylation and specificity of drug transport.

Materials and Methods

Materials. [^{3}H]Colchicine and [^{32}P]orthophosphoric acid were obtained from New England Nuclear. [^{3}H]Vinblastine, [^{3}H]actinomycin D, [^{14}C]adriamycin, and L-[^{35}S]methionine were from Amersham. TPA (12-*O*-tetradecanoylphorbol 13-acetate), PDBU (phorbol 12,13-dibutyrate), and sodium

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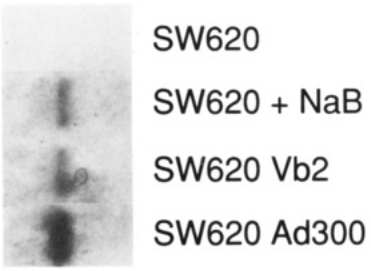

FIGURE 1: Western blot analysis. P-Glycoprotein levels in membrane fractions were determined using the monoclonal antibody C219. Because of the differences in P-glycoprotein levels, different amounts of protein were loaded for the cells with lower levels of expression than for SW620 Ad300 cells: SW620 (200 μg); SW620 + NaB (200 μg); SW620 Vb2 (200 μg); SW620 Ad300 (20 μg).

butyrate were Sigma products.

Cell Lines. Multidrug-resistant sublines were derived by selecting SW620 cells with either vinblastine or adriamycin. The vinblastine-selected subline, SW620 Vb2, was derived by increasing the vinblastine concentration in small increments, and is maintained in 2 ng/mL vinblastine. This subline has a low level of Pgp which is comparable to that detected in sodium butyrate-treated SW620 cells by immunoblotting (Figure 1) and by Northern analysis (the SW620 Vb2 mRNA level is 93% that of SW620 cells treated with sodium butyrate). Studies of these cells demonstrate a 20% reduction in vinblastine accumulation, with a 3.5-fold increase in vinblastine resistance, compared to parental cells (Herzog et al., 1992). The adriamycin-selected subline was derived by stepwise exposure of SW620 cells to adriamycin and is designated SW620 Ad300. This subline is maintained in 300 ng/mL adriamycin and demonstrates a classical multidrug-resistant phenotype with broad cross-resistance, increased Pgp expression, decreased drug accumulation, and partial reversibility of the multidrug resistance phenotype by verapamil (Lai et al., 1991). The levels of *mdr*-1 mRNA expression in this cell line are 146-fold higher than those of parental SW620 cells and increased 2-fold after incubation in 2 mM sodium butyrate.

Drug Accumulation and Efflux Studies. Accumulation studies were performed as previously described (Fojo et al., 1985). SW620 cells were plated in six-well dishes 3 days before accumulation studies, with one-third as many cells plated in the wells not treated with sodium butyrate, to achieve comparable confluency. Cells were treated with 2 mM sodium butyrate for 3 days. When the effect of phorbol ester treatment was evaluated, sodium butyrate was removed, and fresh medium containing 200 nM TPA or PDBU was added for 4 h. Addition of TPA to cells continuing in sodium butyrate gave the same result as removing sodium butyrate. Cells were rinsed twice and preincubated in sodium butyrate-free assay medium for 15 min. The accumulation assays were performed with 2×10^6 dpm of drug per well, equivalent to 14 nM [^{3}H]vinblastine, 30 nM [^{3}H]actinomycin D, 28 nM [^{14}C]-adriamycin, and 66 nM [^{3}H]colchicine.

Drug efflux studies were performed by incubating cells in 2×10^6 dpm of [^{3}H]vinblastine or [^{3}H]colchicine per well for 1 h prior to the efflux assay; under these conditions, steady state is not achieved. After a 15-s wash to remove drug-containing medium, sequential exchanges of medium were performed at the indicated time points, and the amount of drug in the medium was determined by scintillation counting.

Cytotoxicity Studies. Cytotoxicity studies were performed with cells pretreated with or without 2 mM sodium butyrate. Cells were incubated with either vinblastine or colchicine for 3 h and then allowed to grow for an additional 7 days in sodium butyrate-free medium before being counted.

Metabolic Labeling. Untreated cells or cells treated with 2 mM sodium butyrate were labeled as previously described (Richert et al., 1988) with 0.1–1 mCi of [^{32}P]orthophosphoric acid in 4 mL of phosphate-free DMEM without serum over 4 h, or with 1 mCi of [^{35}S]methionine in 4 mL of methionine-free DMEM without serum for 16 h. Sodium butyrate was added for up to 72 h prior to harvesting of the cells. Sodium butyrate treatment, except where indicated, continued during all cell labeling. When the effect of phorbol ester treatment was evaluated, 200 nM TPA or PDBU was added for 4 h prior to harvesting. Both sodium butyrate and TPA were present in the medium during the last 4 h of [^{35}S]-methionine labeling; however, sodium butyrate was removed and TPA was present alone during the 4-h [^{32}P]orthophosphate labeling. After cells were harvested in RIPA buffer (1% Triton X-100/0.1% $NaDodSO_4$/1% sodium deoxycholate/0.15 M NaCl/20 mM Tris-HCl, pH 7.2), protein was assayed by a commercial assay (Bio-Rad), and immunoprecipitation was carried out with a polyclonal antibody raised against recombinant protein fragments (Tanaka et al., 1990). A minimum of 1000 μg of protein was immunoprecipitated from the SW620 cells, and 350 μg of protein from the SW620 Ad300 cells. Subsequently, the product was analyzed on a 7% polyacrylamide gel.

RESULTS

Effect of Sodium Butyrate on the Transport of Four Drugs Comprising the Multidrug Resistance Phenotype. Figure 2 presents the results of studies comparing drug accumulation, drug efflux, and cytotoxicity in SW620 cells treated with or without 2 mM sodium butyrate for 3 days. The experiments in the upper panel extend the observations previously reported for vinblastine (Mickley et al., 1989) by examining three additional drugs transported by Pgp. The studies were performed with cells rinsed free of sodium butyrate by two media exchanges over 15 min, prior to the drug accumulation assay. [^{3}H]Vinblastine accumulation increased 15–57% following addition of sodium butyrate, as previously reported, even though Pgp expression increases 25-fold under these conditions (Mickley, 1989). Similar observations were made when [^{3}H]actinomycin D and [^{14}C]adriamycin accumulation was measured. With both of these agents, an increase of 27–104% above the base-line value was observed. These results suggested that after sodium butyrate neither the newly induced protein nor the low endogenous level of Pgp properly transported these three drugs, leading to an increase in drug accumulation.

The data with these three drugs also suggested that the accumulation of different drugs may be affected to different degrees by sodium butyrate. With colchicine, this observation was confirmed—transport persisted after sodium butyrate treatment. In each of six separate experiments, the addition of sodium butyrate was associated with a fall in [^{3}H]colchicine accumulation, an observation which suggested that after the addition of sodium butyrate the induced Pgp was able to transport colchicine. Confirmation of these differences in drug accumulation was provided by the drug efflux experiments shown in the middle panel of Figure 2. The results show that the observed changes in accumulation are secondary to differences in drug efflux. [^{3}H]Vinblastine efflux is decreased to 69.7 ± 0.7% after sodium butyrate, a value which agrees with the increase in drug accumulation, while [^{3}H]colchicine efflux increased to 127 ± 3% after sodium butyrate, also in agreement with the observations showing decreased colchicine accumulation. Furthermore, in examination of the cytotoxicity

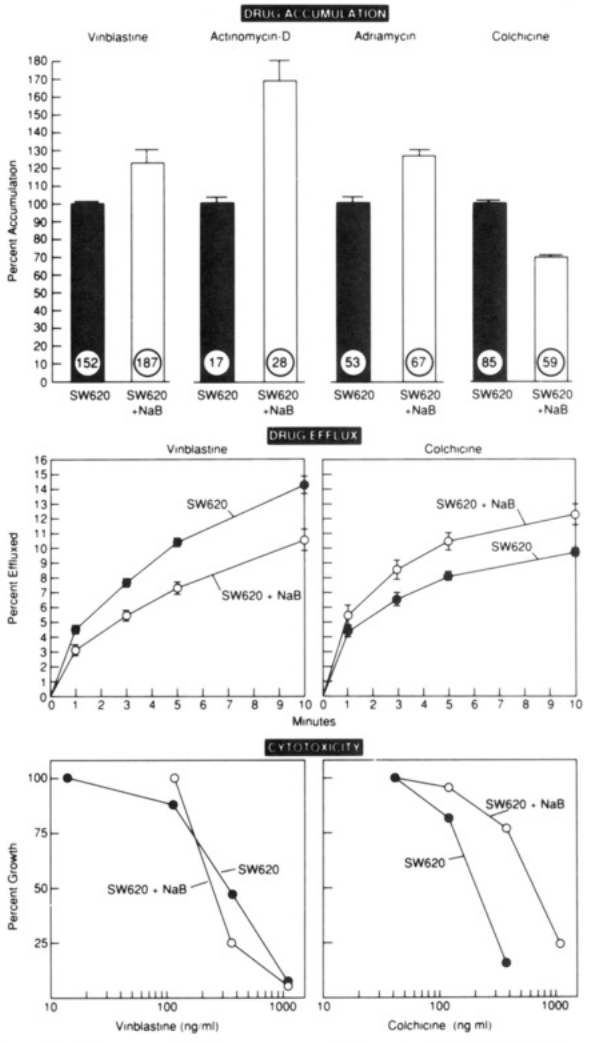

FIGURE 2: (Top panels) Drug accumulation studies in SW620 cells showing the effect of sodium butyrate (NaB) on vinblastine, actinomycin D, adriamycin, and colchicine accumulation. Accumulation in untreated SW620 cells is arbitrarily assigned a value of 100% for all drugs, but differences in absolute amounts are indicated by the numbers within the bars, which represent the femtomoles of drug per 10^5 cells to the nearest 1%. Accumulation of vinblastine, actinomycin D, and adriamycin increased to values of 123 ± 7.5%, 169 ± 13.3%, and 127 ± 2.2%, respectively. Colchicine accumulation decreased to 70 ± 1.2% of that in untreated SW620 cells. (Middle panels) Comparison of vinblastine and colchicine efflux in SW620 cells before and after treatment with 2 mM sodium butyrate. The standard deviations are depicted for each point. Drug efflux in the sodium butyrate-treated cells compared to untreated SW620 cells was retarded for vinblastine to 69.7 ± 0.7% and increased for colchicine to 127 ± 3%, in agreement with the changes in drug accumulation. (Lower panels) Effect of sodium butyrate on vinblastine and colchicine cytotoxicity in SW620 cells. Vinblastine sensitivity increased slightly or remained unchanged, while colchicine tolerance increased. NaB, sodium butyrate.

of these agents for SW620 cells, treatment with sodium butyrate increased or did not significantly change vinblastine sensitivity as shown in the lower left-hand panel, but did increase colchicine resistance by a factor of 3–4, suggesting that the decrease in colchicine accumulation enhanced resistance. Cytotoxicity studies are difficult to interpret because they measure not only the effect of sodium butyrate on Pgp but also other factors that can contribute to drug sensitivity.

Effect of Sodium Butyrate on Pgp Function in a Multidrug-Resistant Subline. To further examine whether these differences were mediated through Pgp, cells with higher levels of Pgp were needed. A multidrug-resistant subline derived from parental SW620 cells which expresses 146-fold higher

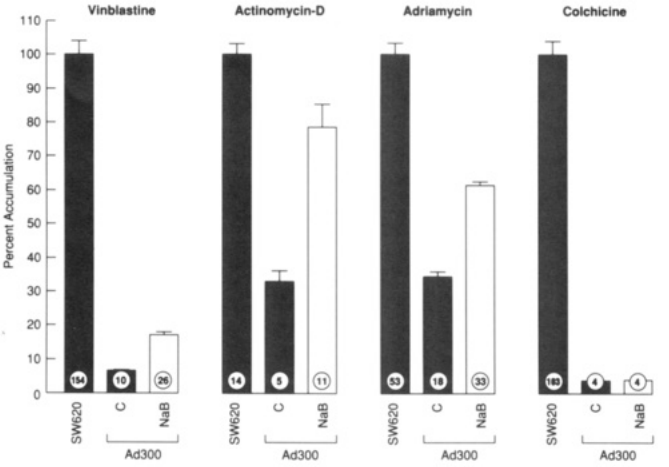

FIGURE 3: Drug accumulation studies in SW620 and SW620 Ad300 cells. The effect of treatment with 2 mM sodium butyrate for 72 h on the accumulation of four drugs is examined. Accumulation in untreated SW620 cells is arbitrarily assigned a value of 100% for all drugs, but differences in absolute amounts are indicated by the numbers within the bars, which represent the femtomoles of drug per 10^5 cells to the nearest 1%. As with SW620 cells, sodium butyrate increased vinblastine, adriamycin, and actinomycin D accumulation in SW620 Ad300 cells. Colchicine accumulation was not increased by sodium butyrate. C, control; NaB, sodium butyrate.

levels of *mdr*-1 was studied without and after treatment with sodium butyrate. [^{3}H]Vinblastine accumulation in SW620 Ad300 is decreased to 6.3% of that in parental SW620 cells. Sodium butyrate increased accumulation 2.6-fold to 17% of parental levels, as shown in Figure 3. Just as in the parental cells, sodium butyrate also increased the accumulation of adriamycin, and actinomycin D. However, in the case of colchicine, accumulation is not affected by sodium butyrate. An actual decrease in colchicine accumulation in SW620 Ad300 was not measured as it was in the parental cells after sodium butyrate. However, any decrease in accumulation in these cells would be difficult to measure because the accumulation in the resistant subline is so low to begin with (3.8 ± 0.3% of parental).

Effect of Phorbol Esters on Drug Transport in Sodium Butyrate-Treated SW620 Cells and Multidrug-Resistant Sublines. Since treatment of SW620 cells with sodium butyrate leads to a decrease in the activity of several protein kinases including pp60$^{c\text{-}src}$ and p56^{lck} (Foss et al., 1989), the possibility existed that sodium butyrate could modulate drug accumulation by changing the phosphorylation of Pgp. Previous studies in multidrug-resistant KB cells have shown that protein kinase C (PKC) phosphorylates Pgp and increases drug transport in these cells (Chambers et al., 1990). Therefore, the effect of protein kinase C activation by phorbol ester was examined in SW620 and SW620 Ad300 cells treated with sodium butyrate.

The effect of phorbol ester treatment on drug accumulation is shown in Figure 4. All accumulation values are related to that in untreated SW620 cells, which was assigned a value of 100%. In SW620 cells, vinblastine accumulation was not significantly affected by treatment with 200 nM TPA. Sodium butyrate, as before, increased vinblastine accumulation to 157% of control in this experiment. Sodium butyrate treatment of SW620 cells followed by 200 nM TPA for 4 h immediately prior to the assay reduced accumulation to 84% of SW620, a level similar to that found in multidrug-resistant SW620 Vb2 cells. SW620 Vb2 cells have Pgp levels which are similar to those found in SW620 parental cells after sodium butyrate treatment (Figure 1). Thus, the reduction to 84% indicates

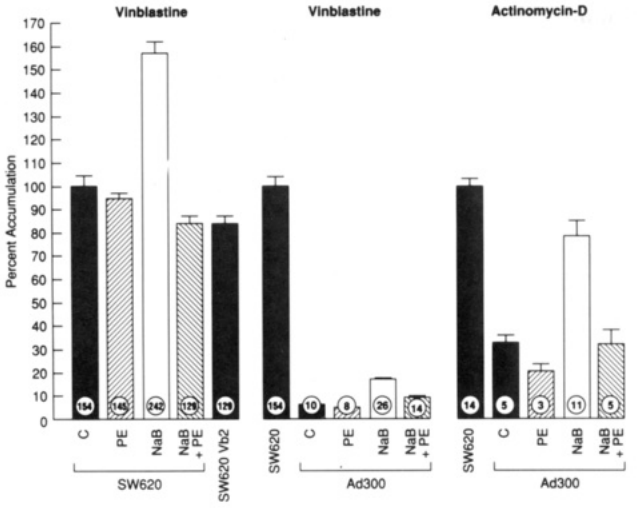

FIGURE 4: Effect of phorbol esters on drug accumulation in SW620, SW620 Vb2, and SW620 Ad300 cells. Accumulation in SW620 cells is arbitrarily assigned a value of 100% for all drugs, but differences in absolute amounts are indicated by numbers within the bars, which represent the femtomoles of drug per 10^5 cells to the nearest 1%. Results with SW620 Ad300 and SW620 Vb2 are expressed relative to SW620. Drug accumulation in cells without (■, ▨) and with (□, ▧) sodium butyrate pretreatment is shown both with (▨, ▧) and without (■, □) 4 h of incubation in 200 nM TPA. Left panel: Effect of 200 nM TPA on vinblastine accumulation in sodium butyrate-treated and untreated SW620 cells. Addition of TPA for 4 h to sodium butyrate-treated SW620 cells decreased accumulation to a value comparable to that in SW620 Vb2 cells, which express Pgp at similar levels. Middle and right panels: Effects of 200 nM TPA on vinblastine and actinomycin D accumulation in SW620 Ad300 cells. NaB, sodium butyrate; PE, phorbol ester; C, control; NaB + PE, cells pretreated for 3 days with sodium butyrate prior to treatment with TPA for 4 h.

that the Pgp in the sodium butyrate-treated SW620 cells can transport vinblastine after TPA treatment as well as that in selected cells with low levels of Pgp expression, such as the SW620 Vb2 cells. Similar results were obtained when actinomycin D accumulation was examined (not shown). The observations made in the SW620 Ad300 cells were similar to those in the SW620 cells, as shown in the panels on the right. In control SW620 Ad300 cells, TPA treatment appears to enhance transport. Four hours at 200 nM TPA decreased vinblastine accumulation from 6.3 to 4.9% and actinomycin D accumulation from 32.7 to 20.5% in SW620 Ad300 cells. In sodium butyrate-treated SW620 Ad300 cells, TPA reduced drug accumulation to levels similar to those in untreated SW620 Ad300 cells: from 17% to 9% for vinblastine and from 78% to 30% for actinomycin D. Similar results were obtained whether TPA was added for 2 or 4 h, and whether 200 nM TPA or 200 nM PDBu was added to butyrate-pretreated cells. In the experiments shown here, TPA was added to cells after removal of sodium butyrate. In separate experiments, addition of TPA to cells remaining in sodium butyrate gave similar results.

Effect of Sodium Butyrate on Pgp Phosphorylation. The alterations in drug accumulation after phorbol ester treatment suggested that changes in phosphorylation could mediate the effect of sodium butyrate on Pgp function. Figure 5 demonstrates the results of [^{35}S]methionine and [^{32}P]orthophosphoric acid labeling of cells after the various treatments followed by immunoprecipitation with a polyclonal antibody raised against recombinant Pgp peptides (Tanaka et al., 1990). The results obtained with SW620 cells and the two drug-resistant sublines labeled metabolically with [^{32}P]orthophosphoric acid are shown in panels A and B, and the results from labeling with [^{35}S]-methionine are shown in panels C and D. Panel C demonstrates the increase in Pgp level after sodium butyrate treatment in SW620 cells and panel D the increase in Pgp level after sodium butyrate treatment in SW620 Ad300 cells. The [^{35}S]methionine experiment also demonstrates that the levels of Pgp were not changed significantly by treatment with phorbol ester in either cell line. As seen in panel A, phosphorylated Pgp was not detected in SW620 cells treated with sodium butyrate although intense phosphorylation was observed in SW620 Vb2 cells. As shown in the [^{35}S]-methionine-labeled protein in panel C (and previously in Figure 1), SW620 Vb2 cells have levels of Pgp comparable to those found in the sodium butyrate-treated SW620 cells and thus serve as a control for the sensitivity of immunoprecipitation. Treatment with 200 nM TPA or PDBU for 4 hours increases phosphorylation of the protein, although the levels detected do not reach those of SW620 Vb2 cells. The figure also shows the disappearance of phosphorylated protein in SW620 Vb2 cells after the addition of sodium butyrate. Similar results were observed when SW620 Ad300 cells were treated with

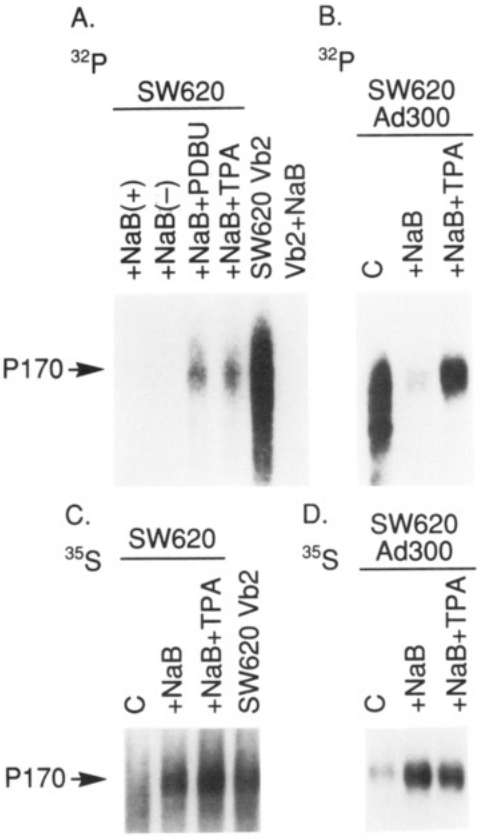

FIGURE 5: Metabolic labeling of SW620 cells and its multidrug-resistant sublines with [^{32}P]orthophosphoric acid (A, B) and [^{35}S]-methionine (C, D). (A and B) Cells treated with or without sodium butyrate for 72 h were labeled with either 1 mCi (A) or 100 μCi (B) of [^{32}P]orthophosphoric acid in 4 mL of phosphate-free DMEM without serum over 4 h. Cells treated with 200 nM TPA had the phorbol ester added to the medium at the same time as the [^{32}P]-orthophosphoric acid (4-h treatment). Similar results were obtained with 2-h phorbol ester treatment. (C and D) Cells were labeled for 16 h with [^{35}S]methionine. Cells treated with sodium butyrate were maintained in sodium butyrate during the period of labeling. Phorbol ester was added only during the last 4 h. No difference was detected after phorbol ester treatment in most experiments. Autoradiogram exposure times were (A) 22, (B) 18, (C) 13, and (D) 2.5 h. In panel A, cells were pretreated with sodium butyrate (NaB), and the (+) and (−) refer to its presence or absence, respectively, during the ^{32}P labeling period. In panels B–D, NaB indicates pretreatment with sodium butyrate for 72 h and the presence of sodium butyrate during the labeling period. C, control, refers to cells not treated with sodium butyrate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDBU, phorbol 12,13-dibutyrate.

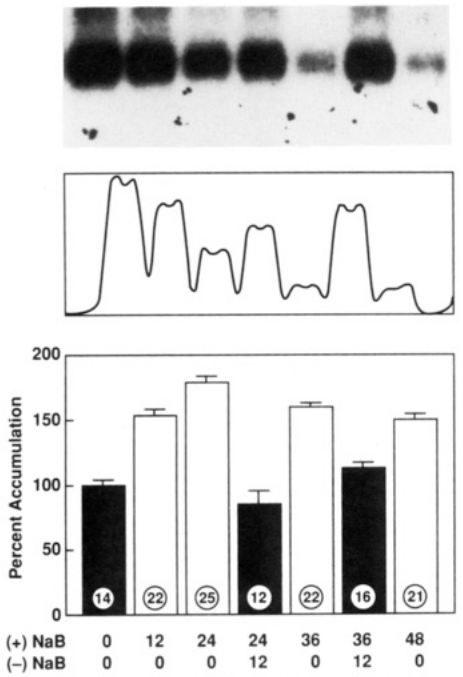

FIGURE 6: Vinblastine accumulation and Pgp phosphorylation studies in SW620 Ad300 cells. Metabolic labeling with [^{32}P]orthophosphoric acid was performed as in Figure 4 except that cells were labeled with 0.4 mCi of [^{32}P]orthophosphoric acid in 5 mL of phosphate-free DMEM without serum over 2 h. The upper panel depicts Pgp phosphorylation, which is quantitated by densitometry in the middle panel. Simultaneous assay of vinblastine accumulation is shown in the lower panel. Accumulation of untreated SW620 Ad300 cells has been arbitrarily assigned a value of 100%, with the femtomoles of vinblastine per 10^5 cells indicated by the numbers within the bars. Addition of sodium butyrate to Ad300 cells results in a progressive decrease in Pgp phosphorylation and a gradual increase in vinblastine accumulation. Both of these effects are reversed 12 h following sodium butyrate withdrawal after either 24 or 36 h of treatment with sodium butyrate. (+) NaB, hours of pretreatment with 2 mM sodium butyrate; (−) NaB, hours in sodium butyrate-free medium prior to cell harvest or accumulation assay.

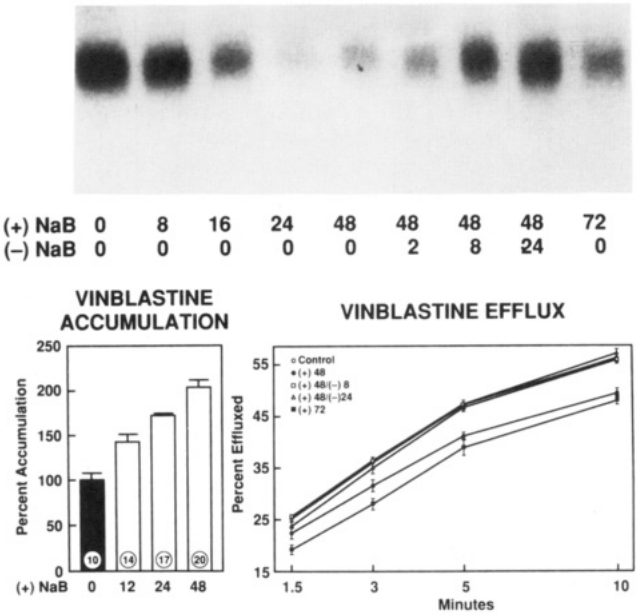

FIGURE 7: Studies of P-glycoprotein phosphorylation, vinblastine accumulation, and vinblastine efflux in SW620 Ad300 cells. Experiments were performed as described in Figure 6. The upper panel depicts Pgp phosphorylation. The lower panel presents the results of drug accumulation on the left and drug efflux on the right. Addition of sodium butyrate to Ad300 cells results in a progressive decrease in Pgp phosphorylation that is reversible after removal of sodium butyrate. These changes are accompanied by changes in vinblastine accumulation and efflux, with increased accumulation observed following sodium butyrate treatment and increased drug efflux seen after its withdrawal. (+) NaB, hours of treatment with 2 mM sodium butyrate; (−) NaB, hours in sodium butyrate-free medium.

sodium butyrate and TPA (panels B and D). Phosphorylated Pgp is readily detectable in the untreated cells, but the level is decreased after sodium butyrate treatment and restored by TPA. The magnitude of the decrease following sodium butyrate is greater when the increase in [^{35}S]methionine-labeled Pgp is considered. Sodium butyrate resulted in a generalized decrease in protein phosphorylation which is not significantly changed by treatment with phorbol ester, indicating that the effect of TPA is more specific than that of sodium butyrate (data not shown). Although the phorbol ester results suggested that the levels of protein kinase C could be affected by sodium butyrate, immunoblotting failed to detect changes in levels (K. Leach, personal communication).

Effect of Sodium Butyrate Withdrawal on Pgp Phosphorylation and Function. To clarify the association between the changes observed in drug accumulation after sodium butyrate and its effect on Pgp phosphorylation, simultaneous phosphorylation and vinblastine accumulation studies were performed. In paired samples, the effect of sodium butyrate addition and removal was studied. As shown in Figure 6, a decrease in Pgp phosphorylation is observed as early as 12 h after addition of sodium butyrate, with further decreases observed at 24, 36, and 48 h. Removal of sodium butyrate for 12 h results in partial restoration of Pgp phosphorylation. A densitometry plot is shown for the autoradiogram, with a 2-fold decrease in phosphorylation of Pgp observed by 24 h. These results are compared to the results of vinblastine accumulation, shown in the lowest panel. Cells for both the accumulation and the phosphorylation studies were plated and treated simultaneously, and the [^{3}H]vinblastine accumulation was assayed during [^{32}P]orthophosphate labeling. Although [^{3}H]-vinblastine accumulation is about 6% of parental SW620 in the SW620 Ad300 cells, in the figure, untreated SW620 Ad300 cells have been assigned a value of 100% for clarity. Sodium butyrate treatment results in an increase in accumulation, first measurable at 12 h. Withdrawal of butyrate results in a decrease in accumulation, approximating levels in untreated cells within 12 h of withdrawal at any time point.

To confirm these findings, to examine earlier time points, and to determine whether these changes in drug accumulation were in fact due to alterations in efflux, another paired experiment was performed (Figure 7). As before, accumulation is shown increasing after the addition of sodium butyrate (lower left panel), while Pgp phosphorylation is decreased 7-fold by 48 h. In cells withdrawn from sodium butyrate for 2, 8, and 24 h following incubation for 48 h in sodium butyrate, phosphorylation and efflux studies were performed. By 8 h, Pgp phosphorylation approximates that in control cells. As with vinblastine accumulation, the efflux results parallel the phosphorylation. Compared with untreated cells, a decrease in phosphorylation is associated with decreased efflux (48- and 72-h time point) while the increase in phosphorylation at both 8 and 24 h out of sodium butyrate is associated with increased efflux, and the efflux curves are almost superimposed with that of the control.

Discussion

This study presents evidence that the differentiating agent sodium butyrate can decrease phosphorylation of Pgp and suggests that this in turn can affect the specificity of drug transport. Confirming our previous report, sodium butyrate

increased Pgp expression in SW620 human colon cancer cells without a concomitant increase in vinblastine transport (Mickley et al., 1989). The studies presented here extend that observation to demonstrate impaired transport of adriamycin and actinomycin D but, conversely, preservation of colchicine transport. Thus, accumulation of colchicine is decreased, while adriamycin, actinomycin D, and vinblastine accumulation is increased. Results with the SW620 Ad300 multidrug-resistant subline mirror those in the parental cells. Treatment with phorbol esters resulted in less restricted drug transport. Studies of [^{32}P]orthophosphate labeling of Pgp demonstrated decreased phosphorylation after sodium butyrate, and rapid restoration after either phorbol ester treatment or removal of sodium butyrate. These observations suggest that the level of Pgp expression may not predict a given level of drug transport.

Preferential colchicine resistance was previously observed in a colchicine-resistant KB cell line with a mutation which resulted in a change in amino acid residue 185 from glycine to valine (Choi et al., 1988). In that cell line, vinblastine transport was present, but exceeded by colchicine, and this difference was attributed to the mutation. The present study demonstrates that after sodium butyrate treatment, there is preferential colchicine transport, which is thought to be mediated by changes in Pgp phosphorylation in the SW620 cells and sublines, which have a wild-type or "vinblastine specific" sequence at residue 185 (unpublished data). These results thus represent two different means by which drug specificity could change. In the KB cells, the amino acid substitution reportedly resulted in preferential colchicine resistance while in the present study changes in Pgp phosphorylation achieved a similar result. The differences in accumulation observed in the SW620 cells as well as the drug-resistant subline after the addition of phorbol esters suggest that changes in drug resistance could develop without a change in Pgp level and conversely that large changes in levels may not completely predict the direction of change in resistance or the drug accumulation profile. Different drugs may have different affinities for transport given a particular state of phosphorylation.

How drug transport is changed by phosphorylation remains to be determined, although it does not appear that the decrease in phosphorylation inhibits drug binding. Azidopine binding to Pgp in SW620 cells treated with sodium butyrate is equivalent to that in SW620 Vb2 cells, and in both can be competed only by pharmacologic doses of vinblastine, and less so by colchicine (Mickley et al., 1989; and data not shown). The azidopine binding result suggests that binding but not transport of certain drugs may occur. Binding without transport has been previously reported for progesterone (Yang et al., 1990).

Analysis of the primary sequence of Pgp indicates the presence of at least 37 potential phosphorylation sites including 14 potential PKC sites, some or all of which could act to modulate drug transport (Barioch, 1990). Both phosphoserine and phosphothreonine (but not phosphotyrosine) have been demonstrated in the few models where phosphoamino acid analysis of Pgp has been performed (Center, 1983; Roy & Horwitz, 1985; Hamada et al., 1987). The work of various investigators suggests that more than one Pgp site can be phosphorylated and that different kinases are able to do this (Chambers et al., 1990; Mellado & Horwitz, 1987; Staats et al., 1990; Fine et al., 1988). Thus, the decrease in drug accumulation and the increase in phosphorylation observed in sodium butyrate-treated cells after TPA treatment need not occur as a result of phosphorylation of the same sites dephosphorylated by sodium butyrate. Since protein kinase C (PKC) levels apparently do not change with sodium butyrate and phorbol esters act predominantly by activating PKC, the results suggest that in our model system, different phosphorylation sites modulate drug transport. Alternatively, there may be gradations in the percentage of Pgp molecules phosphorylated. TPA was able to enhance drug transport in both sodium butyrate-treated and untreated cells. To the extent that differences in Pgp phosphorylation affect transport of individual drugs differently, the profile of cross-resistance could be altered significantly.

Beginning with the work of Center, a number of observations have been made regarding the phosphorylation of Pgp, including (1) an increase in Pgp phosphorylation associated with enhanced drug sensitivity resulting from treatment with verapamil, *N*-ethylmaleimide, or trifluoperazine (Center, 1983; Hamada et al., 1987); (2) stimulation of protein kinase C by phorbol esters, resulting in increased Pgp phosphorylation, increased drug resistance, and decreased drug accumulation (Chambers et al., 1990; Fine et al., 1988); (3) demonstration that Pgp phosphorylation is enhanced by cAMP and that Pgp can be phosphorylated by the catalytic subunit of protein kinase A (Mellado & Horwitz, 1987); and (4) characterization of a unique protein kinase which phosphorylates Pgp but is not a cAMP-dependent kinase or protein kinase C (Staats et al., 1990). These observations suggest regulation of Pgp similar to that of other previously described membrane receptors including the β-adrenergic receptor and rhodopsin, the light receptor, in which phosphorylation by multiple kinases has been observed (Sibley et al., 1987). In conjunction with the results in the present study, they provide a working hypothesis for understanding how the specificity of transport could be regulated in Pgp and other similar proteins.

In summary, the present study suggests that Pgp phosphorylation may affect the specificity of drug transport. With malignant transformation or transiently in normal tissues, an increase in protein kinase activity and subsequent Pgp phosphorylation could confer to Pgp a broader transport capability. Likewise, in cancer cells, resistance could be acquired as Pgp levels increased in a cell with active protein kinases. These findings may help to further understand the role of Pgp in clinical drug resistance.

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Annexin I-Mediated Vesicular Aggregation: Mechanism and Role in Human Neutrophils†

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ABSTRACT: Whole cytosol isolated from human neutrophils was found to accelerate the Ca^{2+}-dependent fusion of phospholipid vesicles with neutrophil plasma membranes as measured by several fluorescence resonance energy transfer lipid dilution assays or by the fate of an encapsulated aqueous soluble fluorophore. The Ca^{2+} (threshold of 2–10 μM) and protein concentration dependencies for fusion mediated by purified human neutrophil annexin I (lipocortin I), recombinant annexin I and des(1–9)annexin I showed behavior similar to that of whole cytosol. A monoclonal antibody against the N-terminal region of annexin I strongly inhibited the action of isolated annexins as well as whole cytosol, indicating that annexin I is the major activity of this type in whole neutrophil cytosol and that it functions even in this complex mixture of proteins. Residual Ca^{2+}-dependent fusion activity in the absence of cytosol or annexin I was not inhibited by several antibodies against annexin I, implicating an as yet unknown protein. Kinetic analysis of liposomal fusion showed that annexin I, as in the case of synexin, accelerates aggregation of vesicles but not the actual fusion event per se. The disposition of annexin I in liposomal aggregates was studied by monitoring binding of the protein with a pyrene-phospholipid and by simultaneously monitoring vesicular aggregation by turbidity. An antibody to the N-terminus of annexin I inhibited vesicular aggregation but not binding, suggesting that initial binding of annexin I is similar to that of annexin V. A relatively small proportion of the bound annexin was involved in intervesicular linkage, and no exchange of bound annexin to subsequently added vesicles was observed. The lack of extensive contact between lipids of aggregated vesicles was supported by a lack of energy transfer between phospholipid probes on separate aggregating vesicles. Covalent linkage of maleimidyl or photoaffinity phospholipid derivatives with annexin I in vesicular aggregates did not allow complete disaggregation of vesicles by EDTA, suggesting that monomers of annexin I can contact two membranes simultaneously at the point of intervesicular linkage. These data are discussed in terms of possible models for the structure of this site.

The annexins (Geisow et al., 1987) are a class of proteins that bind to phospholipid membranes in a Ca^{2+}-dependent manner and may be involved in intracellular fusion processes such as exocytosis, a hypothesis supported by the ability of many of the annexins to aggregate and mediate fusion of various vesicles (Creutz et al., 1978; Hong et al., 1981, 1982a,b; Meers et al., 1987, 1988a,b; Drust & Creutz, 1988; Ali et al., 1989). Annexin I has recently been shown to mediate Ca^{2+}-dependent fusion of phosphatidylserine liposomes alone (Blackwood & Ernst, 1990) and fusion of phosphatidylserine/phosphatidylethanolamine liposomes with neutrophil plasma membranes (Oshry et al., 1991). Since there are other annexins in whole neutrophil cytosol (Meers et al., 1987; Ernst et al., 1990) as well as potential inhibitors, it is important to establish the function of annexin I in this complex system. Of the annexins, only synexin has been characterized kinetically (Meers et al., 1988a) to determine that it accelerates Ca^{2+}-

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