| | 1.0 μM [$^3$H]Ado influx rate (pmol per s per $10^6$ cells) |
|---|---|
| Control (0.1% vehicle) | 0.31 |
| 100 nM PMA, 0.5 h | 0.31 |
| 2 h | 0.24 |
| 4 h | 0.23 |
| 8 h | 0.14* |
| 16 h | 0.11* |
| 24 h | 0.13* |

**Table 6. Rates of [$^3$H]Ado influx in CEM cells treated with 100 nM PMA for 0.5 to 24 h.** Rates were calculated from progress curves in Fig. 12 (Aii). *Significantly different from control values ($P$<0.05).

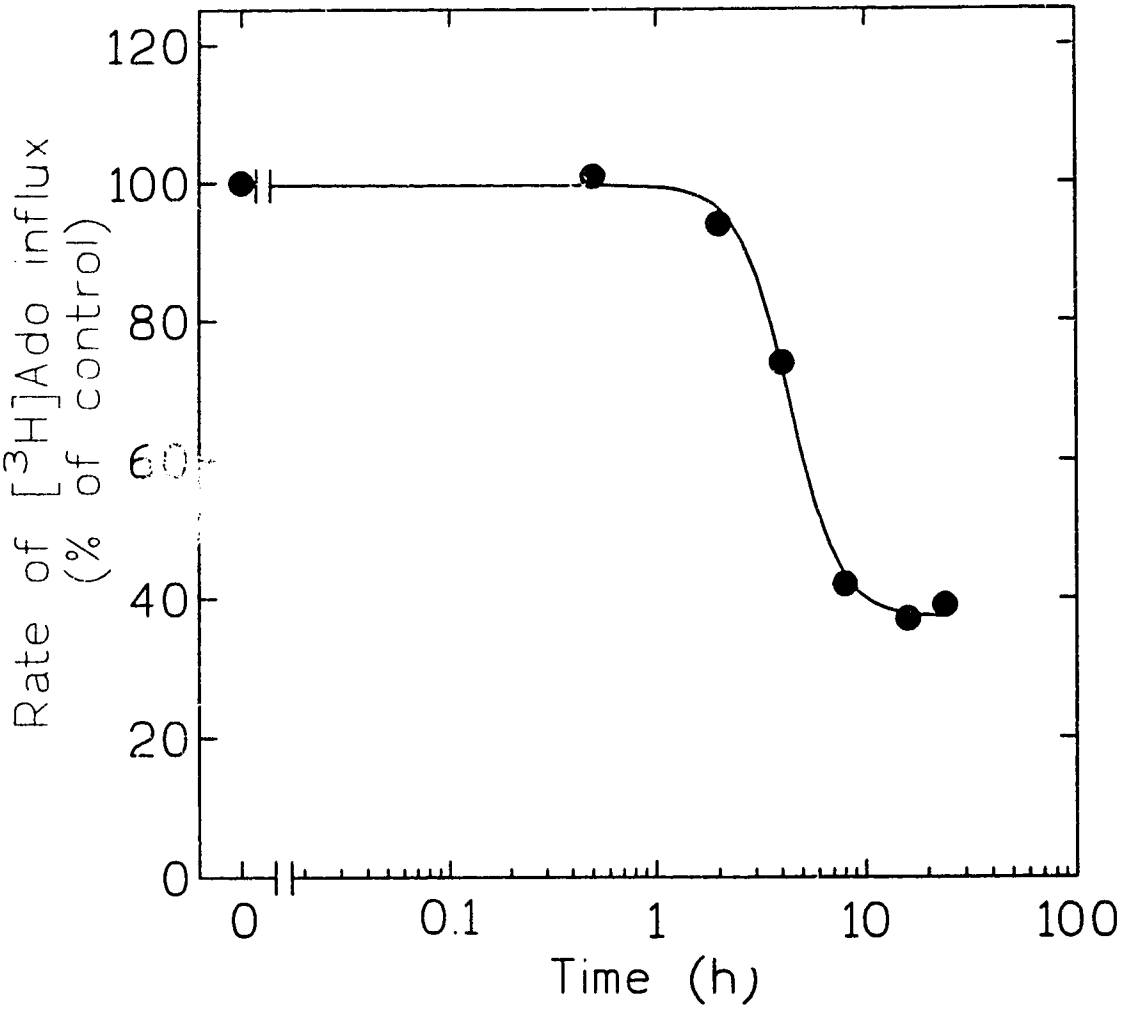


**FIG. 12 (B). The effect of PMA treatment duration on the influx rate of 1 μM [$^3$H]Ado in CEM cells.** Rates of influx of Ado, obtained as the slopes of progress curves in Fig. 12 (Aii), are expressed as percent of rate in control (vehicle-treated) cells, and plotted against duration of PMA treatment. The inward flux of [$^3$H]Ado in control cells was 0.31 pmol per s per $10^6$ cells.

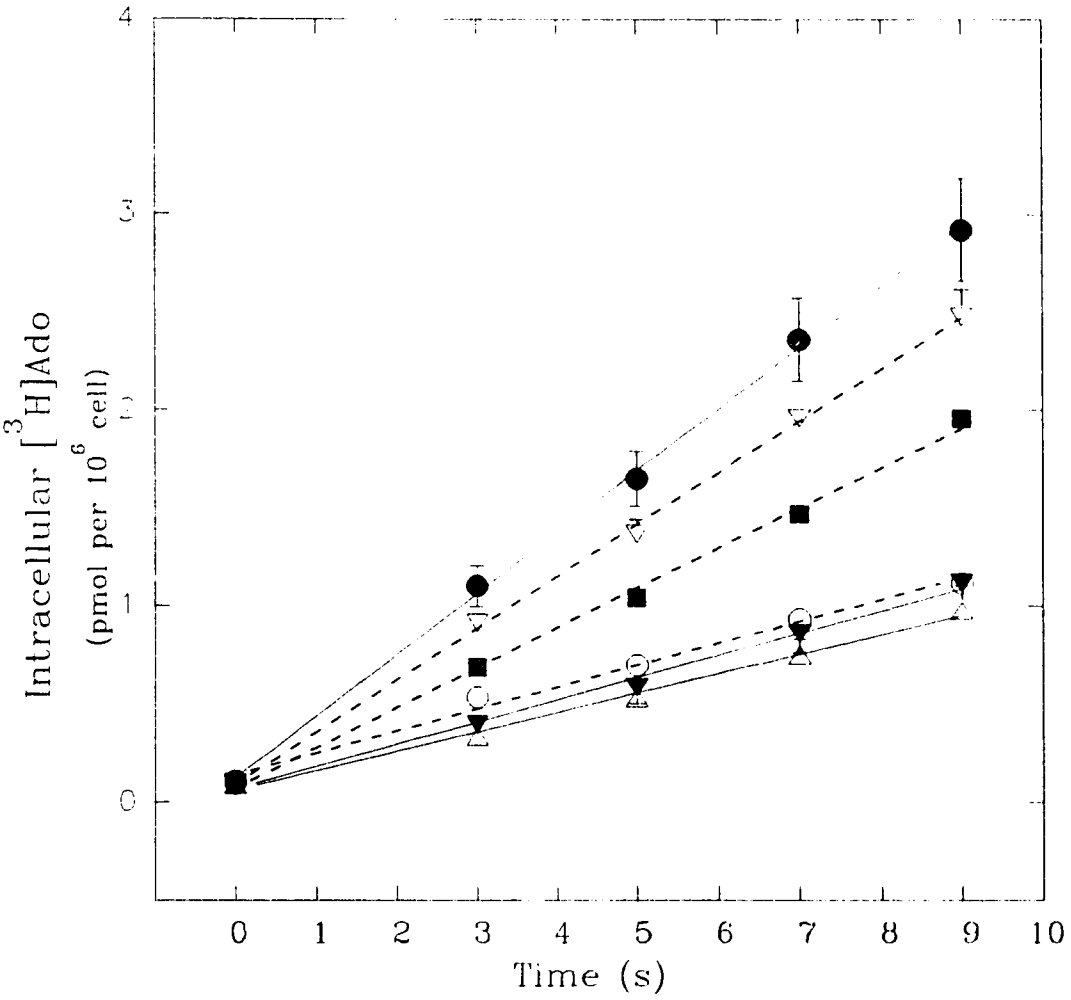


**FIG. 12 (C). Concentration-dependence of PMA effect on the influx of 1 μM [$^3$H]Ado in CEM cells.** Cells were treated for 16 h with 0.1% (v/v) DMSO (vehicle) (●), or 1 (▽), 10 (■), 100 (○), 200 (▼) or 500 nM (△) PMA. Subsequently, cells were washed and resuspended in RPMIt. Uptake of [$^3$H]Ado was measured as described under "Materials and Methods". Each point is the mean ± S.E.M. of 10 separate experiments, performed in triplicate.

| | 1.0 μM [$^3$H]Ado influx rate (pmol per s per $10^6$ cells) |
|---|---|
| Control (0.1% vehicle) | 0.31 |
| [PMA] (nM) 1 | 0.28 |
| 10 | 0.21 |
| 100 | 0.13* |
| 200 | 0.12* |
| 500 | 0.10* |

**Table 7. Rates of [$^3$H]Ado influx in CEM cells treated for 16 h with 1-500 nM PMA.** Rates were calculated from progress curves in Fig. 12 (C). *Significantly different from control values ($P$<0.05).

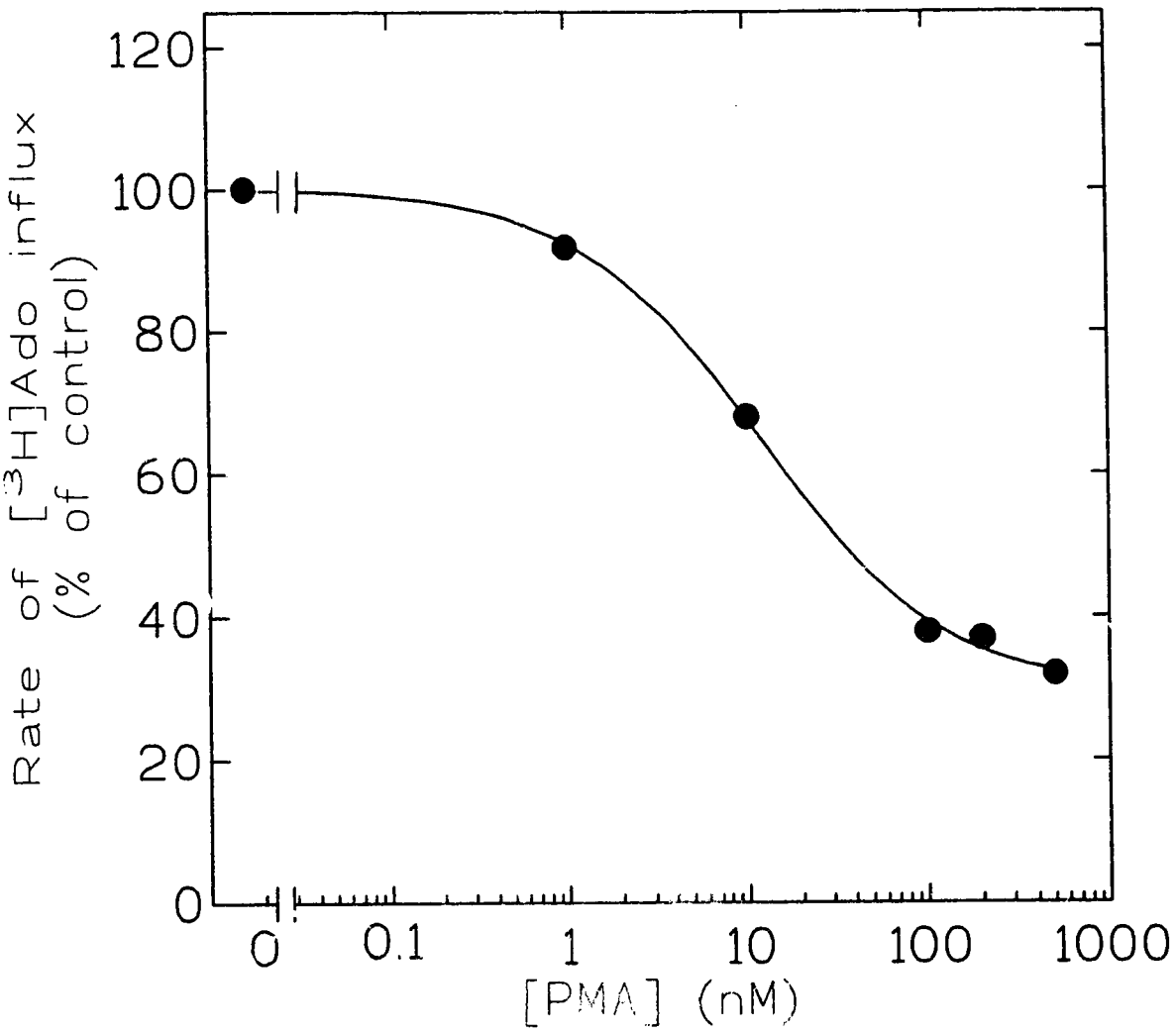


**FIG. 12 (D). The effect of increasing concentrations of PMA on the rate of 1 μM [$^3$H]Ado influx in CEM cells.** Rates of influx obtained as the slopes of progress curves in Fig. 12 (C), are expressed as percent of rate in control (vehicle-treated) cells and plotted against concentration of PMA. The inward flux of [$^3$H]Ado in control cells was 0.31 pmol per s per $10^6$ cells.

This is demonstrated as the nearly horizontal portion of the curve in Fig. 12D, at concentrations of PMA that were 100 nM and higher.

### 3.3.6. Effect of treatment of CEM cells with STA on inward fluxes of Ado

Uptake of 1 μM [$^3$H]Ado was measured in cells treated with STA to determine whether reduction of $B_{max}$ for NBMPR binding in STA-treated cells was associated with changes in nucleoside transport activity. Results in Fig. 13A show that rate of Ado influx in STA-treated cells was reduced in a treatment time-dependent manner. Rates of influx in control (vehicle-treated) and in STA-treated cells are shown in Table 8. The effect of treatment of cells with STA was significant after a prolonged (8 h or more) of incubation of cells with the PKC inhibitor. When the data of Fig. 13A are presented as percent of Ado influx rate in control cells and plotted against treatment period, the length of time of treatment of cells with STA that caused 50% reduction in rate of Ado influx was determined to be 5 h (Fig. 13B). Also obvious from the graph in Fig. 13B is the fact that rate of Ado influx in STA-treated cells was reduced to only about 40% of rate in control cells by the 24 h treatment period.

Preincubation of CEM cells with STA also resulted in concentration-dependent decrease in the rate of Ado influx into STA-treated cells (Fig. 13C). The rates of influx are represented in Table 9. The decline in rates of Ado influx occurred following incubation of cells with STA at concentrations that are known to inhibit PKC suggesting that a STA-PKC interaction underlies the decline in NT activity observed in STA-treated cells. In Fig. 13D, the data of Fig. 13C have been plotted as Ado influx rates (percent of control) against concentration of STA. The rate of Ado influx continued to decline to 20% of value in control (vehicle-treated) cells at STA concentration of 400 nM (Fig. 13D).

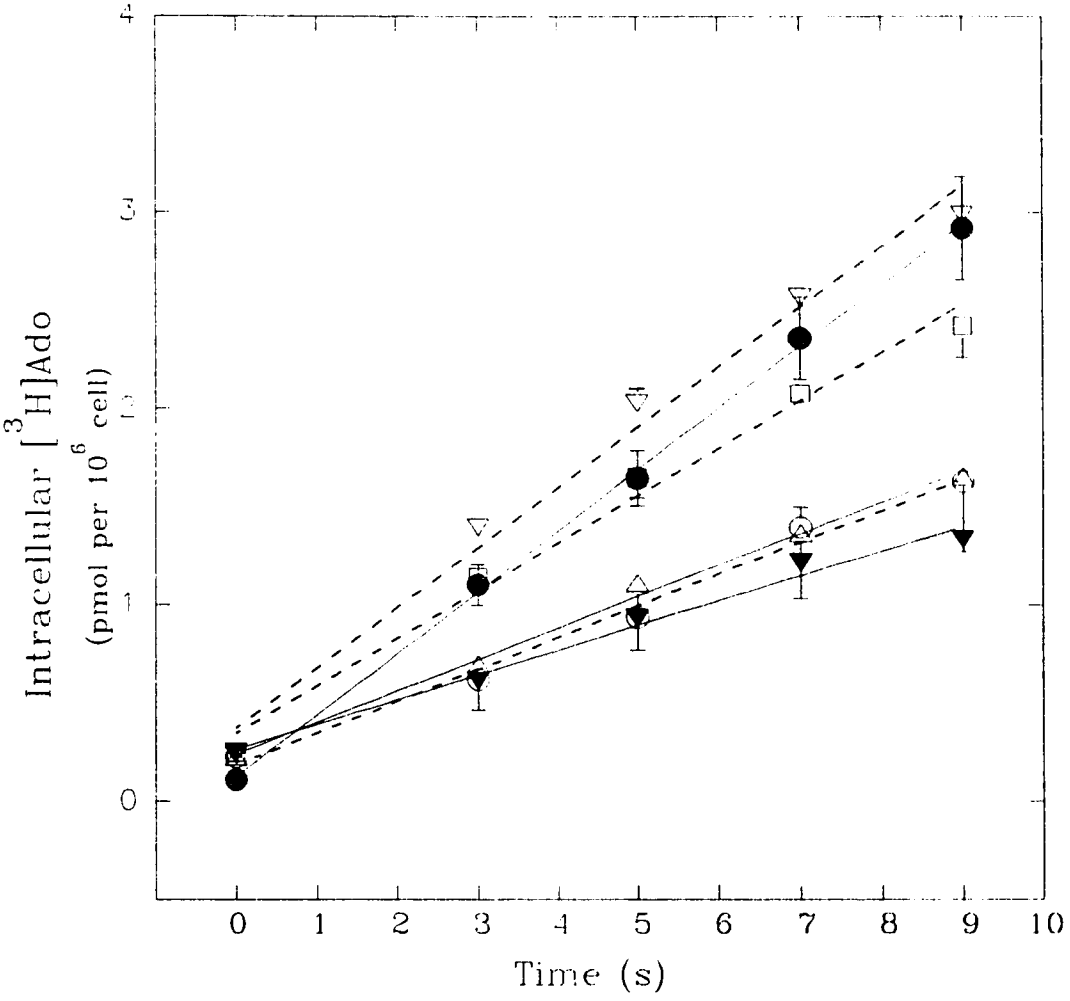


**FIG. 13 (A). Time-dependence of STA effect on the influx of 1 μM [$^3$H]Ado in CEM cells.** Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), or 100 nM STA for 0.5 (▽), 4 (□), 8 (○), 16 (△) or 24 h (▼). After these times, cells were washed and resuspended in RPMIt. Uptake of [$^3$H]Ado was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 10 separate experiments, each performed in triplicate.

| | 1.0 μM [$^3$H]Ado influx rate (pmol per s per $10^6$ cells) |
|---|---|
| Control (0.1% vehicle) | 0.31 |
| 100 nM STA, 0.5 h | 0.30 |
| 4 h | 0.25 |
| 8 h | 0.16* |
| 16 h | 0.15* |
| 24 h | 0.11* |

**Table 8. Rates of [$^3$H]Ado influx in CEM cells treated with 100 nM STA for 0.5 to 24 h.** Rates were calculated from progress curves in Fig. 13 (A). *Significantly different from control values ($P$<0.05).

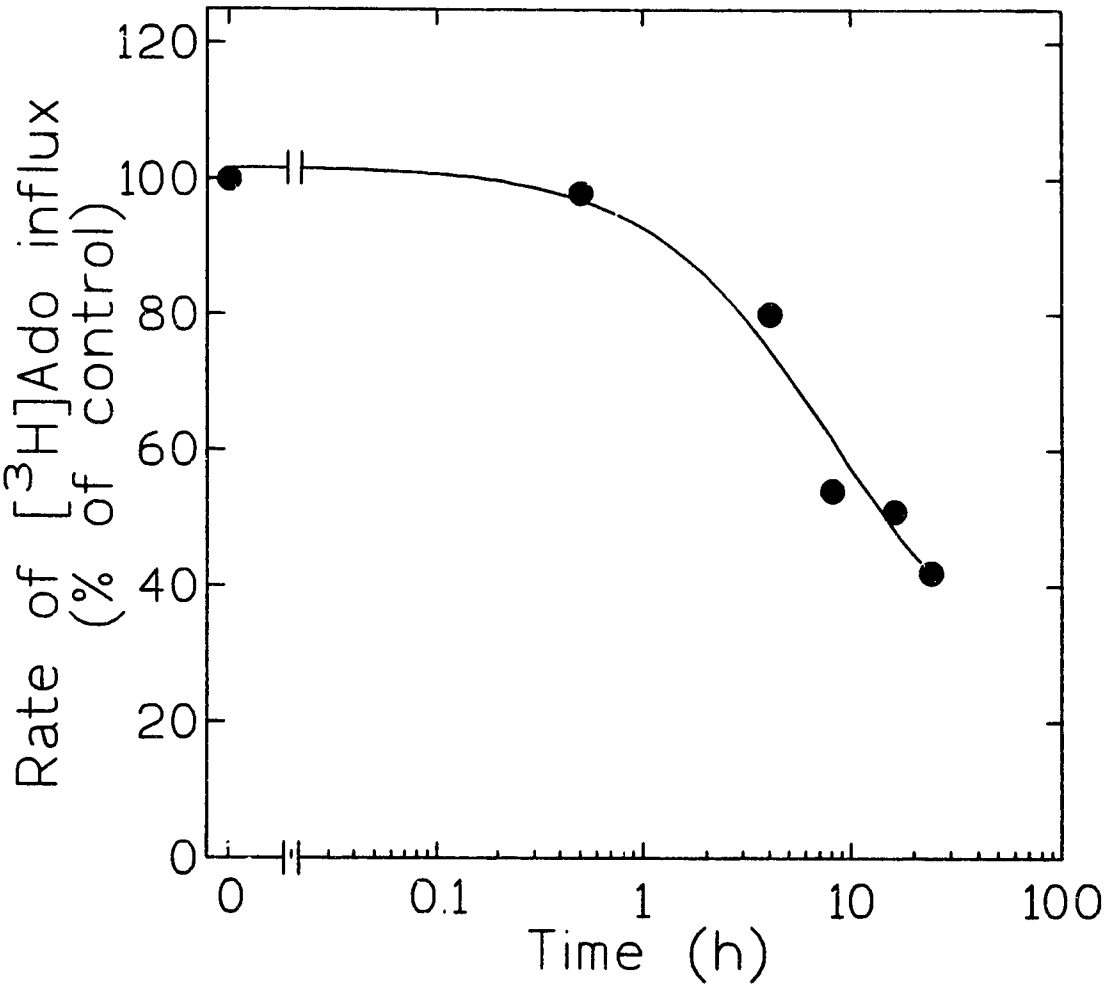


**FIG. 13 (B). The effect of STA treatment duration on the influx of 1 μM [$^3$H]Ado in CEM cells.** Rates of influx obtained as the slopes from progress curves in Fig. 13 (A), are expressed as percent of rate in control (vehicle-treated) cells, and plotted against duration of STA treatment. The inward flux in control cells was 0.31 pmol per s per $10^6$ cells.

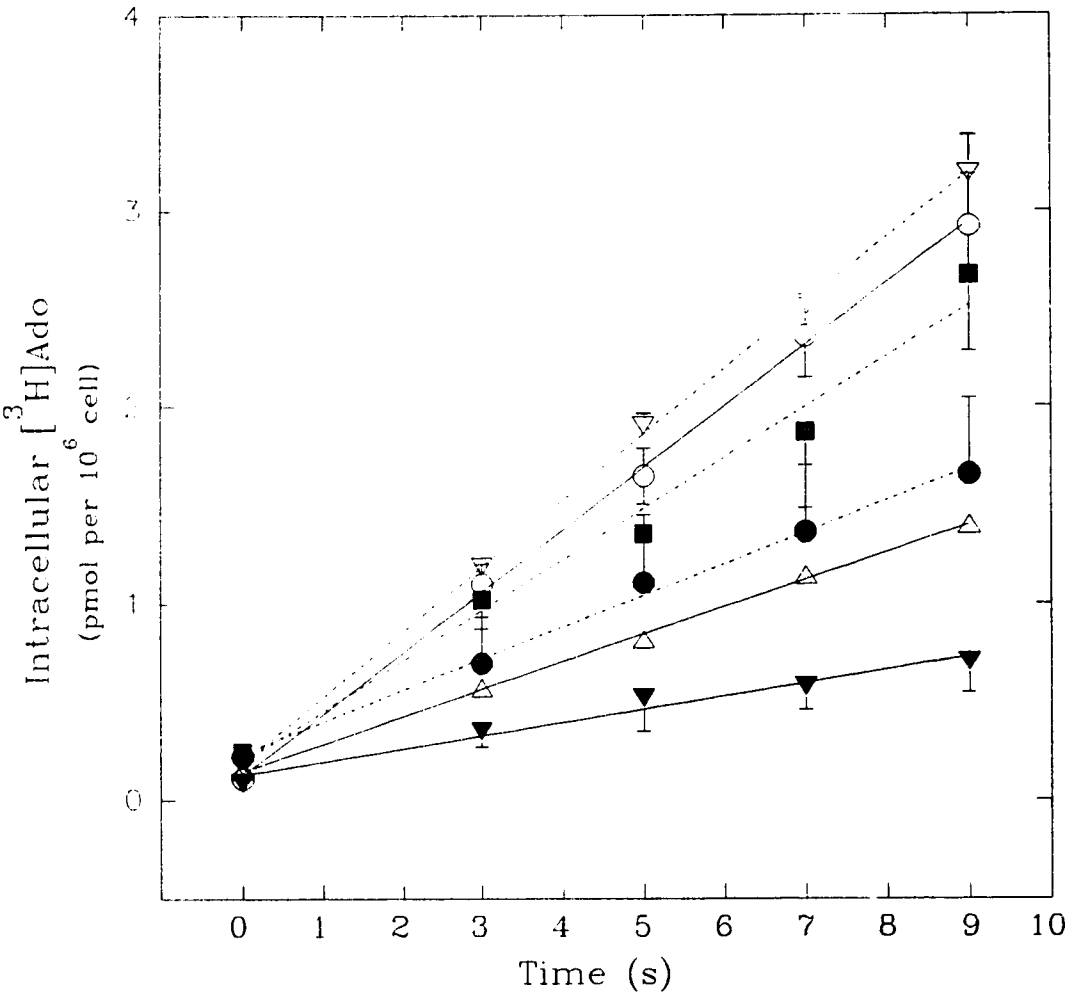


**FIG. 13 (C). Concentration-dependence of STA effect on the influx of 1 μM [$^3$H]Ado in CEM cells.** Cells were treated for 16 h with 0.1% (v/v) DMSO (vehicle) (O), 1 (∇), 10 (■), 100 (●), 200 (Δ) or 400 nM (▼) STA. Subsequently, cells were washed and resuspended in RPMIt. Uptake of [$^3$H]Ado was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 10 separate experiments, each performed in triplicate.

| | 1.0 μM [$^3$H]Ado influx rate (pmol per s per $10^6$ cells) |
|---|---|
| Control (0.1% vehicle) | 0.31 |
| [STA] (nM) 1 | 0.32 |
| 10 | 0.26 |
| 100 | 0.15* |
| 200 | 0.13* |
| 400 | 0.07* |

**Table 9. Rates of [$^3$H]Ado influx in CEM cells treated for 16 h with 10-400 nM STA.** Rates were calculated from progress curves in Fig. 13 (C). *Significantly different from control values ($P$<0.05).

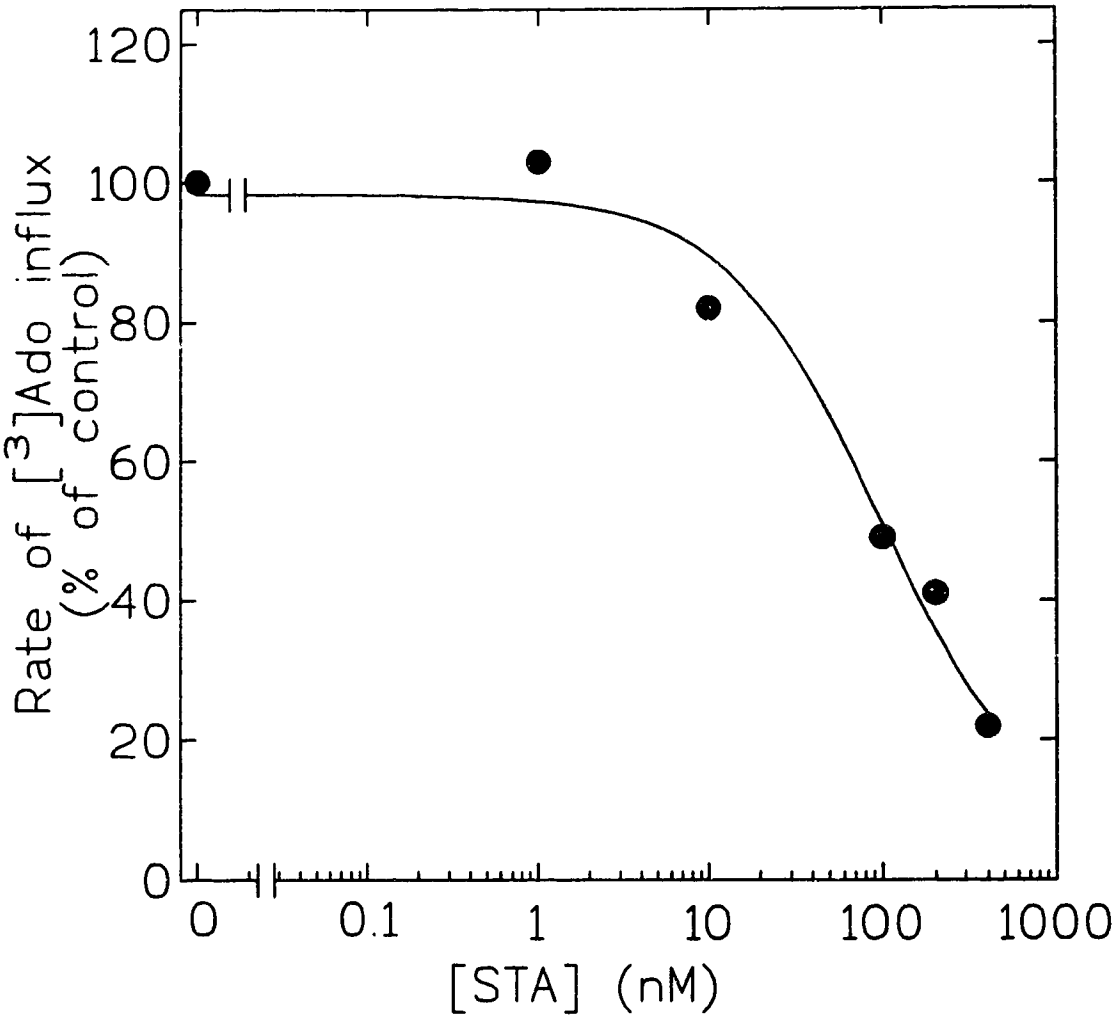


**FIG. 13 (D). The effect of increasing concentration of STA on the rate of influx of 1 μM [$^3$H]Ado in CEM cells.** Rates of influx of [$^3$H]Ado obtained as slopes of progress curves in Fig. 13 (C), are expressed as percent of rate in control (vehicle-treated) cells, and plotted against concentration of STA. The inward flux in control cells was 0.31 pmol per s per $10^6$ cells.

### 3.3.7. Effect of PMA and STA on $V_{max}$ and $K_m$ of ado influx

In studies so far described on the effects of PMA or STA treatment of cells, on the rate of Ado influx, a single concentration of Ado (1 0 μM) was measured The scope of the study was therefore broadened to assess the effects of treatment with PMA or STA on the kinetic constants for Ado influx, $K_m$ and $V_{max}$. We were particularly interested in determining whether the effects of PMA- and STA-treatment on the $V_{max}$ for Ado influx were similar to their effects on $B_{max}$ for NBMPR binding.

Rates of Ado influx were measured at graded [$^3$H]Ado concentrations from 1 to 500 μM. Fluxes were measured in vehicle-treated cells (control), and 16-h, 100 nM PMA- or STA-treated cells. Inward fluxes were calculated as the slopes of lines fitted to data by linear regression, to data that were initial rates of [$^3$H]Ado uptake. Direct plots of rates of Ado uptake against concentration of Ado in control cells, PMA- or STA-treated cells are represented in Fig. 14A. The concentration-dependence of Ado influx was hyperbolic, suggesting that the transport mechanism conformed to Michaelis-Menten kinetics. Incubation of cells for 16 h with 100 nM each of STA or PMA, resulted in a decline in the $V_{max}$, with incubation of cells in the presence of PMA causing a greater reduction than that caused by STA.

The data of Fig. 14A were transformed and replotted in the form of a Hanes plot (Fig. 14B). Values of $V_{max}$ (determined as the inverse of the slope) were 15.2 pmol/$10^6$ cells/s, 11.9 pmol/$10^6$ cells/s, and 8.7 pmol/$10^6$ cells/s for DMSO, vehicle-treated cells (control), 16-h, 100 nM STA-treated, and 16-h, 100 nM PMA-treated CEM cells respectively. $K_m$ values determined as the x-intercept, were 88.2, 97.3, 91.5 μM respectively for control cells, STA- and PMA treated cells. These observation are consistent with the findings in studies (above) of equilibrium binding of NBMPR in drug-treated cells, in which preincubation of cells with STA or PMA decreased $B_{max}$ for NBMPR binding without significantly affecting the equilibrium dissociation constant, $K_D$.

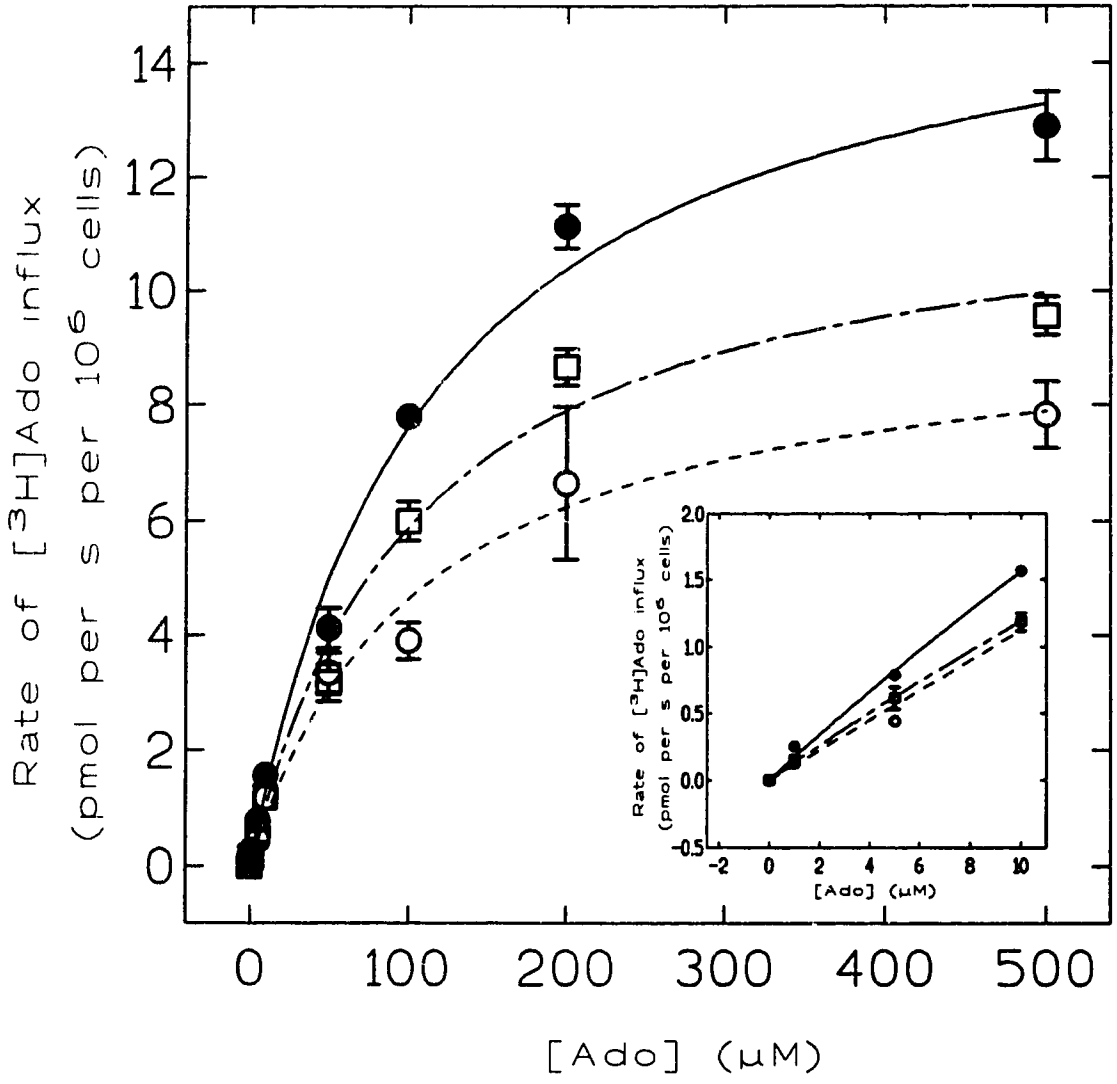


**FIG. 14 (A). Effect of treatment of cells with PMA or STA on the kinetic constants of [$^3$H]Ado influx in CEM cells.** Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), or 100 nM PMA (O) or STA (□), for 16 h. Subsequently, cells were washed and resuspended in RPMIt for measurements of uptake of [$^3$H]Ado. For each concentration of Ado of 1 to 500 μM, fluxes were obtained as the slopes of progress curves that describe [$^3$H]Ado uptake from 3 to 7 s, as described in "Materials and Methods". Each point is the mean ± S.E.M. of 4 separate experiments, each performed in triplicate. Inset is the expanded form of the plot of rate of [$^3$H]Ado influx against [Ado] for concentrations from 0 to 10 μM.

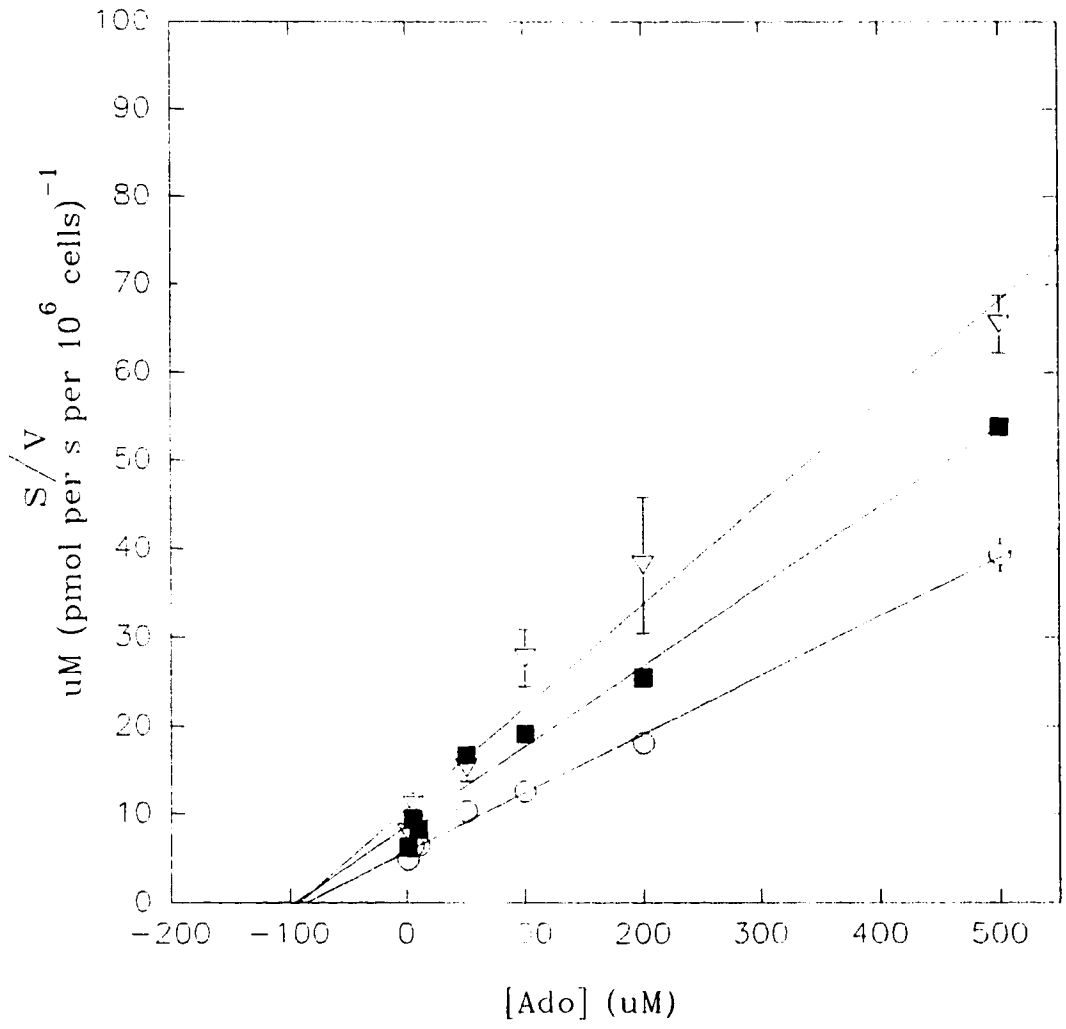


**FIG. 14 (B). Hanes plot of the effect of treatment of CEM cells with PMA or STA on $V_{max}$ and $K_m$ of [$^3$H]Ado influx.** Data from Fig. 14 (A), which represent rates of [$^3$H]Ado influx in cells treated for 16 h with 0.1% (v/v) DMSO (vehicle) (O), 100 nM PMA (∇) or STA (■), are represented as a Hanes plot. The $V_{max}$ and $K_m$ values are determined from the inverse of slopes of the regression lines and the x-intercept respectively.

### 3.3.8. Are the effects of treatment of cells with PMA and STA on equilibrium binding of NBMPR and rate of Ado influx in drug-treated CEM cells reversible following subculture?

Our observations showed that prolonged treatment of cells with PMA or STA reduced $B_{max}$ for NBMPR binding, as well as a decline in rate of Ado influx. With this in mind we decided to study both PMA- and STA-treated cells over a period of several days to determine whether the changes in nucleoside transport activity that followed 16-h treatments of cells with these agents were reversible. In this study, therefore, cells were treated with 0.1% (v/v) DMSO (vehicle), or with 100 nM of PMA or STA for 16 h. After treatments, cells were washed, and measurement of influx of 100 μM [$^3$H]Ado and equilibrium binding of [$^3$H]NBMPR were carried out on portion of the cell population while the remaining cells were resuspended in drug-free medium. After 24 h of subculture, measurements of [$^3$H]Ado influx and equilibrium binding of [$^3$H]NBMPR were repeated and the remaining cells again subcultured in drug-free medium for further 24 h. This pattern was repeated until after 72 or 96 h.

Results in Table 10 indicate, as seen previously, that following 16-hr treatment of cells with 100 nM PMA, both the $B_{max}$ for NBMPR binding and rates of influx of 100 μ M Ado were decreased: $B_{max}$ decreased to 33% of the control value, while rate of influx of 100 μM Ado was reduced to 36% of control. In these and subsequent experiments, 100 μM [$^3$H]Ado was used, a concentration similar to the $K_m$ for Ado influx, to provide a more sensitive measure of the loss of transporter activity. Washout of PMA after 16-h treatment, and subsequent subculture of cells in drug-free medium did not reverse the PMA-induced decline in $B_{max}$ for NBMPR binding or in rate of Ado influx, which remained at about 30% of the control value (Table 10). The $K_D$ for NBMPR binding was not significantly altered.

| | [$^3$H]NBMPR BINDING | | 100 μM Ado influx rate (pmol per s per $10^6$ cells) |
|---|---|---|---|
| | $B_{max}$ (molecules per cell x $10^{-4}$) | $K_D$ (nM) | |
| Control (0.1% vehicle) | 17.6 ± 0.9 | 0.41 | 13.5 ± 0.6 |
| 100 nM PMA, 16 h | 5.8 ± 1.1* | 0.43 | 4.8 ± 1.2* |
| after washout: | | | |
| 24 h | 5.9 ± 1.6* | 0.39 | 3.5 ± 2.1* |
| 48 h | 4.6 ± 1.3* | 0.33 | 3.5 ± 1.9* |
| 72 h | 5.2 ± 0.4* | 0.34 | 3.3 ± 0.2* |
| 96 h | x | | 3.6 ± 0.4* |
| 100 nM STA, 16 h | 6.4 ± 0.9* | 0.42 | 5.0 ± 0.8* |
| after washout: | | | |
| 24 h | 6.0 ± 0.5* | 0.38 | 4.3 ± 1.0* |
| 48 h | 5.4 ± 0.5* | 0.39 | 2.8 ± 1.2* |
| 72 h | 5.3 ± 0.7* | 0.43 | 4.5 ± 0.9* |
| 96 h | x | | 3.5 ± 0.7* |

**Table 10. Levels of $B_{max}$ for [$^3$H]NBMPR binding and rates of [$^3$H]Ado influx in PMA- or STA-treated CEM cells.** Cells were treated with 100 nM of PMA or STA for 16 h. Subsequently, cells were washed and portions of the suspension were used for measurements of equilibrium binding of NBMPR as well as Ado influx rates. The remaining cells were resuspended in fresh culture medium at 1 x $10^5$/ml, and subcultured over 3-4 days. On each day up to day 4, portions of the cell culture were harvested for measurements of Ado influx rate and equilibrium binding of NBMPR. Values are the means ± S.E.M. of two separate experiments performed in triplicates. *Significantly different from control values ($P$<0.05). x: not determined.

A similar trend was observed in STA-treated cells after treatment and following STA washout, resuspension and subculture in drug-free medium. After a 16 hr incubation of cells with 100 nM STA, $B_{max}$ for NBMPR binding was reduced to 36% of control while influx rate of 100 μM Ado was reduced to 37% of control. Again, $K_D$ was not significantly altered (Table 10). After washout of STA, drug-induced decline in $B_{max}$ for NBMPR binding and rates of Ado influx did not return to control values, but remained at about 30% of control values. These results shows that the effects of PMA and STA on nucleoside transporters persisted for at least 3 days after drug removal, suggesting that a long-lasting change in binding sites numbers as well as transporter activity had occurred.

### 3.3.9. Effect of treatment of CEM cells with other agents on rate of ado influx

The involvement of PKC in the STA-mediated decrease in Ado influx rate was addressed, since STA is not specific for inhibition of PKC (74). Ado influx rates were measured in cells pretreated for 16 h with HA1004, a PKA and cGMP-dependent protein kinase inhibitor, genistein, a tyrosine kinase inhibitor, and H7, another non-specific PKC inhibitor. Results in Fig. 15A show that the rate of influx of 100 μM Ado in cells pretreated with HA1004 or genistein did not differ from the rate obtained in vehicle-treated cells (control) (Fig. 15A). Rates of Ado influx in control cells and in cells treated with HA1004 or genistein were 13.5, 13.8 and 14.4 pmol per s per $10^6$ cells respectively. In contrast, rates were significantly reduced in cells treated with STA or H7, from the control rate of 13.5 to 5.0 and 3.8 pmol per s per $10^6$ cells respectively (Figs. 15A and B). These results indicate that STA or H7 did not affect Ado influx through interaction with PKA, cGMP-dependent protein kinase, or tyrosine kinase. Also shown in Figs. 15A and B is the observation that in cells pretreated with 4α-PDD, an inactive phorbol ester, rate of influx of 100 μM Ado did not change significantly, in contrast with cells preincubated for 16 h with 100 nM each of PMA or PDBu, in which rates of influx of 100 μM Ado decreased from control (13.5 pmol per s per $10^6$ cells) rate to 4.8 and 3.5 pmol per s per

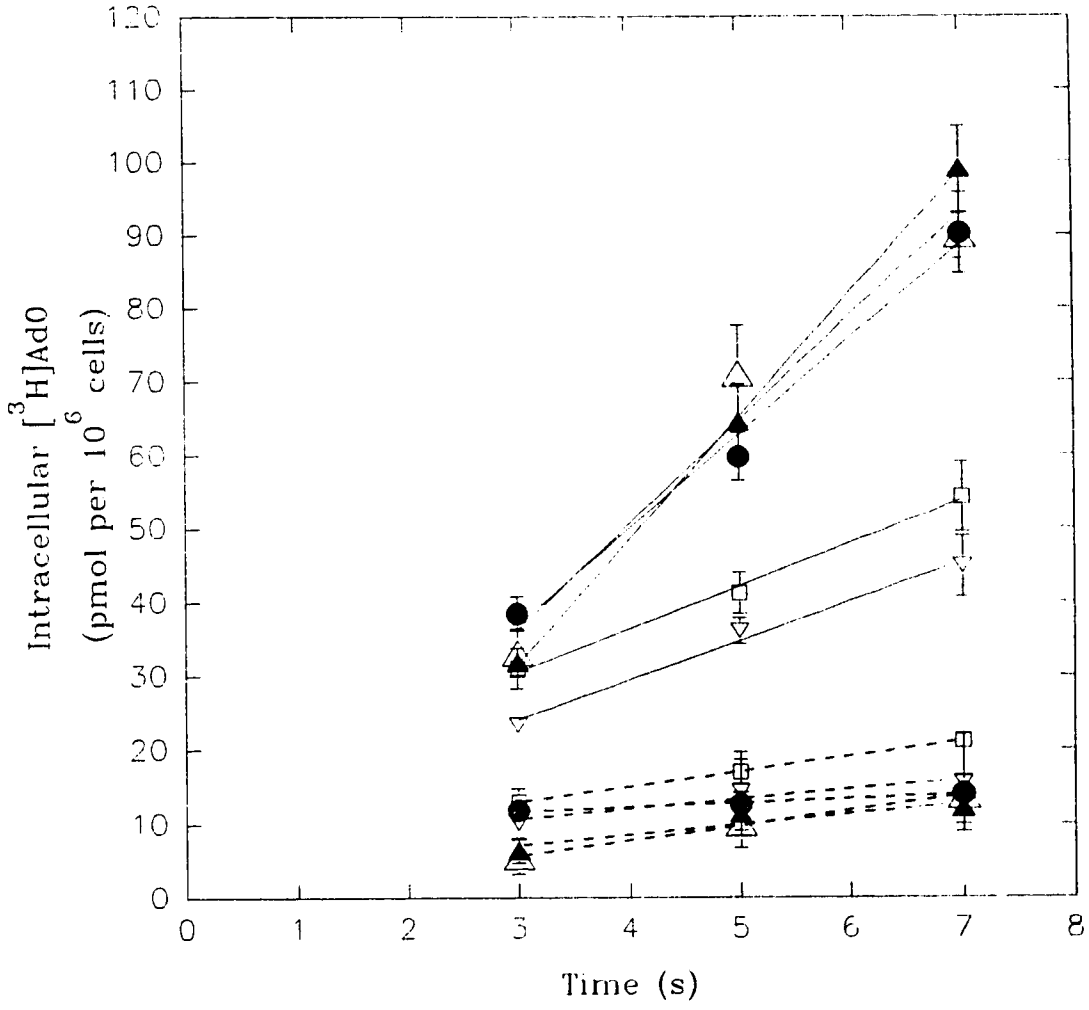


**FIG. 15 (A). Time-course of uptake of 100 μM [$^3$H]Ado in CEM cells treated with various agents.** Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), 40 μM HA1004 (▲), 50 μM genistein (Δ), 100 nM PMA (▽) or 100 nM STA (□) for 16 h. Subsequently, cells were washed and resuspended in RPMIt. Uptake of [$^3$H]Ado (solid lines) was measured as described in "Materials and Methods". Measurements of uptake of [$^3$H]Ado in the presence of 5 μM NBMPR (dashed lines) were also carried out in cells from each group. Each point is the mean ± S.E.M. of 4 separate experiments, each performed in triplicate.

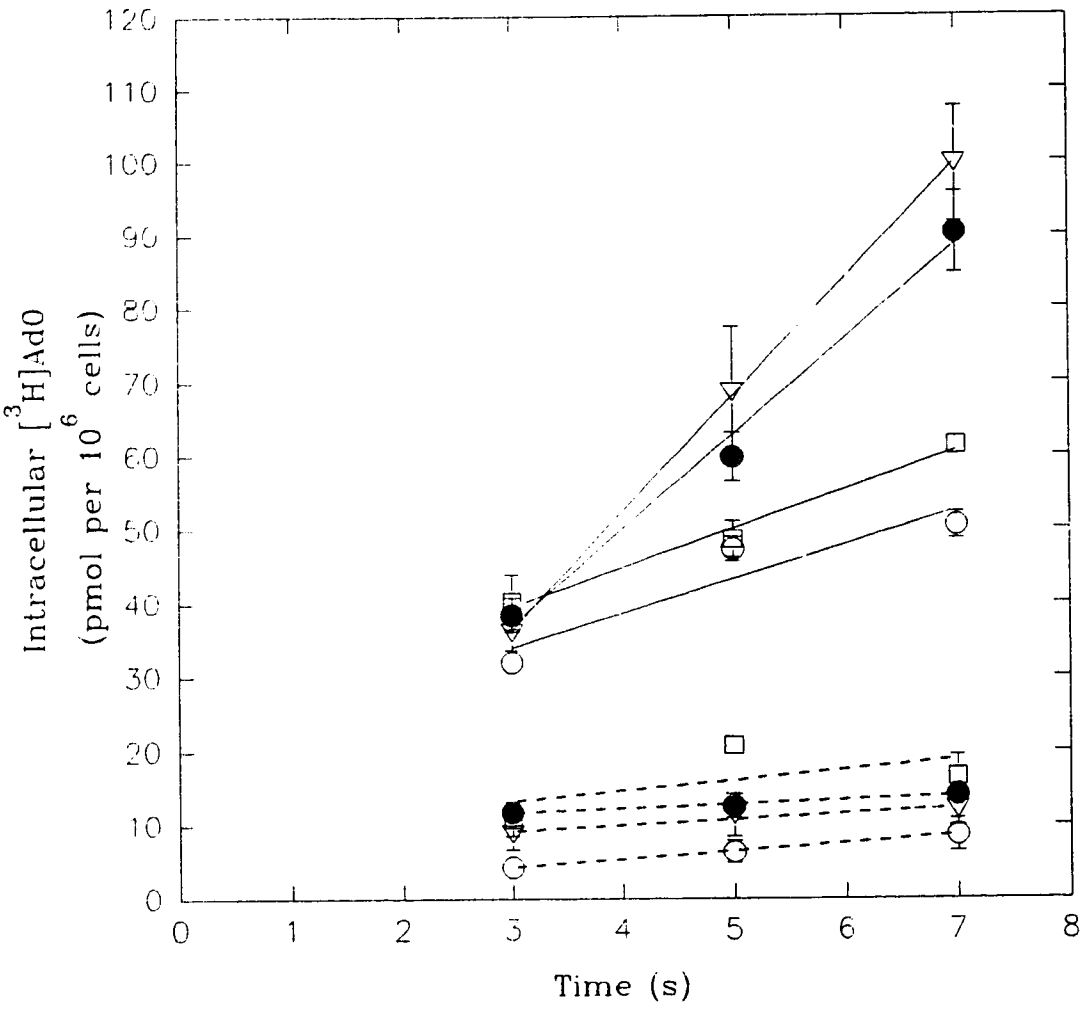


**FIG. 15 (B). Time-course of uptake of 100 μM [$^3$H]Ado in CEM cells treated with various agents.** Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), 100 nM 4α-PDD (▽), 100 nM PDBu (○) or 40 μM H7 (□), for 16 h. Cells were subsequently washed and resuspended in RPMIt. Uptake of [$^3$H]Ado in the presence (dashed lines) or in the absence (solid lines) of 5 μM NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 4 separate experiments, each performed in triplicate.

$10^6$ cells respectively. Time course of [$^3$H] Ado (100μM) uptake into control cells, and into 16-h drug-treated cells in presence of 5 μM NBMPR are also represented in Fig. 15A and B. The reduction in rates of Ado influx to near zero in the presence of NBMPR is in agreement with literature reports that only NBMPR-sensitive facilitated diffusion nucleoside transporters are expressed in CEM cells (102). The data in Fig. 15A and B show that pretreatment of cells with PMA, STA, or other agents did not induce NBMPR-insensitive transporter activity in CEM cells.

In summary, it is clear that prolonged treatment of CEM cells with PMA or with STA, resulted in substantial decline in $B_{max}$ for NBMPR binding and in Ado influx rates. Since prolonged incubation was necessary for PMA effect to be observed, it is not likely that PMA-induced PKC activation is the underlying basis for the changes observed, because PMA-induced PKC activation may be achieved in minutes (40, 90, 91). Besides, prolonged treatment of cells with phorbol ester is known to result in PKC down-regulation (6, 29, 44). Additional support for the idea that PKC down-regulation underlies the observed changes in nucleoside transporter activity is the fact that treatment of cells with STA achieved similar results to those observed following prolonged treatment with PMA, since the effects of down-regulation of PKC and PKC inhibition should be similar.

### 3.3.10. Effect of short treatment of cells with PMA on equilibrium binding of NBMPR and rate of ado influx

The reduction in the NBMPR binding site numbers and nucleoside transporter activity in CEM cells had occurred as a result of prolonged exposure of cells to PMA, and thus suggested that down-regulation of PKC was the basis for the change. In another approach to study the effect of PKC activity changes on nucleoside transporter activity, CEM cells were exposed to PMA for short times (minutes) with the aim of activating PKC, and subsequently, the number of high affinity NBMPR binding sites and transporter activity were measured. In cells treated with 100 nM PMA for 10 min, the $B_{max}$ for

NBMPR binding was not significantly different from that obtained in control (vehicle-treated) cells (Fig. 16A). Values of $B_{max}$ in control (vehicle-) cells and in cells treated with 100 nM PMA for 10 min are 17.4 x $10^4$ and 17.0 x $10^4$ molecules/cell respectively; $K_D$ value was unchanged at 0.39 nM.

The uptake of [$^3$H]Ado measured in vehicle-treated cells and in cells treated with 100 nM PMA, for 5 or 10 min were not significantly different. Rates of Ado influx in control cells were 12.7 pmol per s per $10^6$ cells, while rates measured in cells treated for 5 or 10 min, with 100 nM PMA were 12.9 and 13.1 pmol per s per $10^6$ cells respectively. Also shown is the uptake of [$^3$H]Ado control cells and PMA-treated cells in presence of 5 μM NBMPR which nearly completely blocked the influx of Ado, suggesting that the transporter was still NBMPR-sensitive and also that no inhibitor-insensitive transporter had been induced during treatment with PMA. These results also indicate that a short time of incubation of cells with PMA did not result in significant changes in NT activity.

### 3.3.11. Effect of PMA on NT activity following treatment of cells with STA for 16 h

Another approach to investigate the role of PKC in NT activity was based on the idea that cells treated for 16 h with STA would lose PKC activity, and that such cells, if exposed to PMA for a short period of time, might regain normal levels of PKC activity, and might show a reversal of the STA-mediated decrease in transporter activity. The rate of 100 μM [$^3$H]Ado influx following treatment of cells with 100 nM STA for 16 h, and subsequently with 100 nM PMA for 10 min, was 3.1 which was not significantly different from the rate measured in 16-h, 100 nM STA-treated cells, which were subsequently treated with 0.1% (v/v) DMSO (vehicle) of 1.8 pmol per s per $10^6$ (Fig. 17). Both values of the rates were significantly different compared with that obtained in control (vehicle-treated) cells, of 13.8 pmol per s per $10^6$ cells. The rates of Ado influx measured in the presence of 5 μM NBMPR, showed that after treatment of cells with PMA or STA, the transporter was still sensitive to inhibition by NBMPR.

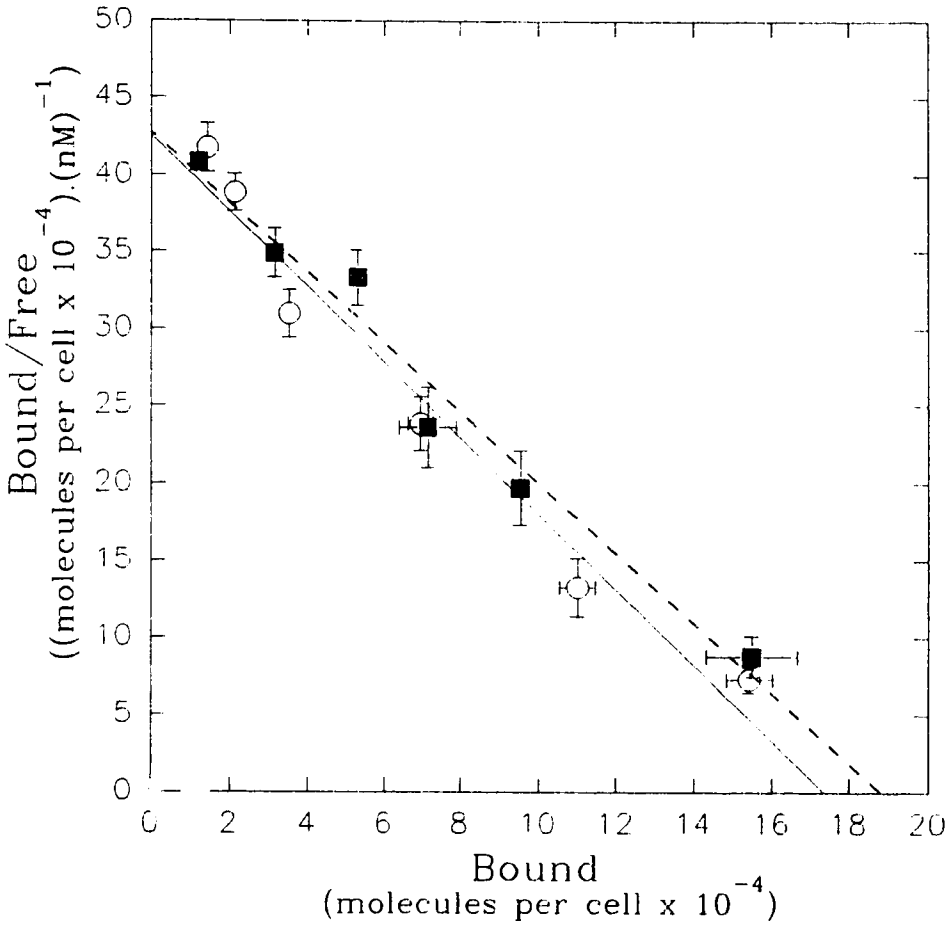


**FIG. 16 (A). Scatchard analysis of the effect of short treatment duration with PMA on equilibrium binding of [$^3$H]NBMPR in CEM cells.** Cells were treated with 0.1% (v/v) DMSO (vehicle) (O) or 100 nM PMA (■) for 10 min. Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [$^3$H]NBMPR was measured as described in "Materials and Methods". Results are from at least 3 separate experiments, each performed in triplicate.

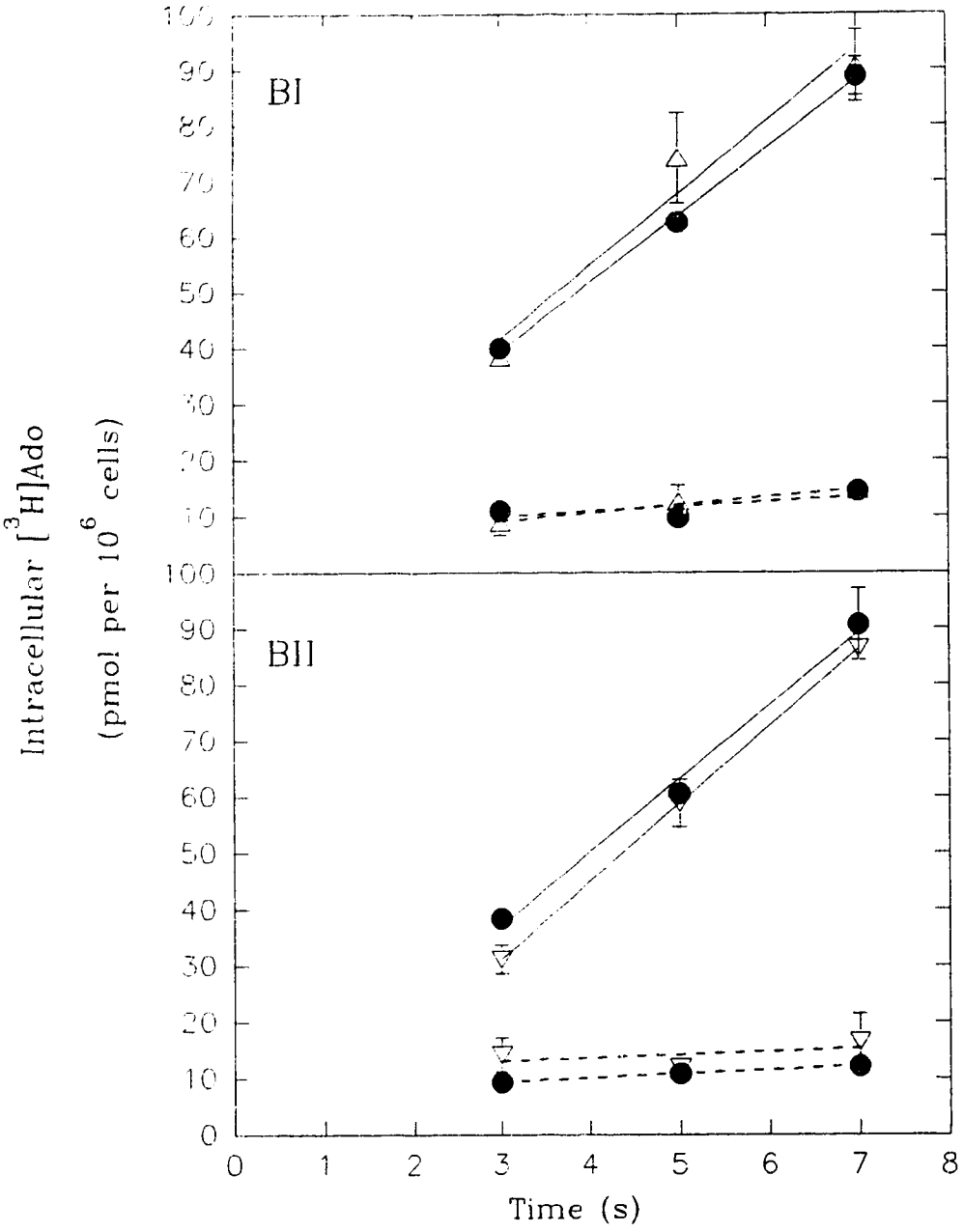


**FIG. 16 (B). Effect of short treatment duration with PMA on the influx of 100 μM [$^3$H]Ado influx in CEM cells.** Cells were treated with 0.1% (v/v) DMSO (vehicle) (●), or 100 nM PMA for 5 min (panel B[I]) (Δ), or 10 min (panel B[II]) (∇) prior to measurements of uptake of [$^3$H]Ado in the presence (dashed lines) or in the absence (solid lines) of 5 μM NBMPR. Each point is the mean ± S.E.M. of 4 separate experiments, each performed in triplicate.

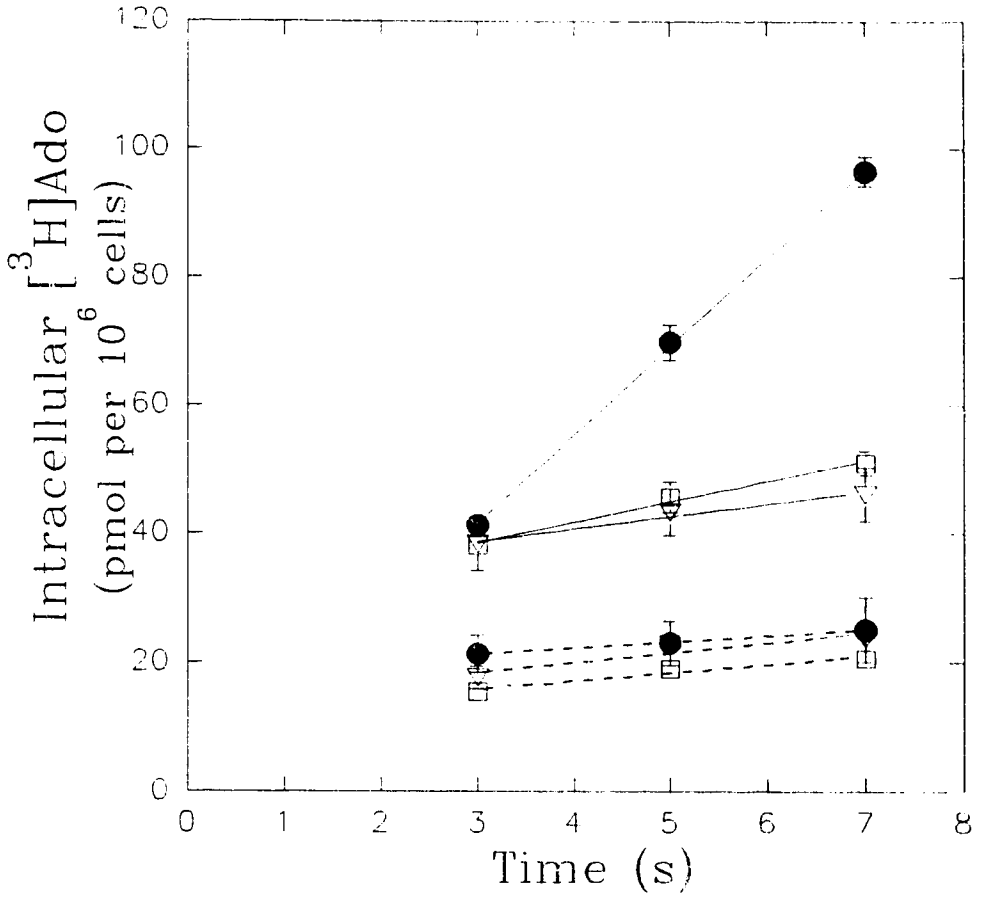


**FIG. 17. Effect of PMA on the time-course of uptake of 100 μM[$^3$H]Ado in STA-treated CEM cells.** Cells were treated with 100 nM STA for 16 h prior to treatment with 0.1% (v/v) DMSO (vehicle) (□) or 100 nM PMA for 10 min (▽). For another control, cells were treated with 0.1% (v/v) DMSO (vehicle) for 16 h prior to a second treatment with vehicle for 10 min (●). Following these treatments, cells were washed, resuspended in fresh growth media and allowed to rest at 37°C for 2 h. Subsequently, cells were washed and resuspended in RPMIt and measurements of uptake of 100 μM [$^3$H]Ado in the presence (dashed lines) and in the absence (solid lines) of 5 μM NBMPR were carried out as described in "Materials and Methods". Each point is the mean ± S.E.M. of 3 separate experiments, each performed in triplicate.

In studies so far, it was observed that modulation of PKC resulted in reduction in $B_{max}$ of NBMPR binding and in Ado influx rate. These changes were evident only when PKC was inhibited or down-regulated. This means that for the regulation of the number high affinity NBMPR binding sites and nucleoside transporter activity, PKC activity is required. Furthermore, conditions that were capable of activating PKC did not result in increased $B_{max}$ of NBMPR binding or Ado influx rate. Therefore, NT activity and binding sites numbers for NBMPR on transporter protein may be at the maximum in CEM cells when basal PKC activity is unperturbed.

## 3.4. EFFECT OF OKADAIC ACID (OKA) AND CALYCULIN A (CLA) ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND INWARD FLUXES OF ADO IN CEM CELLS

The results so far indicated that a decrease in PKC activity was the cause for the reduction in number of high affinity NBMPR binding sites and nucleoside transporter activity. Thus it was reasoned that PKC-mediated phosphorylation steps may be required in the mechanism of maintaining the highest level of nucleoside transporter. Since cycles of phosphorylation and dephosphorylation are known to regulate the activity of some cellular enzymes, it was thought that inhibition of the dephosphorylation step may provide some further insights on the dependence of NT activity on PKC-mediated phosphorylation. Therefore experiments were conducted using protein phosphatase inhibitors that would block dephosphorylation steps. Effects of treatment of cells with protein phosphatase inhibitors okadaic acid (OKA) and calyculin A (CLA), on the equilibrium binding of NBMPR and rate of Ado influx were measured. Results in Fig. 18A show that treatment with OKA alone did not change $B_{max}$ of NBMPR binding. However, in cells treated for 1 h with 100 nM STA, $B_{max}$ was reduced to 7.1 x $10^4$ molecules/cell; in cells treated with both STA and OKA, $B_{max}$ was 12.9 x $10^4$ molecules/cell, which was

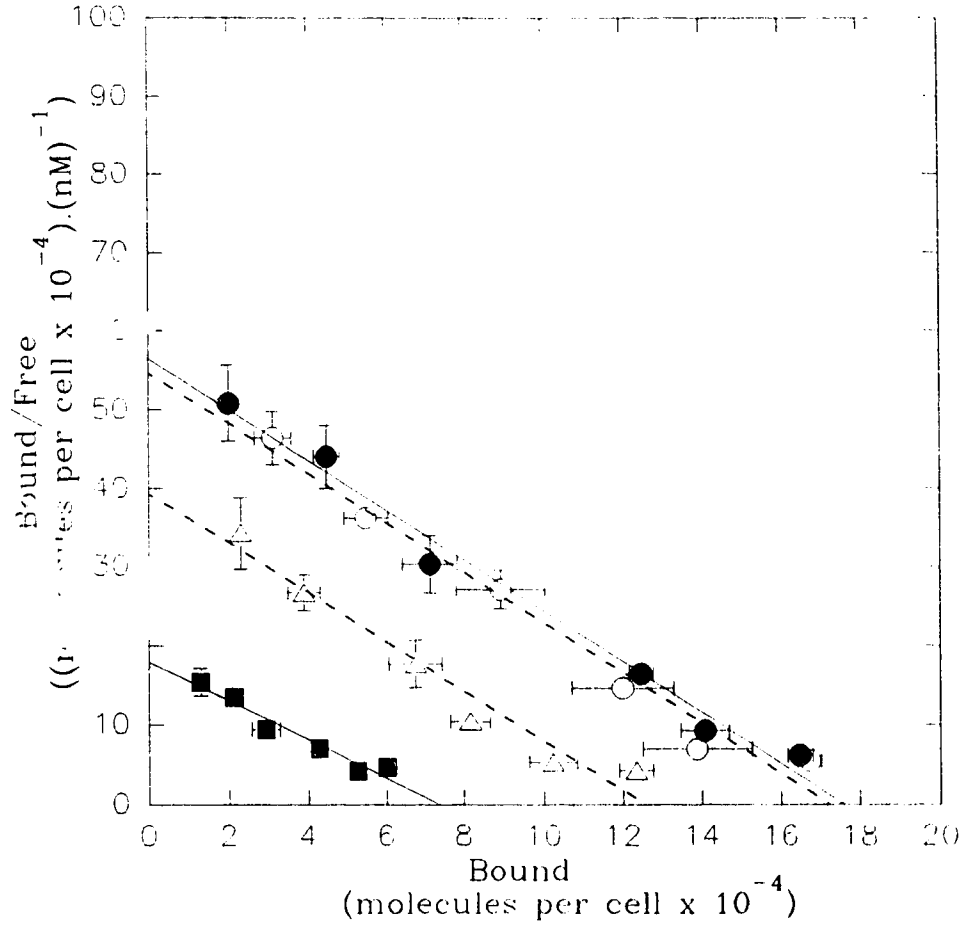


**FIG. 18 (A). Scatchard analysis of the effect of treatment of CEM cells with OKA on the equilibrium binding of [$^3$H]NBMPR.** Cells were treated for 16 h with 0.1 % (v/v) DMSO (vehicle) (●, solid line), 10 nM OKA (O, dashed line), 10 nM OKA plus 100 nM STA (Δ, dashed line) or 100 nM STA (■). Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [$^3$H]NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of at least 5 separate experiments, each performed in triplicate.

cells preincubated for 16 h with both STA (100 nM) and OKA (10 nM), the decrease in $B_{max}$ observed in STA-treated cells was partially blocked by the presence of OKA. In near the control value of 17.4 x $10^4$ molecules/cell (Fig. 18 .).

The observation that OKA alone did not have any effect on $B_{max}$ agrees with the concept that NT may be maximally active at basal PKC activity, and that any further enhancement in the PKC-mediated phosphorylation, or in the level of putative phosphorylated protein (either transporter protein or some other protein), would not result in elevation in NT activity. The latter case would be achieved by inhibition of protein phosphatases. Furthermore, the finding that OKA partially blocked STA-mediated decline in $B_{max}$ of NBMPR binding sites when present in cells in combination with STA, shows that blockade of protein phosphatase-mediated dephosphorylation might keep a putative phosphoprotein at a level that is adequate for maintenance of NBMPR binding sites and NT activity, even though PKC may be inhibited by STA. This also confirms that protein phosphorylation and dephosphorylation are necessary steps in the regulation of NT activity. Similar results were obtained when cells were incubated simultaneously with 100 nM STA and 10 nM CLA for 6 h. In cells treated for 6 h with 10 nM CLA, $B_{max}$ of NBMPR binding was not significantly different from $B_{max}$ value obtained in 0.1% (v/v) ethanol, vehicle-treated cells (16.4 x $10^4$ molecules/cell for cells treated with CLA, and 17.4 x $10^4$ molecules/cell in control cells) (Fig. 18B). However, when cells were treated for 6 h with both CLA and STA (in the same solution), STA-mediated decline in $B_{max}$ of NBMPR binding was not seen. The value of $B_{max}$ in cells treated for 6 h with STA was 10.8 x $10^4$ molecules/cell, while $B_{max}$ in cells simultaneously treated with CLA and STA was 15.3 x $10^4$ molecules/cell (Fig. 18B). The difference in the abilities of OKA and CLA to prevent the STA-induced decline in $B_{max}$ may be due to the fact that CLA is equally potent in inhibiting protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), while OKA is more potent against PP2A than PP1 (180-182). The results of the study

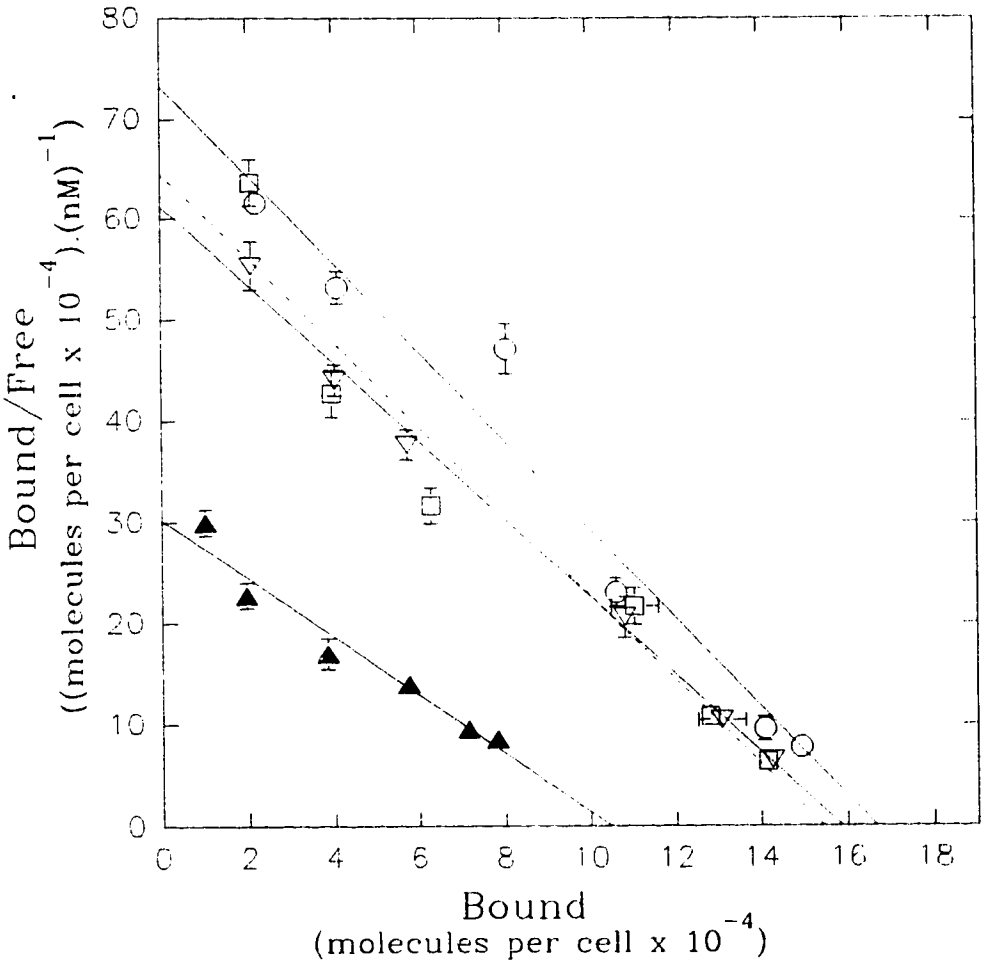


**FIG. 18 (B). Scatchard analysis of the effect of treatment of CEM cells with CLA on the equilibrium binding of [$^3$H]NBMPR.** Cells were treated for 6 h with 0.1% (v/v) ethanol (vehicle) (O), 10 nM CLA (□), 10 nM CLA plus 100 nM STA (▽) or 100 nM STA (▲). Subsequently, cells were washed and resuspended in RPMIt and equilibrium binding of [$^3$H]NBMPR was measured as described in "Materials and Methods". Each point is the mean ± S.E.M. of 3 separate experiments, each performed in triplicate.

involving CLA agree with the study with OKA, and further support the concept that phosphorylation and dephosphorylation are essential steps in the regulation of the cellular abundance of NBMPR binding sites and NT activity.

The effects of the two phosphatase inhibitors on rate of Ado influx in STA-treated cells were also examined. OKA alone did not have significant effect on rate of influx of Ado. Contrary to expectations, however, incubation of cells with both OKA and STA together did not block the STA-induced decrease in rate of Ado influx (Fig. 18C). Rates of influx of Ado in vehicle-treated and in OKA-treated cells, were 12.4 and 12.5 pmol per s per $10^6$ cells respectively. Rates of Ado influx in STA-treated cells, and in cells treated simultaneously with STA and OKA, were 4.18 and 4.17 pmol per s per $10^6$ cells respectively (Fig. 18C). Also shown in Fig 18C is Ado uptake in vehicle-treated cells, and in drug-treated cells, measured in the presence of 5 μM NBMPR, which blocked uptake of Ado, indicating that the transporter remained NBMPR-sensitive.

Results in Fig. 18D also show that CLA alone did not have any effect on rate of Ado influx, however, CLA completely reversed STA-mediated decrease in Ado influx rate. The rate of Ado influx in cells exposed for 6 h to 100 nM STA was 6.3 pmol per s per $10^6$ cells, and this was increased to 11.8 pmol per s per $10^6$ cells in cells simultaneously treated with 10 nM CLA and 100 nM STA. Rate of Ado influx in control cells (0.1% (v/v) ethanol, vehicle-treated cells) was 13.9 pmol per s per $10^6$ cells, while the rate in cells treated for 6 h with 10 nM CLA was 13.7 pmol per s per $10^6$ cells. As was stated before, the differences in the effects of OKA and CLA on rate of Ado influx may be due to the fact that CLA is equally potent against PP1 and PP2A while OKA is more potent against PP2A than PP1 (180-182). Fluxes of Ado measured in vehicle-treated and in CLA-treated cells, in the presence of 5 μM NBMPR showed that the transporter remained NBMPR-sensitive in control cells and in cells treated with CLA and that no inhibitor-insensitive transporters had been induced (Fig. 18D). Rates measured in the presence of NBMPR were minimal.

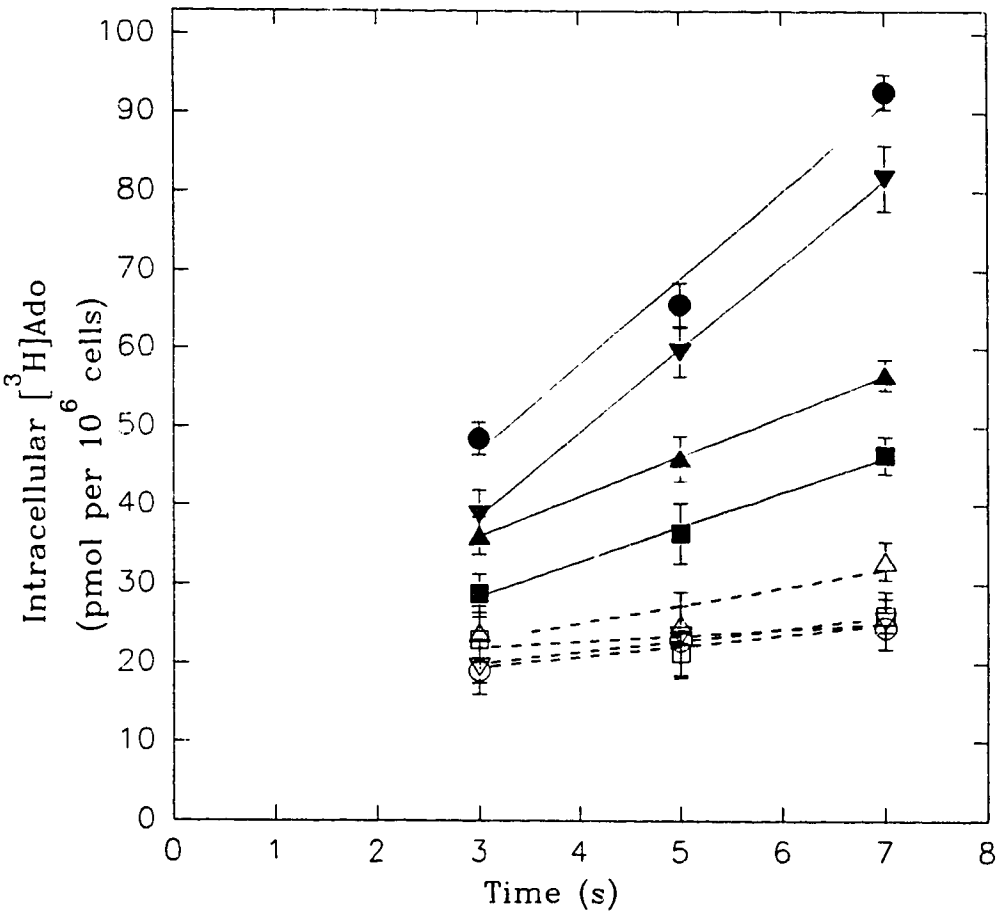


**FIG. 18 (C). Effect of treatment of cells with OKA on time-course of 100 μM [$^3$H]Ado uptake in CEM cells.** Cells were treated with 0.1% (v/v) DMSO (vehicle) (●, O), 10 nM OKA (▼,▽), 10 nM OKA plus 100 nM STA (■,□) or 100 nM STA (▲, △) for 16 h. Subsequently, cells were washed and resuspended in RPMIt and uptake of [$^3$H]Ado was measured as described in "Materials and Methods". Closed and open symbols represent uptake of [$^3$H]Ado measured in the absence and in the presence of 5 μM NBMPR respectively. Each point is the mean ± S.E.M. of 3 separate experiments, each performed in triplicate.

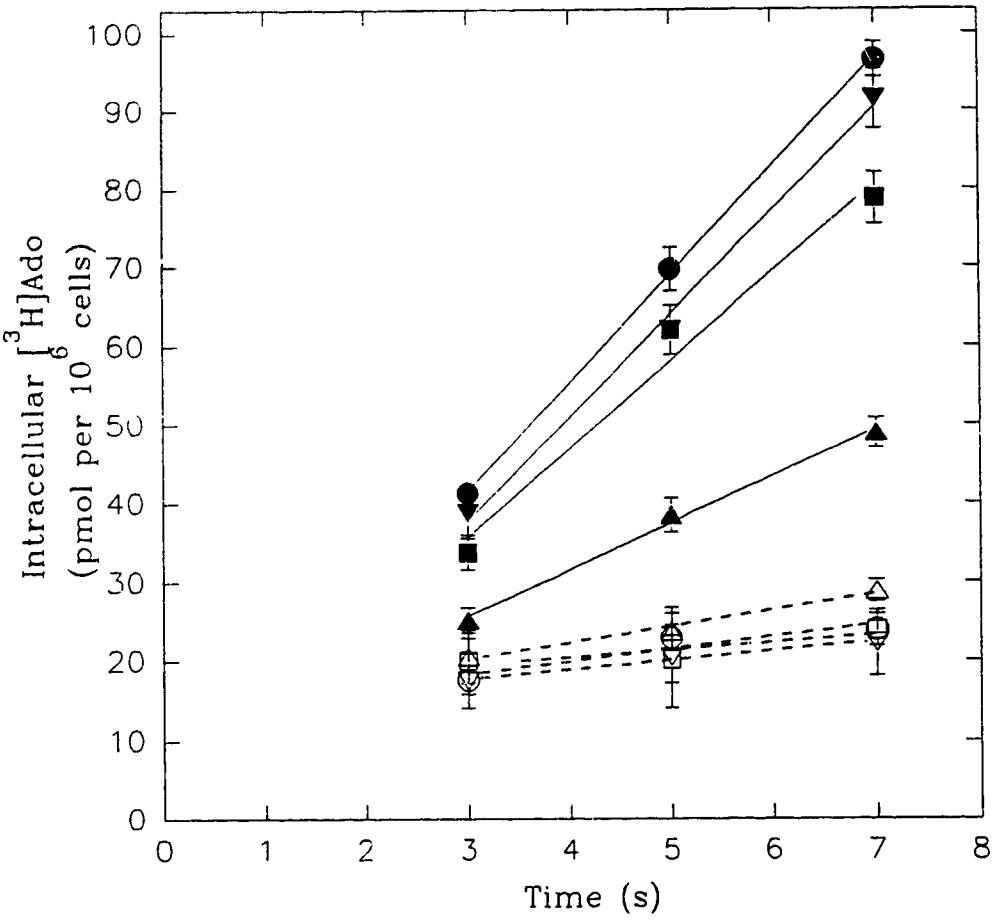


**FIG. 18 (D). Effect of treatment of cells with CLA on time-course of 100 μM [$^3$H]Ado uptake in CEM cells.** Cells were treated with 0.1% (v/v) DMSO (vehicle) (●, O), 10 nM CLA (▼,▽), 10 nM CLA plus 100 nM STA (■,□) or 100 nM STA (▲,△) for 6 h. Subsequently, cells were washed and resuspended in RPMIt and uptake of [$^3$H]Ado was measured as described in "Materials and Methods". Closed and open symbols represent uptake of [$^3$H]Ado measured in the absence and in the presence of 5 μM NBMPR respectively. Each point is the mean ± S.E.M. of 3 separate experiments, each performed in triplicate.

# 4. DISCUSSION

## 4.1. NON-PKC SPECIFIC EFFECTS OF STAUROSPORINE ON INOSITOL PHOSPHATE SIGNAL TRANSDUCTION IN HUMAN PLATELETS

### 4.1.1. STA STIMULATES HYDROLYSIS OF PHOSPHATIDYL INOSITOL 4,5-BISPHOSPHATE IN HUMAN PLATELETS

The effects of STA, a non-specific PKC inhibitor, on the inositol phosphate signal transduction was studied to assess the non-PKC-specific effects of this inhibitor. Results of the studies described herein, which have already been published (207), indicate that micromolar concentrations of STA cause hydrolysis of polyphosphoinositide in intact human platelets. STA induced both time- and concentration-dependent formation of $InsP_3$ (Fig. 2) (207). At STA concentrations of 1-2 μM, production of $InsP_3$ plateaued after 15 s, in contrast to levels produced in response to the physiological agonist, thrombin (Fig. 2), which is known to peak at 10 s and rapidly decline back to basal levels again (89).

Physiologically, hydrolysis of $PIP_2$ is achieved by the action of phosphoinositide-specific phosphoinositidase, PLC, in response to agonist (4). This produces Ins $(1,4,5)P_3$ and DAG (4, 61), two second messengers that mobilize intracellular $Ca^{2+}$ from storage sites (46, 62) and activate PKC, respectively (45, 62). In this study, STA, a PKC inhibitor, was found to act like an agonist, stimulating the hydrolysis of $PIP_2$ with the formation of $InsP_3$ (Fig. 2) (207). STA-induced formation of $InsP_3$ was sustained (Fig. 2). This might be due to the simultaneous inhibition of PKC-induced degradation of $InsP_3$. PKC is known to regulate two key enzymes that metabolize $Ins(1,4,5)P_3$, $InsP_3$ kinase and $InsP_3$ 5-phosphatase (89), which convert $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ and $Ins(1,4)P_2$ respectively. The idea that STA-induced $InsP_3$ formation is via activation of PLC-mediated $PIP_2$ hydrolysis is further supported by the observation that neomycin

completely blocked STA-induced $InsP_3$ production (Fig. 3) (207). Neomycin is known to inhibit PLC-mediated hydrolysis of $PIP_2$ by blocking PLC (91). By blockade of PLC, STA-induced activation of PLC-$PIP_2$ hydrolysis was cirumvented.

The possibility that STA might be mediating an indirect activation via thromboxane $A_2$ ($TXA_2$) generation is unlikely. This is because work in the Wong laboratory that assayed for the $TXA_2$ metabolite, $TXB_2$, by radioimmunoassay did not detect significant levels of the metabolites (207). STA may directly act on a molecular target anywhere along the phosphoinositide pathway preceeding PLC, to eventually cause activation of $PIP_2$ hydrolysis, as the results of the study with neomycin (Fig. 3) would also suggest.

The possibility of a G protein being the site of action of STA was investigated using the non-hydrolyzable GTP analog, GTPγS, in permeabilized platelets. The results provide further evidence substantiating the hypothesis that the STA-induced formation of $InsP_3$ occurs via activation of the PLC-$PIP_2$ pathway. In the study of STA effects on [$^3$H]-inositol labeled saponin-permeabilized human platelets, results show that the PKC inhibitor caused increased formation of $InsP_3$ above basal levels similar to the observation in intact platelets (Fig. 4). Furthermore, in the presence of GTPγS, STA caused an increased formation of $InsP_3$ higher than levels observed when either one of the two agents was used alone (Fig. 4). The observed effect of the two agents albeit simultaneously, did not suggest synergism between the actions of the two agents. This suggests that the two agents may act at the same molecular target along the PLC-$PIP_2$ signal transduction pathway. In such a situation, the same molecular target would be activated by both agents with the action of each agent contributing to the activation process. GTPγS, a non-hydrolyzable GTP analog, would persistently activate G proteins (186, 187), causing activation of PLC-mediated hydrolysis of $PIP_2$ in the absence of a physiological agonist.

Based on this observation, it was conceived that STA may be affecting the G protein that couples the agonist receptor to PLC, which is also the same molecular target

for the action of GTPγS. Or perhaps STA may affect some other target, a receptor, which in turn would alter the G protein function to activate PLC activity. However, experiments conducted later (discussed in section 4.1.3.), and direct evidence reported by Kanaho and others (214) indicate that STA directly activates the G protein which would consequently lead to PLC-induced hydrolysis of $PIP_2$ and $InsP_3$ formation.

Studies carried out to measure levels of Ins(1,4,5)$P_3$ by a Ins(1,4,5)$P_3$ radioligand binding assay, showed that STA caused significant formation of Ins(1,4,5)$P_3$ in intact platelets (Fig. 5). Levels of Ins(1,4,5)$P_3$ induced by STA were, however, lower than levels of $InsP_3$ measured in [$^3$H]myo-inositol labeled platelets by anion exchange chromatography (Figs. 2, 3). One factor that may account for this difference is the fact that $InsP_3$ levels measured by anion exchange chromatography include all isomers of $InsP_3$ while only one isomer, Ins(1,4,5)$P_3$, was measured by the radioligand binding assay. Another reason may be the fact that $InsP_3$ levels measured by anion exchange chromatography may contain some contaminants of inositol mono- and bis-phosphates (184) although washing using the appropriate buffers should remove these contaminants.

Contrary to results observed here, Murphy and others (215) have reported no effect of 1 μM STA on Ins(1,4,5)$P_3$ levels in rabbit platelets. This difference in results may be accounted for by species differences.

### 4.1.2. EFFECT OF STA ON $[CA^{2+}]_i$ IN HUMAN PLATELETS

A puzzling observation was the fact that STA induced a weak elevation in $[Ca^{2+}]_i$ in platelets (Fig. 1) compared to the levels of $InsP_3$ induced by this agent (Fig. 1). The possibility that STA blocks STA-induced Ins(1,4,5)$P_3$ from mobilizing intracellular $Ca^{2+}$ is ruled out due to the fact that the PKC inhibitor potentiated $Ca^{2+}$ mobilization induced by Ins(1,4,5)$P_3$ formed as a result of thrombin-induced $PIP_2$ hydrolysis (Fig. 1). Various reasons could be invoked to explain the weak $[Ca^{2+}]_i$ mobilization. First, the levels of

$Ca^{2+}$-mobilizing second messenger, Ins(1,4,5)$P_3$, induced by STA are lower than those observed in response to thrombin (Fig. 1). Second, initial increases in $[Ca^{2+}]_i$ triggered by platelet agonists like thrombin are known to be amplified by phospholipase A2 activation and $TXA_2$ formation (47, 61, 216). This signal leading to amplification may be absent in the STA-mediated responses since it has been found that STA does not activate $TXA_2$ formation (207). Third, it has recently been reported that agonist-induced mobilization of $Ca^{2+}$ may have a small contribution from $Ca^{2+}$ influx. This observation, made by Geiger and others (217), showed that even after inhibition of thrombin-stimulated $Ca^{2+}$ mobilization through stimulation of PKA or cGMP-dependent kinase, there was still observed a small influx of $Ca^{2+}$ (217). The additional influx of extracellular $Ca^{2+}$ may be absent in STA-mediated $[Ca^{2+}]_i$ mobilization. For these reasons, STA-induced $InsP_3$ formation may not produce a $[Ca^{2+}]$-mobilizing response to the same degree as would be observed for thrombin. Unlike physiological agonists, STA may not invoke other processes required to augment any mobilization of $[Ca^{2+}]_i$ mediated by the STA-induced formation of $InsP_3$.

### 4.1.3. STA CAUSES GTPASE ACTIVATION IN HUMAN PLATELET MEMBRANES

In the search for answers as to how STA might mediate these responses, the observation that treatment of platelets with STA and GTPγS simultaneously caused formation of levels of $InsP_3$ higher than did either one of the two suggested that both agents might be acting at the same site and possibly at the G protein level (Fig. 4). On the basis of this, studies were carried out to investigate any stimulatory effect of STA on high affinity GTPase activity in platelet membranes. Results showed that STA caused both time- and concentration-dependent stimulation of high affinity GTPase activity in platelet membranes (Fig. 6). STA-induced GTPase activity plateaued at 10 min, and was

significant for STA concentrations from 0.5 μM to 5.0 μM (Fig. 6). Soon after this work was completed, Kanaho and his group published studies on the effect of STA on the activity of $G_i$ that had been purified and reconstituted into phospholipid vesicles (214). They showed that STA activated GTPase activity of reconstituted $G_{i1}$ in a concentration-dependent manner, with a maximal 4- to 5-fold increase at 50-100 μM STA (214). In the present study, we have investigated the effect of STA on the native G protein in the membranes of platelets. The present observations, together with those of Kanaho and others (214) clearly demonstrate that at micromolar concentrations, the PKC inhibitor would activate membrane bound G protein. Other PKC inhibitors, such as H7, and K252a, were not found to induce any significant levels of GTPase activity in platelet membranes (Fig. 6). It seems, therefore, that this effect may be unique to STA.

There is some evidence that $PIP_2$ hydrolysis in platelets may also be induced by a tyrosine kinase-activated phospholipase C-γ (218). It is unlikely that STA activated this pathway in the current study since STA itself has been found to inhibit tyrosine kinase activity, at nanomolar concentration (74, 218). There are other reported effects of STA, including inhibition and activation of subsets of serine/threonine kinases in platelets, at 1 μM STA (219). Because of these non-PKC specific effects of STA, results of studies on the role of PKC using STA must be interpreted with caution, particularly if the PKC inhibitor was used at micromolar concentrations.

### 4.1.4. EFFECT OF STA ON PLATELET RESPONSES

STA was found to have no significant effect on platelet secretion (results not shown) or on aggregation (Fig. 7). It appears that at micromolar concentrations, STA both activated and inhibited steps in signal transduction pathways in human platelets. The overall observed effect would therefore depend on the physiological response measured. The lack of effect on platelet secretion or aggregation in our study is likely the result of

the fact that this drug concurrently inhibits PKC. This is largely in keeping with the known synergistic roles played by PKC and $Ca^{2+}$ in such cellular processes. By the use of calcium ionophores or PKC activators such as phorbol esters or membrane-permeable DAGs, it has been established that both Ins(1,4,5)$P_3$ and DAG/PKC are involved in the cascade of reactions that culminates in platelet physiological responses (44, 45, 62, 220). Even though STA may stimulate PLC-induced $PIP_2$ hydrolysis, the minimal level of $[Ca^{2+}]_i$ mobilized, coupled with its potent inhibitory effect on PKC would be insufficient to induce any physiological responses of platelets, in agreement with published results (4, 89). The contribution of each second messenger arm of the $PIP_2$/PLC pathway has been assessed only qualitatively and is not precisely known. For example, several groups have shown that the DAG/PKC pathway is prominently involved in the induction of secretion and secondary aggregation rather than with the earlier events of shape change and primary aggregation (61, 208, 221). Results of this study show that 1 to 2 μM STA alone did not cause platelets to aggregate (Fig. 7, traces b, c), neither did it cause any significant secretion in platelets (results not shown). It can be reasoned that at these concentrations of STA, PKC was completely inhibited and that the transient rise in $[Ca^{2+}]_i$ was far below the threshold level needed to trigger platelet aggregation.

On the other hand, PKC inhibition would account for the partial suppression of thrombin-induced, and total suppression of PMA-induced aggregation by STA (Fig. 7, traces c, g). The former agrees with previously published results (4). As implied by present evidence, thrombin-triggered shape change occurred mainly through $Ca^{2+}$-dependent mechanisms and thus was not blocked by preincubating platelets with STA (Fig. 7 , traces b, c).

## 4.2. EFFECTS OF MODULATORS OF PKC ON CELL PROLIFERATION

CCRF-CEM (CEM) cells used in these studies were maintained in the logarithmic growth phase. The phorbol ester, PMA, and the PKC inhibitor, STA, both affected

proliferation of CEM cells. The PMA- and STA-induced cell proliferation changes were assessed using two approaches, cell counting by Coulter counter, and determination of number of viable cells by the MTT assay. Both techniques yielded similar results, namely, that the cell proliferation rate was reduced following a 16-h treatment with the phorbol ester; in contrast, STA induced cell death and reduced cell proliferation rate during and following 16-h treatment with the PKC inhibitor (Fig. 8A-C). The presence of 0.1% DMSO (vehicle) in cell growth media did not alter the doubling time of CEM cells. Thus, changes in proliferation of CEM cells in this study could not be attributed to the presence of the vehicle.

Following the 16-h treatment of CEM cells with PMA, the cell doubling time was increased 6.5-fold (doubling time for control cells, 21.5 h); cell death occurred during the 16-h treatment with STA, and thereafter (Fig. 8A). The PMA-induced decrease in number of viable cells (as percent of control) was delayed, occurring only after a 16-h treatment with the phorbol ester (Fig. 8B). In contrast, STA caused a decrease in number of viable cells during the 16-h treatment (Fig. 8C). Since PMA and STA both interact with PKC, the latter is, therefore, implicated in the mechanism underlying the PMA-induced decline in cell proliferation, or the STA-mediated decrease in cell numbers. This implies that PKC activity may be required for cellular proliferation (93, 94, 133, 222-224). During the 16-h treatment with PMA (that is, from $T_{-16}$ to $T_0$, in Fig. 8A, B), there was no significant decline in cell proliferation even though PKC activity was down-regulated (as observed in Fig. 9, discussed later). Thus, down-regulation of PKC may have preceded PMA-induced reduction in cell proliferation rate. The decline in cell proliferation observed following the 16-h treatment with PMA and that induced by STA may reflect the beginning of PMA- or STA-induced differentiation, since the two PKC modulators have been reported to induce cell differentiation (93, 94, 129, 133, 150, 151, 154, 222-224, also see 225 for review).

Various cell types have been induced to differentiate using phorbol esters such as PMA (TPA). The induction of differentiation of cells in studies following prolonged

treatment with PMA, a condition similar to that used in current studies has been reported. For instance, PMA induced differentiation of K562 cells following 24-h treatment (151), and also of HL-60 cells after 48 h (93), or 72 h of treatment (129). Also observed were induction of differentiation and phenotypic changes in the T-leukemic cell lines RPMI 8402, HPB-MLT cells, and in the T-lymphoblastoid lines, CCRF-CEM, MOLT-4 and MOLT-3 cells, following 2 to 4 days treatment with PMA (94, 133, 222). Similarly, Guerrin and others (223) reported PMA-induced differentiation of human breast cancer cells MCF-7, 6 days after treatment with the phorbol ester. These reports, together with these observations, show that PMA caused a decline in cell proliferation and induced differentiation of proliferating cells, all of which occurred several hours after treatment of cells with phorbol ester, thus supporting the view that down-regulation of PKC is the cause of the changes in cell proliferation and the induction of differentiation.

The idea that persistent activation of PKC resulted in a decline in cell proliferation or induction in differentiation is also supported by the observations of studies using membrane-permeable DAGs. Results of studies using membrane-permeable DAGs, 1-oleoyl-2-acetylglycerol and 1,2,-dioctanoylglycerol, indicated that DAGs could not induce any monocytic differentiation of human myeloid leukemia cells although these agents do activate PKC (see 225 for review, 231,. DAGs are incapable of persistently activating PKC since they are rapidly broken down. Therefore, down-regulation of PKC which could not be elicited by the DAGs, may have been responsible for the induction of differentiation of cells in studies where prolonged treatment with phorbol ester has led to decreased cell proliferation or induced differentiation. The onset of PMA-induced differentiation may be characterized by partial or complete block of cell proliferation (224). It was reported that almost complete inhibition of cell proliferation characterized the PMA-induced differentiation of human erythroleukemia, K562 cells by PMA (224).

The low percent of viable PMA-treated cells relative to control (vehicle-treated) cells (observed with either increasing concentration of PMA or increasing duration of

subculture) in Fig. 8B does not, however, suggest PMA cytotoxicity. This observation could be explained on the basis that during the 24-96 h subculture of previously PMA-treated cells, proliferation rate of these cells decreased (Fig. 8A), which, in relation to control (vehicle-treated) cells, would be evident as decreased numbers of viable cells as percent of control (Fig. 8B).

The effects of phorbol esters on cell proliferation and differentiation are varied among different cell types. Notwithstanding the PMA-mediated decrease in cell proliferation in the present study and induction of differentiation observed in the studies of others (129, 94, 222, and references therein), phorbol ester-induced inhibition of differentiation of cells has also been reported including phorbol ester-mediated blockade of differentiation of erythroleukemia (226), neuroblastoma (227), chondroblasts (228), 3T3-preadipocytes (229), and myoblasts (230). This shows that the cell type would influence the outcome of phorbol ester treatment of cells.

Results of the current study also show that STA induced a decline in cell numbers during the 16-h incubation and shortly afterwards (Fig. 8A, C). The decreased cell numbers were more pronounced during the 16-hr incubation than after drug removal. As inhibition of PKC by STA would be achieved in minutes as previous studies suggest (4, 83, 89, 90, 183-185 and see 225 for review), reduction in cell numbers caused by STA during the 16-h incubation implicates PKC in the underlying mechanism. Reduction in cell numbers during the 16-h treatment period and shortly afterward indicates that STA-induced cell death predominated over cell proliferation, but neither of the two processes predominated during the next 20 to 96 h after treatment.

Blockade of cell cycle progression by STA-mediated PKC inhibition may be the basis for the STA-induced changes in CEM cell proliferation rate. STA-induced cell cycle arrest and/or differentiation of human lung epithelial EKVX cells at G1 (150), of human leukemia K562 cells (151), and of nontransformed mammalian cells at G1, or G1 and G2 (152), have been reported. It was also reported by Baltuch and others (223) that altered

PKC activity was the basis for hyperproliferative state of glioma cells, and that STA, at concentrations that inhibit PKC, inhibited glioma proliferation. Also, in murine leukemia A65 cells, another PKC inhibitor, H7, raised the proportion of cells in the G0/G1 phase and decreased the percentage of cells in the S phase, without having much effect on cells in the G2/M phase (233). On the other hand, STA may also inhibit some other protein kinases involved in cell cycle progression to cause changes in the latter and in cell proliferation. For example, it was reported that STA-induced cell cycle arrest at G2, in normal and transformed mammalian cells, was due in part to inhibition of p34cdc2 kinase, a cell cycle kinase (234). The possibility that STA-induced decrease in cell numbers may be by inhibition of PKA or tyrosine kinase is also not ruled out since the PKC inhibitor inhibits these other two protein kinases with comparable $IC_{50}$ values (83).

Individual PKC isozymes may play specific roles in cellular proliferation and in induction of differentiation. For example, prolonged (for 24-48 h) treatment of cells with PMA showed that PKC-β activity was necessary and sufficient for phorbol ester-induced growth arrest and differentiation of HL-60 promyelocytic cells and that the phorbol ester effect on PKC-α and PKC-δ was insufficient for the induction of differentiation (235). Other studies also showed that in human-derived A549 lung and MCF-7 adenocarcinoma cell lines, TPA or bryostatin caused complete down-regulation of PKC-ε, and at 100 times higher concentrations, the PKC activators down-regulated PKC-α with growth arrest (236). In contrast, it was found in another study that PKC-δ, and to a lesser extent, PKC-ε and PKC-ζ play a role in the induction of terminal differentiation of murine erythroleukemia cells (237). Still in other studies, PKC-α, -βII, and -ζ isozymes were observed to be expressed in proliferating human erythroleukemia K562 cells, and the levels of these isozymes were reduced following PMA-induced differentiation (238). These and other observations show that individual PKC isozymes may be responsible for proliferation and differentiation in some cells. The ability of PKC modulators to alter cell proliferation or induce differentiation may thus be affected by the types of isozymes of

PKC present in particular cells. The existence of PKC-β isozyme in CEM cells has been reported (239). It may therefore be reasoned that the PKC-β and any other isozyme present in cells used in the present study are susceptible to modulation by PMA or STA, a modulation which resulted in changes in cell proliferation rate.

## 4.3. DOWN-REGULATION OF PKC ACTIVITY IN CEM CELLS FOLLOWING TREATMENT WITH PMA

Since observations in the present studies showed that a reduction in cellular abundance of NBMPR binding sites and nucleoside transporter activity followed prolonged treatment of cells with phorbol ester (to be discussed later), it was suggested that phorbol ester-mediated PKC down-regulation was the cause for the decrease in transporter activity. Thus, changes in NT activity following prolonged treatment of cells with phorbol ester could be attributed to the reduced PKC activity. In the literature, persistent activation of PKC by phorbol ester was reported to result in degradation of the kinase by $Ca^{2+}$-activated proteases, such as calpain (2, 3, 32). For example, prolonged incubation of cells with phorbol esters has been reported to result in low PKC activities (240-243). In one study, exposure of neuronal cells to phorbol ester for 3 h decreased PKC activity by 80-90% of control (205). It was therefore necessary that we assayed the activity of PKC in cells treated with PMA for prolonged periods.

Results from the current study show that following a 16-h treatment of CEM cells with PMA, the activity of PKC was significantly reduced in both cytosolic and membrane fractions (Fig. 9). Total basal PKC activity in control (vehicle-treated) cells of 162 ± 38 pmol per min per mg protein in the present study is less than levels observed in another cultured cells, renal carcinoma SMKT-R3 cells, of 586 ± 145 pmol per min per mg protein (244). Differences in cell types may account for the difference in values observed. Treatment of CEM cells with 100 nM PMA for 16 h caused 96.4% decrease in total PKC

activity in our study which compares favorably with the of 93.7% in total PKC activity caused by treatment of SMKT-R3 cells with 10 nM PMA for 12 h (244). By inference, phorbol ester-PKC down-regulation may be the underlying cause for the decrease in cell proliferation rate that followed the 16 h treatment with PMA, and the decreased cellular abundance of NBMPR binding sites and NT activity (discussed below).

The response of PKC levels to prolonged treatment of cells with PMA may be influenced by the cell type. Thus, decreased PKC activities were observed following 3, 10, 12 or 24 h of treatment with phorbol ester of neuronal cells (205), Swiss-3T3 fibroblasts (240), rat soleus muscle (241), or HepG2 cells (242) respectively. In our study, we have observed that PKC activity was drastically reduced following 16-h treatment of CEM cells with PMA.

## 4.4. EFFECTS OF PKC MODULATORS AND PROTEIN PHOSPHATASE INHIBITORS ON NUCLEOSIDE TRANSPORTER ACTIVITY AND THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES IN CEM CELLS

### 4.4.1. THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND NT ACTIVITY IN CEM CELLS TREATED WITH PKC MODULATORS

To gain further insight into the role of PKC in regulation of cellular abundance of NBMPR binding sites and NT activity, and also to clarify the obscurity surrounding this subject, we have conducted studies to assess changes in abundance of NBMPR binding sites and rate of transporter influx following treatment of CEM cells with the PKC

modulators, PMA or STA. CEM cells employed in these studies were maintained in the logarithmic growth phase.

### 4.4.1.1. EQUILIBRIUM BINDING STUDIES OF NBMPR

The cellular abundance of NBMPR binding sites ($B_{max}$) and the binding affinity, $K_D$, in control (vehicle-treated) CEM cells in the current study were 17.4 ± 0.9 x $10^4$ molecules/cell and 0.39 nM respectively (Fig. 10A). The reported values of $B_{max}$ and $K_D$ for these cells reported in the literature are 33 x $10^4$ molecules/cell, 0.5 nM (245), and 17.7 x $10^4$ molecules/cell ($K_D$ was not given) (246). The value of $B_{max}$ of NBMPR binding in CEM cells in the current study is similar to literature values (246). The differences in reported $B_{max}$ values might be attributable to the use of different clones of CEM cells in each study.

Incubation of CEM cells in the presence of PMA resulted in time- and concentration-dependent decreases in $B_{max}$ of NBMPR binding without any changes in equilibrium dissociation constant, $K_D$ (Fig. 10A -10D, Tables 1 and 2). Significant changes in $B_{max}$ were observed following longer times, 8 h or more, of incubation of cells with PMA. Since it was observed that in CEM cells treated for 16 h with PMA, PKC activity was drastically reduced (discussed in section 4.3, Fig. 9), lower $B_{max}$ value observed in cells treated for 16 h with PMA is the consequence of PMA-induced PKC down-regulation. The decrease in $B_{max}$ for NBMPR binding is in agreement with the findings of Lee and others (93) who showed that prolonged (48 h) incubation of HL-60 cells with PMA resulted in decline in NBMPR-sensitive nucleoside transport. In that report (93) however, PMA-induced PKC activation was suggested as the basis for the decline, contrary to observations in the literature that prolonged incubation of cells with PMA would down-regulate PKC (2, 3, 6, 29). In spite of this statement, the decline in NBMPR-sensitive nucleoside transporter activity as observed by Lee and others (93) following prolonged treatment of HL-60 cells with PMA agrees with our result. Our

results, however, contrast with the observation that PKC activation by PMA (10 min treatment) resulted in decreased $B_{max}$ of NBMPR binding in chromaffin cells (95). In the current study, conditions that are capable of activating PKC did not affect equilibrium binding of NBMPR (Fig. 16A), while PKC down-regulation resulted in a decrease in number of high affinity NBMPR binding sites. The decline in $B_{max}$ of NBMPR binding may be indicative of the beginning differentiation of PMA-treated cells, since proliferating cells express higher levels of NBMPR binding sites than non-proliferating, differentiated cells (108-113). Moreover, the PMA-induced decrease in $B_{max}$ of NBMPR binding occurred prior to the PMA-mediated decrease in cell proliferation (Fig. 8A, B). This is deduced from the results that significant decrease in the number of high affinity NBMPR binding sites occurred during 8 or 16 h treatment periods (Fig. 10A) in contrast with decreased cell proliferation of PMA-treated cells that was evident only following 16 h treatment period (Fig. 8A).

Our observations and those of others (93, 94) suggest that PKC down-regulation is an event that leads to a decrease in the number of high affinity NBMPR binding sites and NT activity. However, *N,N*-dimethylformamide (DMF), an agent which may or may not interact with PKC, induced a decline in high affinity NBMPR binding sites and NT activity in HL-60 cells (114). Supposing that DMF does not interact with PKC, this would suggest that other mechanism(s) for regulating NT activity may exist. Since N,N-dimethylformamide concurrently induced differentiation of HL-60 cells in the study in question, it may imply that decline in NT activity could occur with induction of differentiation in the absence of PKC modulation if DMF was not capable of modulating PKC activity. For the reason that non-proliferating, differentiated cells have been shown to possess fewer NBMPR binding sites than proliferating, undifferentiated cells (108-113), cells may possess a biological mechanism by which cellular abundance NBMPR binding sites and activity of NT may be reduced prior to or during differentiation.

Since the current study showed that the decrease in NBMPR binding sites in PMA-treated cells was the consequence of PKC down-regulation, it came as no surprise that a short time of incubation (30 min) of CEM cells with phorbol ester would produce no changes in $B_{max}$ of NBMPR binding (Fig. 10A). While PKC is activated during short treatment periods, that is in minutes (225), the protein kinase would not be down-regulated during such intervals. This implies that the duration of exposure of CEM cells to PMA is a critical factor in determining the outcome of the phorbol ester treatment. The observation that a short period of treatment of cells with PMA did not result in any change in equilibrium binding of NBMPR contrasts that of Delicado and others (95). In the latter study, a 10 min treatment of chromaffin cells with 100 nM PMA led to decreased $B_{max}$ of NBMPR binding and NT activity (95). Differences in cell types might be the cause for the differences in these observations.

The cellular abundance of NBMPR binding sites and NT activity have been shown to vary with the cell cycle phase. The number of NBMPR binding sites in cells are positively correlated with the fraction of cells in S-phase (247). For instance, an increase in NT activity was observed when cultures of S1 macrophages synchronously entered S-phase (248). It is unlikely, however, that in our study, PMA decreased the cellular abundance of NBMPR binding sites and NT activity by arresting cells in any particular phase of cell cycle since changes in $B_{max}$ for NBMPR binding and NT activity induced by PMA occurred during the 16-h treatment, prior to changes in cell proliferation rates, which occurred only following 16-h PMA treatment (Figs. 8A and 10A).

Significant decreases in $B_{max}$ of NBMPR binding following treatment of CEM cells with PMA occurred at concentrations of phorbol ester that are relevant to PMA-PKC interaction. The concentration of PMA that caused a 50% decrease in $B_{max}$ of NBMPR binding, $IC_{50}$, is 9.2 nM (Fig. 10C, D), which is an effective concentration for PMA-PKC interaction (29, 249), thus further supporting the idea that decreases in NT activity and

$B_{max}$ for NBMPR binding observed in PMA-treated CEM cells are associated with PMA-induced PKC down-regulation.

To strengthen the view that PMA-mediated down-regulation of PKC was the cause of the decline in $B_{max}$ of NBMPR binding in current study, we investigated the effect of another PKC-active phorbol ester, phorbol 12, 13-dibutyrate (PDBu), on equilibrium binding of NBMPR in drug-treated CEM cells. Treatment of CEM cells with PDBu for 16 h caused a significant reduction in $B_{max}$ of NBMPR binding without significant change in $K_D$ (Fig. 10E). The similarity of results suggests that as for PMA, PKC down-regulation following the prolonged treatment period with PDBu caused the decrease in $B_{max}$. In contrast, incubation of CEM cells with 4α-phorbol 12, 13-didecanoate (4α-PDD), a phorbol ester that does not activate PKC (210), did not produce significant changes in $B_{max}$ or $K_D$ (Fig. 10E), also supporting the idea that decreases in NT activity and number of high affinity NBMPR binding as a result of treatment of CEM cells with PMA were PKC-mediated.

In summary, evidence from our study suggests that the phorbol ester, PMA, induces a decrease in $B_{max}$ of NBMPR binding without change in the equilibrium dissociation constant, $K_D$. No change in $B_{max}$ could be elicited following short treatment period of cells with PMA. Thus, PMA-induced PKC down-regulation may underlie the phorbol ester-mediated decline in cellular abundance of NBMPR binding sites.

Treatment of CEM cells with STA, an inhibitor of PKC activity, caused both time- and concentration-dependent decreases in $B_{max}$ of NBMPR binding sites without any significant changes in $K_D$ (Figs. 11A-11D, Tables 3 and 4). Thus, both PMA and STA exhibited similar effects. This means that PKC down-regulation underlies the PMA-induced decrease in $B_{max}$ of NBMPR binding while inhibition of PKC activity by STA is the cause for the decreased in $B_{max}$ in cells treated with either agent. These observations

are in agreement with those made by others using phorbol ester-induced down-regulation or STA-induced inhibition of PKC to achieve the same purpose (116-118). For example, down-regulation or inhibition of PKC similarly inhibited insulin effects on 2-deoxyglucose uptake (116), caused a significant reduction in α-thrombin-induced formation of phosphatidylethanol in human umbilical vein endothelial cells (117), and diminished IgE-mediated histamine release in skin mast cells (118). Based on such reported findings, as well as ours, it is be said that the effects of PMA or STA, in the current study, are the results of the suppression of PKC activity by the two modulators.

The STA-induced decrease in $B_{max}$ of NBMPR binding may be related to the decrease in cell proliferation and cell numbers mediated by STA (Fig. 8A, C). This would suggest that STA-induced PKC inhibition caused decreases in NT activity and $B_{max}$ for NBMPR binding and proliferating rate of CEM cells. Thus the decreased NT activity and cellular abundance of NBMPR binding sites induced by STA may be an event that occurred during transition from proliferation to differentiation.

The effect of STA on $B_{max}$ of NBMPR binding was evident following treatment of cells with the PKC inhibitor for 6 h or more (Fig. 11A and Table 3) although, in studies of PKC inhibition, the STA mediated inhibition of the protein kinase has been evident in minutes (4, 83, 88-91, 183-185). The observation of the present study that STA-induced decrease in $B_{max}$ of NBMPR binding was detected only following hours of treatment with STA (Fig. 11A and Table 3) may reflect the fact that the measured parameter is distal to PKC inhibition and may be associated with the events that prepare the cell for proliferation changes and induction of differentiation. The implication of this is that, in spite of the rapidity of achieving STA-mediated PKC inhibition, it may take hours for changes subsequent to PKC inhibition to be noticed. For instance, STA-induced changes in cell cycle progression, inhibition of cell proliferation, and induction of differentiation of cells were observed hours after STA-treatment (150-152, 154). Thus the interval between

application of STA and the observation of induction of differentiation may be influenced by the events following PKC inhibition, that is, cell cycle arrests, growth/proliferation inhibition, etc., which may occur downstream to PKC inhibition. In the same manner, changes in NT activity and high affinity NBMPR binding sites could be downstream to PKC inhibition, implying that it would take much longer than it takes for PKC inhibition before any induced changes may be observed. This would make the effect of STA on NT activity time-dependent.

The STA-induced decrease in $B_{max}$ of NBMPR binding was found to be significant at STA concentrations that are capable of inhibiting PKC. For example, following treatment of CEM cells with 10 nM STA for 16 h, $B_{max}$ of NBMPR binding was reduced to 70% of control (vehicle-treated) cells (control $B_{max}$ is 17.4 x $10^4$ molecules/cell, Table 4). This suggests that the STA-mediated decrease in $B_{max}$ of NBMPR binding sites is a consequence of PKC inhibition although it does not rule out the possibility that at 32 nM, STA could have inhibited PKA ($IC_{50}$, 8 nM), or tyrosine kinase ($IC_{50}$, 6 nM) (74).

Other PKC inhibitors, GF 109203X (bisindolylmaleimide, BIM) and H7, both altered the equilibrium binding of NBMPR in CEM cells treated with either of these agents (Fig. 11E and Table 5). In CEM cells pretreated with BIM or H7 for 16 h, $B_{max}$ of NBMPR binding sites was reduced to about 81% and 58.2% of control (17.4 x $10^4$ molecules/cell) respectively. BIM, like STA, inhibits PKC at the ATP binding site (211). BIM was reported to be a more specific inhibitor of PKC inhibitor than STA, although is less potent than the latter in inhibiting PKC in intact cells (211). H7 on the other hand is less selective and inhibits other protein kinases as well (211). Nonetheless, using these two inhibitors, we have been able to substantiate the STA-mediated decrease in $B_{max}$ of NBMPR binding sites in CEM cells.

The decrease in $B_{max}$ of NBMPR binding sites observed in BIM-treated CEM cells was smaller compared to that observed in STA-treated cells (compare Figs 11A and 11E). This may be due to lower potency of BIM in inhibiting PKC than STA. It is reported that BIM inhibited isolated PKC isozymes with $IC_{50}$ values of 10 to 20 nM (211). However, in intact cells, BIM inhibited PKC-mediated phosphorylation, with $IC_{50}$ values 10- 20-fold higher than values obtained for STA (211). Similarly, platelet responses such as aggregation, were inhibited by BIM with $IC_{50}$ values also 10 - 20-fold greater than for STA (211). Intracellular concentrations of BIM may not be sufficient to effectively inhibit PKC. This may be because, as a derivative of maleimide, in traversing the plasma membrane, BIM may covalently bind to sulfhydryl groups in the plasma membrane (212, 213) which would decrease the concentration of drug inside cells. Micromolar concentrations of BIM are the suggested concentrations for PKC inhibition studies in intact cells (211).

In preliminary experiments (results not shown) we observed that treatment of CEM cells with 4 μM BIM and higher concentrations resulted in decreased cell numbers. For these reasons, we used 2 μM BIM in the current study. This is in line with the idea of studying an agent that would be beneficial in the use of nucleoside analogs as anticancer drugs. Such a compound should affect NT activity at sub-toxic concentrations. We used a concentration that has a reasonably low cytotoxicity but was unable to significantly lower $B_{max}$ of NBMPR binding. Since higher concentrations caused a dramatic decrease in cell numbers, it became apparent that BIM may not be clinically useful since it may be incapable of producing therapeutic benefits at non-toxic concentrations.

H7, on the other hand, was effective at decreasing $B_{max}$ of NBMPR binding sites in drug-treated cells at a concentration relevant to its inhibition of PKC (Table 5), which supports the idea that STA-induced PKC inhibition was the basis for the decrease in $B_{max}$

in STA-treated cells. However, this does not rule out the involvement of PKA or tyrosine kinase in the effects observed for either STA and H7.

To support our suggestion that the effect of STA is a consequence of inhibition of PKC, inhibitors specific for other protein kinases but not PKC, namely, HA1004 and genistein, were employed in this study. HA1004 is an analog of H7, and a more specific inhibitor for PKA and cyclic GMP-dependent protein kinase (250, 251), while genistein is a tyrosine kinase inhibitor (150, 251). Treatment of CEM cells for 16 h with either of these agents did not reduce $B_{max}$ of NBMPR binding in drug-treated cells (Table 5). Thus, neither inhibition of PKA, cGMP-dependent protein kinase nor of tyrosine kinase affected $B_{max}$ of NBMPR binding in CEM cells treated with inhibitors of any of these protein kinases. On the basis of these results, we ruled out the involvement of PKA, cGMP-dependent protein kinase or tyrosine kinase in the STA- or H7-mediated decrease in $B_{max}$ of NBMPR binding. The results of this study support the hypothesis that inhibition of PKC causes a decline in $B_{max}$ of NBMPR binding with no change in $K_D$ in CEM cells treated with PKC inhibitors, STA or H7.

### 4.4.1.2. IS THE TIME-DEPENDENT EFFECTS OF PKC MODULATORS ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND NT ACTIVITY RELATED TO TRANSPORTER TURNOVER?

A nucleoside transport protein turnover cycle as suggested by Torres and others (252), may play a role in the decrease of high affinity NBMPR binding sites and nucleoside transport activity. The NT protein turnover rate in plasma membrane however, is unknown. In the study in question, localized compartments, such as exosomes, which may act as possible carriers of non-functional nucleoside transporters, were observed in

cultures of chromaffin cells (252). This may be cellular machinery to regulate the abundance of NBMPR binding sites and NT activity. NT protein may go through cycles of internalization from membrane, degradation, and subsequent recruitment of new transporter proteins; it may require a long period of time to complete one cycle. This concept seems likely in view of the observation that cytoplasmic pools of some membrane proteins exist, including NBMPR binding sites (253), and CD4 (254). Such observations suggest that a transfer process for specified proteins between membranes and cytoplasmic pools might occur. In support of this concept are the findings of Torres and others (1992) (252) who reported that photolabeling of the nucleoside transporter with [$^3$H]NBMPR, induced a biphasic down-regulation of the transporter protein from the plasma membrane with a first half-life of 2.3 h in chromaffin cells. During this half-life time, the transporter protein was internalized and 50-60% was destroyed while the remainder reappeared in the plasma membrane to undergo a second phase of disappearance cycle with a half-life of 35 h (252). Since NT protein in the plasma membrane of CEM cells may undergo a similar process, that is internalization, degradation and recruitment, the reduction of NBMPR binding by PKC inhibitors, such as STA, may not be evident in minutes, (in spite of its rapid inhibition of PKC) until such a time that the existing transporter proteins have gone through the normal one cycle of internalization and degradation, as suggested by Torres and others (1992) (252). Following this stage, the subsequent recruitment of fresh transporter protein may be impaired because of incompetence of the PKC-dependent biological mechanism required for recruitment. Such a mechanism would become non-functional as a consequence of PKC inhibition. This idea is related to the suggestion (above) that PKC down-regulation induced by PMA would also impair the PKC-dependent recruitment process. It is for this reason that both PMA-induced down-regulation and STA-induced inhibition of PKC would achieve similar results. The effect of STA on cellular abundance of NBMPR binding sites and NT activity thus could be separated into two components: PKC inhibition, and the blockade of PKC-dependent

transduction on the levels of NT activity. While it is unlikely that the first process is time-dependent, the latter is likely to be.

### 4.4.1.3. RATE OF INFLUX OF ADENOSINE IN CEM CELLS

The observed decreases in cellular abundance of NBMPR binding sites in cells treated with PMA or STA suggested that as the number of high affinity NBMPR binding sites was reduced the transport activity would be decreased. As expected, the inward fluxes of Ado declined simultaneously with the decrease in $B_{max}$ of NBMPR binding following treatment of CEM cells with PKC modulators. Kinetic analysis of Ado influx in CEM cells treated with either PMA or STA indicated that treatment of cells with either of these agents decreased the $V_{max}$ of Ado influx into drug-treated cells without affecting the $K_m$. These responses correlated with the effects of PKC modulators on $B_{max}$ and $K_D$ of NBMPR binding.

Inward fluxes of Ado in the current study were measured by initial rate methods. This is a crucial requirement for the reason that only initial rates of uptake are measures of the unidirectional inward flux. Procedures to determine initial rates include determining time-courses of permeant uptake and calculation of the slopes of the tangents to the curves at time zero. Another approach to measurement of initial rates is to measure permeant accumulation over a brief time period during which progress curves are linear, and fitting the data by linear regression. In the current study, time courses were linear during the first 9 s of uptake. Progress curves were fitted to data points by linear regression and slopes were a measure of the initial rate. The intracellular accumulation of permeant was calculated taking into consideration permeant that was present within extracellular spaces in cell pellets.

Of the several NT processes that have been described in mammalian cells (102), only *es* is expressed in CEM cells (102). The transporter has a high affinity for and is inhibited by NBMPR, with $K_D$ of less than 1 nM (255-257). In the current study, it was observed that the sensitivity of the transporter to NBMPR remained the same after treatment of cells with PMA or STA and that such treatment did not induce any other NT activity in cells. This is deduced from the observations that fluxes of Ado measured in the presence of NBMPR were very minimal (Figs. 15A-Fig. 17, Fig. 18C, Fig. 18D).

Treatment of CEM cells with PMA caused both time- and concentration-dependent reductions in the rate of influx of Ado into cells (Fig. 12Aii - 12D, Tables 6 and 7). These changes occurred concomitantly with reduction in abundance of NBMPR binding sites. Both types of changes were observed prior to the onset of reduction in cell proliferation that followed 16-h treatment with PMA (discussed in 4.2.1. above). Depending on which transporters are present in cells, NBMPR occupancy of sites on the NT polypeptide may be correlated with inhibition of nucleoside transport (101, 255-257), suggesting that a direct relation may exist between abundance of NBMPR binding sites and *es* nucleoside transport activity. The presence of only NBMPR-sensitive (*es*) nucleoside transport in CEM cells (102), is consistent with the observation in the current study that decreases in $B_{max}$ of NBMPR binding sites following treatment of CEM cells with PMA is correlated with PMA-mediated decreases in Ado influx rate.

Incubation of CEM cells with 100 nM PMA caused a time-dependent reduction in the rate of Ado influx into cells (Fig. 12Aii, Fig. 12C and Table 6), with significant decreases in transport rate following prolonged treatment periods. As with the NBMPR binding studies, PKC down-regulation (Fig. 9) is correlated with the decline in Ado influx rate. In agreement with our results, are the studies of others showing that prolonged treatment of cells with phorbol ester resulted in reductions in nucleoside transport activity (93, 94). Prolonged treatment of cells with phorbol esters in those studies implicates PKC

in the observed decreases in facilitated diffusion of nucleosides. However, in none of those reports was PKC down-regulation identified as the cause for the changes even though the conditions used (24 to 48 h of treatment of cells with PMA) would result in PKC down-regulation. The PKC assay carried out as part of this project (Fig. 9, section 4.2.2), and studies of others (28, 29), show that prolonged exposure of cells to PMA did down-regulate PKC, thus implicating the latter activity in decreases in nucleoside transport activity observed in PMA-treated cells. Thus, the present study clarifies somewhat the conflicting reports that are associated with the role of PKC in the regulation of cellular abundance of NBMPR binding sites and NT activity. Direct PKC activation by PMA (10 min treatment) decreased Ado influx rate in chromaffin cells (95), and Ado influx in these cells was abolished by PKC down-regulation (95). In contrast, conditions that would activate PKC did not have any effect on NT activity in our studies. These differences in results may be due to different cell types.

Significant decreases in the Ado influx rate were caused by the 16-h treatment of cells with 10 nM PMA and higher (Fig. 12C, Table 7). $IC_{50}$ values for the decrease in rate was 11 nM (Fig. 12D), a concentration capable of causing PKC activation and down-regulation. Similarly, treatment of cells with PDBu led to a significant reduction in Ado influx into cells (Fig. 15), further indicating the involvement of PKC. Treatment of cells with 4α-PDD, a phorbol ester that does not activate PKC (210) and is not capable of down-regulating the kinase, did not change the Ado influx rate, confirming that the effects of PMA or PDBu on NT activity occurred through interaction of the phorbol esters with PKC. The observation that reduction in the rate of Ado influx followed prolonged treatment of cells with PDBu also supports the idea that PKC down-regulation is the underlying mechanism for the decrease in Ado influx.

Rates of Ado influx in DMSO (vehicle)-treated cells and in untreated cells were not different from each other (Fig. 12Aii), showing that treatment of cells with the vehicle

alone did not alter NT activity. Also, rates of Ado influx in 0.1% DMSO (vehicle) -treated cells, or in PMA-treated cells, measured in the presence of 1. μM NBMPR were negligible compared to rates measured in the absence of NBMPR (Fig. 12Aii and 12C), which shows that, as expected, NT in CEM cells is virtually totally sensitive to NBMPR (102), and that no change in the sensitivity of the transporter to NBMPR was induced by PMA treatment. This observation suggests that treatment of CEM cells with PMA did not alter the sensitivity of NT to NBMPR, nor did it induce the expression of another type of Ado transport; in contrast is the report that 48-h treatment of HL-60 cells with PMA induced $Na^+$-dependent uridine influx (93).

As with PMA, treatment of cells with STA caused time- and concentration-dependent decreases in the rate of Ado influx into CEM cells (Fig. 13A-13D, Tables 8 and 9). The effects of STA on the Ado influx rate were apparently a consequence of the inhibition of PKC because 16-h treatments of CEM cells with HA1004 (a PKA and cGMP-dependent protein kinase inhibitor) or genistein, a tyrosine kinase inhibitor, did not lead to significant changes in the rate of Ado influx in CEM cells, while treatment of cells with H7 caused a significant decrease in rate of Ado influx (Fig. 15). Thus, on the basis of these observations, the possibility of STA or H7 mediating effects through inhibition of PKA, tyrosine kinase or cGMP-dependent protein kinase is ruled out. Also, the experiments with H7 support the results obtained with STA, and together suggest that PKC inhibition by STA is the cause for the decrease in number of high affinity NBMPR binding sites and NT activity.

After longer intervals of exposure of CEM cells to STA, the change in rate of Ado influx induced by STA was increased (Fig. 13A, B and Table 8). After 8 h of exposure, rates of Ado influx were reduced to about 50% of rate in DMSO (vehicle) -treated cells, (0.31 pmol per s per $10^6$ cells) (Fig. 13A, Table 8). As discussed above, the time-dependency of the STA effect could be due to the fact that the measured parameter is not

PKC inhibition, but rather it is distal to the inhibition of PKC, and may involve NT protein turnover. Reduction of the Ado influx rate in CEM cells following treatment of cells with STA for 16 h, also showed dose-dependency (Fig. 13C, Table 9).

Some observations have indicated that simultaneous treatment of HL-60 cells with PMA and STA abolished the reductions of facilitated diffusion rates induced by 48-h treatment of cells with PMA (93). This is in contrast to our observation that the simultaneous presence of PMA and STA did not abolish the PMA- or STA-induced decrease in Ado influx rate (results not shown). While the observation of Lee and others (93) is contradictory to current understanding in the literature, based on these ideas, our observation is consistent with such deductions. In theory, PKC down-regulation and inhibition would eliminate PKC activity and therefore should produce similar results. Furthermore it is unlikely that STA or PMA together could mutually prevent effects of these agents, if the effect of STA or PMA is the result of elimination of PKC activity, either by inhibition or down-regulation. Our observation also conflicts with that of Delicado and others (95) who found that down-regulation of PKC produced no effect on NT activity, while treatment with STA caused increase in $B_{max}$ of NBMPR binding in chromaffin cells. Results of our studies, although contrasting with the observations of others, are consistent in the sense that PMA-induced down-regulation of PKC and achieved the same effect as STA.

Effects of treatments of cells with PMA or STA on the kinetic constants of Ado influx were determined with the aim of determining whether changes in $B_{max}$ for NBMPR binding correlated with changes in maximum rate of influx, $V_{max}$, of Ado influx in PMA- or STA-treated cells. In cells treated for 16 h with either PMA or STA, these agents altered $V_{max}$ without altering $K_m$ for Ado influx in CEM cells (Figs. 14A and 14B), suggesting that under these conditions, the number of transporters decreased in parallel with decreases in NBMPR binding sites (Figs. 10A - 11D).

Results of present study are similar to the findings that phorbol ester-induced PKC down-regulation decreased glucose transporter activity (259). Our observations are only partly in agreement with those of Delicado and others (95) who showed concurrent decreases in $B_{max}$ of NBMPR binding and in $V_{max}$ of nucleoside transport in PMA-treated chromaffin cells. However, their observations were made in cells treated with PMA for 10 min, suggesting that PKC activation was the basis of these effects. Down-regulation of PKC activity, in cells in their study, had no effect on [illegible] ity (95).

In summary, there was a decreased maximal rate, $V_{max}$, of influx of Ado into cells with no significant change in $K_m$, following prolonged treatment of cells with phorbol ester or PKC inhibitor. Together with observations made from binding studies, the results suggest that decreased $V_{max}$ is the result of decreased expression (lower $B_{max}$) of functional NT polypeptides, and that no alteration occurred in the affinity of transporter polypeptides for the permeant or the transporter inhibitor, NBMPR.

It has been discussed above that, following prolonged treatment of CEM cells with phorbol ester, cell proliferation rate declined as evidenced by decreased slope of the growth curve (Fig. 8A, 0 to 96 h), and also that there was decreased cellular abundance ($B_{max}$) of NBMPR binding sites and reduced rate of influx of Ado. Reports have attributed changes such as phorbol ester-induced reduction in NT activity, inhibition of cell cycle progression, inhibition of cell proliferation, and induction of differentiation, to PKC activation even though prolonged treatment periods (24 h or more) were used (93, 94, 133, and see 225 for review). One reason for conducting this study was to address this issue of PKC activity following prolonged treatment with phorbol ester. This would provide some evidence to support one or the other claims, as to whether PKC activation or down-regulation is the cause for the PMA-induced decrease in cell proliferation, NT activity and the number of high affinity NBMPR binding sites. Overall, the evidence favors the idea that the decreased $B_{max}$ of NBMPR binding sites and decreased rates of Ado

influx following prolonged treatment of CEM cells with phorbol esters is a result of down-regulation or the inhibition of PKC.

### 4.4.1.4. EFFECT OF "PKC ACTIVATION" ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND NT ACTIVITY IN CEM CELLS

In view of the result that phorbol ester-mediated PKC down-regulation or PKC inhibition resulted in decreases in $B_{max}$ for NBMPR binding, as well as Ado influx rates, studies were carried out to examine any variations in number of high affinity NBMPR binding sites and NT activity in CEM cells following short periods of PMA treatment. The rationale was to stimulate PKC activity and determine the influence of this on NT $B_{max}$ for NBMPR binding and Ado influx rate.

Treatment of CEM cells with 100 nM PMA for 10 min, conditions that would be expected to activate PKC (225), did not produce significant changes in $B_{max}$ of NBMPR binding, $K_D$ (Fig. 16A), or in the inward flux of Ado (Fig. 16Bi and ii). The lack of significant changes in $B_{max}$ for NBMPR binding or Ado influx rate following exposure of cells to PMA for 10 min was interpreted to mean that cellular abundance of NBMPR binding and NT activity were not affected by an increase in PKC activity over this time period. This contrasts with the reports that treatment of chromaffin cells with PMA for 10 min decreased $B_{max}$ of NBMPR binding (95), and that PMA-induced PKC activation led to stimulation of glucose uptake in human erythrocytes (92).

On the basis of this observation, we propose that in the normally proliferating CEM cell, PKC activity is not rate-limiting in the expression of NT which is at its highest

level; that transporter activity could not exceed this level in cells (at basal PKC activity). NT expression may be rate-limited at the level of polypeptide synthesis, so that an increase in PKC activity alone would not up-regulate transporter expression. If this is the case, then activation of PKC may not lead to an increase in NBMPR binding sites or in transport rate. In contrast, if PKC was down-regulated, this would remove PKC-mediated events necessary to maintain the maximal expression of NT, resulting in an overall decrease in $B_{max}$ of NBMPR binding and rate of Ado influx.

Considering the possibility that NT activity may go through a turnover cycle, of degradation and subsequent recruitment of new transporter proteins into the membrane as suggested by Torres and others (252), there are chances that the period of PKC activation, which occurs in minutes (225), may occur several hours before the recruitment of new transporter proteins into the membrane, or before the exposure of transporter binding sites which hitherto may be cryptic in the membrane. As a result, it may be unlikely that measurements of NBMPR binding and Ado influx rates would show any increase, at 5-10 min following phorbol ester treatment of cells. We probed this concept further by conducting experiments that allowed 2 h following 10 min treatment of cells with PMA. Results (not shown) did not show any enhancement in rate of Ado influx into cells, therefore ruling out the idea that the duration of time following PKC activation may not have been adequate to allow activated PKC-mediated process to produce any effect.

### 4.4.1.5. DOES SHORT TREATMENT WITH PMA REVERSE THE STA-REDUCTION OF NT ACTIVITY?

It seems that the activation of PKC following STA-induced PKC inhibition in CEM cells could not reverse the decrease in NT activity caused by STA. When CEM cells

were pretreated with STA for 16 h, prior to treatment with PMA for 10 min, rate of Ado influx remained low, indicating no reversal of STA-induced decrease in Ado influx rate (Fig. 17). The rationale for the 2-h resting period following 10 min PMA-treatment of STA-treated cells was to allow some time for any possible initiation of events by PMA-induced PKC activation, the events which may be necessary to effect reversal of STA-induced effects. The 2-h period was an estimate only and it is possible that the resting period after PMA-treatment may not be adequate for allowing activated PKC-mediated changes to reverse STA-induced decrease in rate of Ado influx.

Events such as recruitment of new transporter proteins into the membrane, or exposure of binding sites which hitherto have been cryptic and could be accessible only after PKC-induced phosphorylation, may be critical for reversal of STA-induced decrease in rate of Ado influx. The concept of transporter recruitment is in line with the observations and suggestions of Torres and others (252), that nucleoside transporter protein may undergo a turnover cycle of internalization, degradation, and recruitment of new transporter proteins into the membrane. In such a situation, if measurements of NT activity and number of high affinity NBMPR binding sites were carried out earlier than the time required for one complete turnover cycle, one might not observe return of NT activity to basal levels, even though PKC activity may have been restored.

The persistence of STA-induced changes in NT could be viewed as related to STA-induced changes in cell proliferation, or differentiation, a view which would also be in line with an observation, that treatment of cells with STA induced long-lasting changes in cell proliferation and cell numbers (see Fig. 8A). Since the decreased NT activity and the number of high affinity NBMPR binding sites induced by STA was long-lasting, it is suggestive that the STA-induced decline in cell proliferation may be related to the STA-induced decrease in NT expression. The implication of this is that reversal of the reduced NT activity and low numbers of high affinity NBMPR binding sites would not be evident

until after several days, should it be associated with the processes related to reversal of proliferation rate to normal.

### 4.4.2. EFFECTS OF PROTEIN PHOSPHATASE INHIBITORS ON THE CELLULAR ABUNDANCE OF NBMPR BINDING SITES AND NT ACTIVITY IN CEM CELLS

Results of the current project show that conditions that activate PKC above basal levels did not increase cellular abundance of NBMPR binding sites and NT activity, while elimination of the kinase activity led to decrease in $B_{max}$ for NBMPR binding and NT activity. On the basis of these observations, we propose a model for the role of PKC in *es* NT expression. In this model, the nucleoside transporter polypeptide is perceived to be phosphorylated in the membrane of the CEM cells. There is absolute requirement for basal PKC activity in maintaining the expression of NT. Activation of PKC does not cause an increase in transporter activity or number of high affinity NBMPR binding sites. The model predicts that PKC-mediated phosphorylation of nucleoside transporter polypeptides, or of some intermediate membrane protein, is necessary for NT activity. However, elimination of PKC activity, either by phorbol ester-induced PKC down-regulation, or by inhibition of PKC, removes the required PKC-induced phosphorylation steps for maintenance of NT expression.

The general pattern among proteins that require phosphorylation for functioning is that they may also undergo dephosphorylation. It was seen as a plausible idea to exploit any possible dephosphorylation steps that transporter polypeptides may undergo. For instance, if NT expression required PKC-mediated phosphorylation and that elimination of PKC activity caused a decrease in expression of NT, it may be possible to circumvent that

decrease by blocking the dephosphorylation step. This is because blockade of dephosphorylation would maintain the normal level of phosphorylated nucleoside transporter polypeptides even though PKC activity may have been inhibited. To test this hypothesis, we have investigated the effects of protein phosphatase inhibitors, okadaic acid (OKA) and calyculin A (CLA), on equilibrium binding of NBMPR and Ado influx rates in CEM cells. OKA and CLA are inhibitors of PP1 and PP2A (175-179).

In cells treated with OKA and CLA, no changes were observed in the $B_{max}$ of NBMPR binding, or in the inward fluxes of Ado (Fig. 18A-D). This observation is in agreement with the proposed model, which predicts that phosphorylation of NT polypeptides is complete under conditions of resting PKC activity; if this model is correct, a decrease in dephosphorylation would not result in enhancement of NT activity or increase in number of high affinity NBMPR binding sites. This observation is similar to the results obtained for short treatment of cells with PMA (which would cause PKC activation), and supports the proposed model.

A key finding was that OKA or CLA blocked the STA-induced decrease in $B_{max}$ of NBMPR binding (Fig. 18A and B). To explain this result, we would have to view the expression of NT as dependent on a cycle of phosphorylation and dephosphorylation, as suggested in the proposed model. Blockade of PKC by STA would result in a deficiency in the phosphorylation step, while dephosphorylation step by protein phosphatases continued. This would ultimately lead to the decreased expression of NT in the presence of STA, as observed throughout this project. In the presence of protein phosphatase inhibitors, OKA or CLA, however, dephosphorylation step was disengaged at the same time that PKC-mediated phosphorylation was blocked by STA, with the result that the number of high affinity NBMPR binding sites in the membrane did not decrease.

Treatment of cells with STA in the presence of CLA also prevented the STA-induced decrease in Ado influx (Fig. 18D), although simultaneous incubation of CEM cells with STA and OKA did not (Fig. 18C). This anomaly may be due to the fact that OKA is more potent against PP2A than PP1 (175-179) while CLA inhibits both phosphatases with equal potencies (177, 179). This may also suggest that PP1, which is poorly inhibited by OKA, may play a greater role in NT polypeptides dephosphorylation than PP2A. If this was the case, then inhibition of PP2A alone may not be sufficient to cause complete blockade of dephosphorylation of transporter polypeptides in which case, complete reversal of STA-induced decreases in $B_{max}$ of NBMPR binding or Ado influx rate may not be achieved.

The idea that the nucleoside transporter protein, and perhaps other transporter proteins, may be phosphorylated has been proposed by Delicado and others (95), who suggested that PKC-mediated phosphorylation might have caused the decrease in $B_{max}$ of NBMPR binding and of nucleoside transport activity. Also in line with this idea are the reports that activation of PKC increased the phosphorylation of glucose transporters and caused increased glucose uptake in erythrocytes (92), and that PKC-mediated modification of $Na^+$-independent glucose transport activity occurred in quiescent mouse fibroblasts (98). These studies, as well as ours, provide some evidence that phosphorylation mediated by PKC is required for expression of membrane transporters. From our study, we can say that protein dephosphorylation plays a role in the activity of the *es* transporter in CEM cells

Quite recently, the amino acid sequence of a $Na^+$-dependent nucleoside transporter, *cit*, from rat jejunum was deduced, and potential PKC-dependent phosphorylation sites were identified (259). The presence of PKC-dependent phosphorylation sites on the nucleoside transporter polypeptides in CEM cells is shown in

these studies; it would be interesting to determine if probes for the *cit* transporter would interact with the *es* transporter.

It could be reasoned that phosphorylation serves as a positive modulation step for NT expression, in contrast to the dephosphorylation step which may serve to decrease NT expression. In that regard, the two contrasting modulatory processes may be a part of the biological cycle of recruitment (and/or expression), removal of transporter from the accessible position in the membrane (or internalization), followed by degradation, and then back to recruitment of new transporters, as suggested by the studies of Torres and others (252). Alternatively, the dephosphorylation step may bring about a decrease in NT expression by causing the dephosphorylated NT to undergo change in conformation which would result in concealment or occlusion of the high affinity NBMPR binding sites. This could result in a decrease in the cellular abundance of accessible NBMPR binding sites, and a decrease in transporter activity.

In summary, use of protein phosphatase inhibitors like OKA or CLA, which block dephosphorylation steps, have shown that attempts to further elevate phosphorylated NT polypeptides or some other protein that is involved in the regulation of NT expression, did not result in increased NT activity or in the cellular abundance of NBMPR binding sites. This would support the proposed model in which, at basal PKC activity, the relevant membrane polypeptides were already phosphorylated. The results also support the view that NT polypeptides (or some other protein) undergoes phosphorylation and dephosphorylation, as the two opposite steps of positive and negative modulation. Blockade of PKC-mediated phosphorylation (by PKC down-regulation or inhibition) would result in lower levels of phosphorylated NT protein, and a consequent decrease in NBMPR binding sites, as well as nucleoside transport rates. This could be blocked by simultaneous treatment of cells with protein phosphatase inhibitors such as CLA. The protein phosphatase inhibitors act to block the negative modulation step of reducing levels

of phosphorylated NT and thus serves as alternate route to maintain levels of phosphorylated NT, by preventing dephosphorylation steps.

## 5. SUMMARY AND CONCLUSIONS

5.1. The effect of STA on inositol phosphate signal transduction was studied in human platelets. The following are the observations:

1. STA at micromolar concentrations caused formation of significant levels of $InsP_3$ in both intact and permeabilized human platelets; this phenomenon was inhibited by neomycin, a PLC inhibitor, in intact platelets
2. The STA-induced mobilization of cytosolic calcium was rather weak although the PKC inhibitor did potentiate the effect of thrombin.
3. STA also induced the formation of low levels of $Ins(1,4,5)P_3$, the $[Ca^{2+}]_i$-mobilizing second messenger.
4. STA did not induce any appreciable effect on platelet aggregation or on platelet secretion (results not shown).
5. Micromolar concentrations of STA stimulated GTPase activity in platelet membranes suggesting that the PKC inhibitor activated the receptor-coupled G protein, in agreement with the findings of Kanaho and others (214).
6. Therefore, at micromolar concentrations, STA would not only inhibit PKC, but also invoke non-specific effects such as the hydrolysis of $PIP_2$, similar to a $Ca^{2+}$- mobilizing agonist.

5.2. Studies of the effects of PMA and STA on proliferation of undifferentiated T-lymphoblastoid CCRF-CEM (CEM) cells showed that both PKC modulators reduced proliferation of CEM cells. The effects of PMA and STA were attributed to down-

regulation and inhibition of PKC, respectively. Using PKC modulators and protein phosphatases inhibitors we have also investigated the role of protein kinase C in the expression of NT in CEM cells. The following are our findings:

1. Both STA and PMA caused concentration- and time-dependent decreases of rate of Ado influx, and of $B_{max}$ of NBMPR binding in CEM cells without any significant change in the $K_D$, following incubation of cells with these agents.
2. Significant changes in $B_{max}$ and Ado influx rate occurred only after prolonged treatments periods with PMA or STA, suggesting that PMA-induced PKC down-regulation, or PKC inhibition by STA, was the underlying mechanism for the observed decreases in number of high affinity NBMPR binding sites and NT activity.
3. Prolonged treatment of cells with PMA or STA significantly reduced $V_{max}$ of Ado influx without appreciable changes in $K_m$; this correlates with the corresponding drug-induced reduction in $B_{max}$ of NBMPR binding sites with no change in $K_D$.
4. Treatments of cells with H7, another inhibitor of PKC, or PDBu, led to decreases in rate of Ado influx and $B_{max}$ of NBMPR binding without significant change in $K_D$.
5. The exposure of cells to genistein, an inhibitor of tyrosine kinase, HA1004, a PKA and cGMP-dependent kinase inhibitor, and 4α-PDD, an inactive phorbol ester, did not result in decline in rate of Ado influx or $B_{max}$ for NBMPR binding.
6. The decreased cellular abundance of NBMPR binding sites and NT activity induced by PMA or STA persisted during a 3-4- day period of subculture of cells following withdrawal of agent.

7. Treatment of cells with PMA for 5 or 10 min, conditions known to activate PKC, did not change in NT activity.

8. Treatment of cells with the protein phosphatase inhibitors, OKA or CLA, did not alter the number of high affinity NBMPR binding sites and NT activity; however, OKA and CLA partially or completely blocked STA-induced decline in $B_{max}$ for NBMPR binding respectively; CLA also completely prevented STA-mediated decrease in rate of Ado influx.

9. Results of this study are consistent with the hypothesis that protein phosphorylation by PKC, and dephosphorylation by protein phosphatases, are involved in the regulation of the number of high affinity NBMPR binding sites and NT activity.

## 6. FUTURE DIRECTIONS

The subject of STA and G protein function is fairly new, with not much information in the literature. The first report that STA may alter G protein activity was made by Kanaho and others (214), around the same period that the present work was carried out. Thus, more exploration of the STA-G protein phenomenon is needed to understand the nature of the interaction between STA and the G protein. Also the types and the fate of the $InsP_3$ that are produced in response to STA treatment need to be identified. It is known that agonist-induced $InsP_3$ undergoes further metabolism with the generation of inositol which is fed back into the inositol pool.

On NT expression and PKC activity, there is also very little information in the literature. Reported findings also show inconsistencies. Unlike phorbol ester- or STA-induced cell growth arrest and differentiation, which has been extensively studied to provide ample evidence for the role of PKC in these processes, PKC and NT expression requires further work. PKC-dependent phosphorylation of NT polypeptides or some other

protein which may be associated with expression of NT remains to be directly proven, although in our study we have used an indirect approach in the form of protein phosphatase inhibitors. Dephosphorylation of the phosphorylated NT polypeptides or the putative phosphoprotein by a protein phosphatase also requires a further direct investigation.

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