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Chronic Somatostatin Treatment Induces Enhanced Forskolin-Stimulated cAMP Accumulation in Wild-Type S49 Mouse Lymphoma Cells But Not in Protein Kinase-Deficient Mutants

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SUMMARY

Many different types of cells exhibit a supersensitivity of adenylate cyclase after chronic treatment with inhibitory drugs; this phenomenon is manifested by enhanced cAMP accumulation upon removal of the inhibitory drug. Acute treatment of wild-type S49 cells with the somatostatin analog SMS 201-995 (SMS) results in inhibition of cAMP accumulation. We have found that chronic SMS treatment of S49 cells results in enhanced isoproterenol- and forskolin-stimulated cAMP accumulation after removal of the SMS. The forskolin-stimulated cAMP synthetic rate was about 57% higher in SMS-pretreated cells (14.22 ± 1.02 pmol of cAMP/10^6 cells/min) than in untreated control cells (9.08 ± 0.84 pmol of cAMP/10^6 cells/min). The time course of forskolin-stimulated intracellular cAMP accumulation is complex, with desensitization of cAMP synthesis and marked egress of cAMP from the cells. We have modeled the forskolin-stimulated cAMP time course to a simple function incorporating the initial synthetic rate and rate constants for desensitization and elimination (degradation plus egress). The mathematical modeling suggests that the difference in forskolin-stimulated cAMP time courses between control and SMS-pretreated cells can be explained on the basis of a difference in initial synthetic rates. We tested the hypothesis that the SMS-induced change in forskolin-stimulated cAMP accumulation is triggered by the decrement in the concentration of intracellular cAMP caused by SMS. We studied two independently isolated mutants of S49 cells that are devoid of cAMP-dependent protein kinase activity (kin^-). Although SMS acutely inhibits cAMP accumulation in both kin^- mutants, neither mutant exhibited an enhanced forskolin-stimulated cAMP synthetic rate after chronic SMS treatment. These results suggest that cAMP-dependent protein kinase is important in the induction of adenylate cyclase supersensitivity in wild-type S49 cells. The mechanistic signal for induction of supersensitivity may be the decreased cAMP accumulation that occurs in response to stimulation of inhibitory receptors, although other hypothetical mechanisms may be invoked.

A variety of different types of cells exhibit an adaptive response to chronic treatment with agents that inhibit adenylate cyclase (1). The response is manifest as an enhanced stimulation of adenylate cyclase in membranes or an increased cAMP accumulation in cells after withdrawal of the inhibitory drug. This phenomenon was originally observed in neuroblastoma × glioma hybrid cells (NG 108-15). Pretreatment of NG cells with morphine for at least 12 hr resulted in an increased prostaglandin E_1-stimulated cAMP accumulation in intact cells and an enhanced basal and prostaglandin E_1-stimulated adenylate cyclase activity in membranes (2, 3). In view of the neuron-like qualities of NG cells, these adaptive changes were proposed to represent a cellular model for narcotic addiction (2). Other inhibitory agonists (muscarinic cholinergic and α_2-adrenergic) also induce a similar supersensitivity of adenylate cyclase in NG cells (3, 4). Furthermore, this supersensitivity is observed in several different cells, such as pituitary cells, adipocytes, and cardiomyocytes, in response to chronic treatment with various drugs that acutely inhibit adenylate cyclase (1). The phenomenon of supersensitive adenylate cyclase after prolonged exposure to inhibitory drugs represents a common cellular adaptation to chronic inhibition of the enzyme (1).

The biochemical signal by which activation of inhibitory receptors leads to the induction of the supersensitive adenylate cyclase response is unknown. Because these receptors all inhibit adenylate cyclase and decrease intracellular cAMP concentrations, a plausible hypothesis is that chronically suppressed activity of cAMP-dependent protein kinase leads to some adaptive change in the adenylate cyclase system. Receptors that inhibit adenylate cyclase frequently have additional effects such as regulation of G proteins, ion channels, and Na^+/H^+ ex-

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ABBREVIATIONS: SMS, somatostatin analog SMS 201-995; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; kin^-, protein kinase-deficient S49 mouse lymphoma cell.

change, which are likely independent of the effects of these receptors on cAMP accumulation (5–8). Therefore, an alternative hypothesis is that these cAMP-independent pathways may be involved in inducing the adaptive response.

One approach to distinguishing between these two hypotheses would be to utilize a cell deficient in cAMP-dependent protein kinase activity. Because virtually all known effects of cAMP are mediated through protein kinase (9, 10), such a cell should be insensitive to any change in cAMP concentration. A cell deficient in protein kinase would enable a test of whether the consequence of adenylate cyclase inhibition, i.e., the decrease in intracellular cAMP, is the signal for the development of the adaptive change in adenylate cyclase. We have used the well characterized protein kinase-deficient mutants of the S49 mouse lymphoma cell (9, 11) to pursue this problem. S49 cells have somatostatin receptors that inhibit adenylate cyclase (12, 13), but the effects of chronic somatostatin treatment and withdrawal have not been reported. This communication describes our experiments that demonstrate the somatostatin-induced adenylate cyclase supersensitivity in S49 cells, as revealed by an enhanced forskolin-stimulated synthetic rate in intact cells. In addition, we have tested two independently isolated protein kinase-deficient S49 mutants for the expression of this response. The results provide evidence for the importance of cAMP-dependent protein kinase in the development of adenylate cyclase supersensitivity.

Methods

Dulbecco's modified Eagle medium (4500 mg of D-glucose/liter), antibiotics, and fungizone were from GIBCO (Grand Island, NY). Horse serum was purchased from the University of California Cell Culture Facility (San Francisco, CA). cAMP antiserum was obtained from Research Products International (Mount Prospect, IL). Somatostatin, (−)-isoproterenol, forskolin, IBMX, and cAMP were from Sigma Chemical Co. (St. Louis, MO). SMS (14) was kindly donated by Sandoz Research Institute (East Hanover, NJ), and RO 20-1724 was donated by Hoffman-La Roche, Inc. (Nutley, NJ).

Cells. S49 mouse lymphoma wild-type cells (24.3.2) and protein kinase-deficient S49 mutant cells [kin$^-$, designated 24.6.1 or Alr (11)], were obtained from the University of California Cell Culture Facility (San Francisco, CA). Another, independently isolated protein kinase-deficient mutant [kin$^-$, designated 24.4.6 or Blr (9, 11, 15)] was kindly provided by Dr. Paul Insel (University of California, San Diego). Both of the kin$^-$ lines are from the class of mutants with no detectable cAMP-dependent protein kinase activity (9). We have confirmed the absence of this activity, assayed as described (16) (data not shown). Kin$^-$ mutant 24.4.6 has been reported to also have decreased cAMP phosphodiesterase activity (9, 11, 17).

S49 cells were cultured in Dulbecco's modified Eagle Medium (4500 mg of D-glucose/liter), supplemented with 1.0 g/liter $NaHCO_3$ and 10% heat-inactivated horse serum, in a humidified atmosphere of 5% CO_2 at 37°. Cells were maintained at a density of 0.5 to 2.0 × 10^6 cells/ml by the daily addition of fresh medium.

Inhibition of isoproterenol-stimulated cAMP accumulation by SMS. The potency and efficacy of inhibition by SMS was compared in S49 wild-type and kin$^-$ mutant cells by measuring the inhibition of isoproterenol-stimulated cAMP production in the presence of phosphodiesterase inhibitors IBMX and RO 20-1724. Cells were washed and resuspended in medium (without $NaHCO_3$ or serum, supplemented with 20 mM Na HEPES, pH 7.4, antibiotics, and fungizone. To 0.090 ml of drug solution, 0.360 ml of cells at 0.4 to 0.5 × 10^6 cells/ml was added to yield final concentrations as follows: 10^{-6} M isoproterenol, 10^{-4} M sodium ascorbate, 5 × 10^{-4} M IBMX, 10^{-4} M RO 20-1724, 0.2 mg/ml bovine serum albumin, and from 10^{-11} to 10^{-6} M SMS. Incubation was at 37° for 10 min (the peak of isoproterenol stimulation in the presence of IBMX and RO 20-1724). The reaction was stopped by adding 0.05 ml of 1.0 N HCl. cAMP was measured by radioimmunoassay (18).

Chronic SMS treatment of cells and cAMP accumulation during time courses of agonist stimulation. S49 cells were harvested in the logarithmic phase of growth and seeded at 0.6 to 0.7 × 10^6 cells/ml as a control group and a group treated with SMS (usually 10^{-6} M) for 24 hr. Preliminary experiments demonstrated that the response to chronic SMS treatment was greater after a 24-hr treatment than after a 4- or 12-hr treatment; these changes did not increase further when the treatment was extended to 48 hr (data not shown). Cells (having grown to a density of 1.5 to 1.8 × 10^6 cells/ml over 24 hr) were harvested and washed three times with medium (as specified above). For washes, cells were pelleted by centrifugation at 500 × g at room temperature for 5 to 10 min and resuspended in a volume of medium equal to that during the chronic treatment. Lastly, the cells were resuspended in medium to a density of about 0.4 × 10^6 cells/ml and equilibrated to 37° in a gyratory shaking bath before treatment with stimulatory hormones. Either isoproterenol or forskolin was added at 100 × final concentration. The final concentration of isoproterenol was 10^{-6} M (with sodium ascorbate at 10^{-4} M), and the final concentration of forskolin was 10^{-4} M; these are maximally effective concentrations in stimulating cAMP accumulation in S49 cells.

Initial synthetic rates (over a 30-sec interval for isoproterenol and a 3- to 5-min interval for forskolin) were estimated from total cAMP (both intracellular and extracellular). At various times after agonist addition, 0.9-ml aliquots of cells were withdrawn and pipetted into tubes containing 0.1 ml of 1.0 N HCl to stop the reaction and extract intracellular cAMP. After a 2500 × g centrifugation to pellet the fixed cells, the 1.0-ml supernatant was designated as total cAMP. The use of total cAMP was necessitated by the difficulty in accurately determining intracellular cAMP at shor time intervals. Although intracellular cAMP has often been assessed for suspended S49 cells by means of rapid, high speed (10–12,000 × g) centrifugation in a Microfuge (12, 15, 19, 20), we found that this results in a large egress of the intracellular cAMP; up to 70 to 80% of the intracellular cAMP appears extracellularly after only 15 sec at 10,000 × g. With the use of lower g forces, e.g., 500 × g for 3 min, as little as 2 to 5% of the total cAMP is extracellular after 2 min of forskolin stimulation. Because the lower speed centrifugation requires more time to pellet the cells (e.g., 3 min), this approach precludes the use of intracellular cAMP determinations in estimating synthetic rates over short time intervals.

In some experiments, we measured intracellular cAMP and the transport of cAMP out of S49 cells with time. In the interest of accuracy and repeatability, we decided to calculate intracellular cAMP from measurements of the total and extracellular cAMP. Thus, in addition to an aliquot for total cAMP at each time point, in those experiments an additional aliquot was withdrawn and pipetted into tubes in an ice-water bath. These aliquots were immediately centrifuged at 500 × g, 0°, for 3 min and cAMP was measured in the supernatant. Intracellular cAMP was calculated as the difference between total and extracellular cAMP.

Data analysis. Analysis of time course data was based on the rate equation (21, 22). The rate of change of cAMP concentration throughout a time course of stimulation by agonist is the difference between the rate of synthesis and the rate of elimination:

$$\frac{d[\text{cAMP}]}{dt} = V_{syn} - V_{elim}$$

$$= V_{syn} - k_{elim}[\text{cAMP}]$$

where V_{syn} is the synthetic rate, V_{elim} the elimination rate, and k_{elim} the first-order rate constant for elimination or turnover (21, 22). At the onset of stimulation, the cAMP concentration is very low, and the rate

of synthesis may be approximated as the rate of change of cAMP with time:

$$V_{syn} = \frac{\Delta[cAMP]}{\Delta t}$$

Thus, the initial synthetic rates were estimated from the slopes of the linear portions of time courses immediately after stimulation.

Egress of cAMP from the cells was assessed by relating the extracellular cAMP to the intracellular cAMP accumulation as follows:

$$[cAMP]_e = k_e \int_0^t [cAMP]_i \, dt$$

where $[cAMP]_e$ and $[cAMP]_i$ are the extracellular and intracellular concentrations, respectively, and k_e in the first-order rate constant for egress (23). Extracellular cAMP concentration at each time point was plotted against the integral accumulation of intracellular cAMP from time 0 to each time point. The slope of this straight line relationship was taken as an estimate of the egress constant k_e, as previously described (23). Elimination of cAMP from cells occurs by egress and degradation (20, 23). By adding exogenous cAMP to suspensions of S49 cells, we determined that the cAMP in the extracellular space is not subject to hydrolysis (data not shown). Therefore, egress and degradation can be considered to be two separate components in the elimination of cAMP from S49 cells.

The forskolin-stimulated intracellular cAMP time courses were modeled to a mathematical expression incorporating the initial synthetic rate and rate constants for desensitization, degradation, and egress. The following assumptions were made: 1) the initial synthetic rate is attained instantaneously; 2) the synthetic rate declines exponentially to zero with time; 3) there is no "resensitization" in the presence of agonist; 4) elimination of cAMP from the cell is composed of two first-order processes, namely, degradation and egress; and 5) the rate constants for desensitization, degradation, and egress are invariant with time. Although there is actually a transient lag in the initial cAMP synthesis after forskolin stimulation (see Results), this lag is negligible for purposes of evaluating the later time course. Including a term for desensitization is based on experimental evidence for desensitization of forskolin stimulation, namely a peak and subsequent decline in cAMP concentration during the time course (see Results). Our experiments also provide evidence that egress is a first-order process and is characterized by a single rate constant throughout the time course of forskolin stimulation (see Results). Degradation after isoproterenol stimulation appears to be a first-order process (20, 22).

Incorporating rate constants into the rate equation

$$\frac{d[cAMP]_i}{dt} = V_{syn} - V_{elim}$$

$$= V_{S_0} e^{-k_{desens} t} - (k_d + k_e)[cAMP]$$

and integrating yields the following solution:

$$[cAMP]_i = \frac{V_{S_0}}{(k_d + k_e) - k_{desens}} (e^{-k_{desens} t} - e^{-(k_d + k_e)t})$$

where V_{S_0} is the initial synthetic rate, k_{desens} is the rate constant for desensitization, k_d is the rate constant for degradation, and k_e is the rate constant for egress. Solutions to the above equation were generated by using an extended least squares modeling program (MKMODEL, by Nick Holford, Auckland, New Zealand) run on an IBM personal computer. For this purpose, k_d and k_e in the equation were combined as a single rate constant of elimination. From intracellular cAMP concentrations at each time point, the equation was solved for three parameters; V_{S_0}, k_{desens}, and $(k_d + k_e)$. Because k_e was calculated independently as described above, the value of k_d could then be determined.

Results are expressed as means ± standard errors and were compared using Student's t tests. Statistical significance was indicated for $p < 0.05$. Straight line data were fitted by linear regression.

Results

Somatostatin inhibits adenylate cyclase activity in S49 cells (12, 13). Our preliminary experiments indicated that somatostatin and the more stable SMS (14) have equal efficacy in inhibiting isoproterenol-stimulated cAMP accumulation in S49 wild-type cells. SMS (10^{-6} M) inhibited isoproterenol-stimulated cAMP accumulation by 56.1 ± 2.7% with an EC_{50} of 1.94 ± 0.2 × 10^{-9} M (five experiments; Fig. 1). To assess the effect of chronic exposure to SMS, S49 cells were incubated with 10^{-6} M SMS for 24 hr and the drug was washed out as described in Methods. The time course of isoproterenol-stimulated total cAMP accumulation in control and SMS-pretreated wild-type cells is shown in Fig. 2. In the case of isoproterenol stimulation, the time course appeared linear for only about 30 sec. Thus, our estimates of the initial isoproterenol-stimulated synthetic rates are based on time points up to 30 sec after agonist addition (Fig. 2, *inset*). Results from nine paired experiments revealed nearly identical initial synthetic rates for control cells (21.47 ± 1.73 pmol of cAMP/10^6 cells/min) and for SMS-pretreated cells (21.80 ± 1.84 pmol of cAMP/10^6 cells/min). Fig. 2 also shows that the peak cAMP accumulation was not different in control and SMS-pretreated cells. However, differences between the two groups were apparent at longer times after isoproterenol addition; at 10 min and longer, paired comparisons revealed significantly greater (up to 2-fold) isoproterenol-stimulated cAMP accumulation in the SMS-pretreated cells compared with control cells. Intracellular cAMP accumulation was also greater in SMS-pretreated cells at these later time points (data not shown).

The dose-response relationship for the SMS-induced increase in isoproterenol-stimulated cAMP accumulation was determined by treating cells with various concentrations of SMS, from 10^{-10} to 10^{-6} M, for 24 hr. The percentage of SMS-induced increase in total cAMP after 40 min of isoproterenol stimulation above that seen in controls is illustrated in Fig. 1. The maximum effect was observed with 10^{-6} M SMS during the pretreatment, and this concentration was used in subsequent experiments. As seen in Fig. 1, SMS appeared to be less potent in inducing enhanced isoproterenol responsiveness (EC_{50} = 1.27 ± 0.74 × 10^{-8} M) than in acutely inhibiting isoproterenol-stimulated cAMP accumulation (EC_{50} = 1.94 ± 0.2 × 10^{-9} M).

We next examined forskolin-stimulated cAMP accumulation in controls and cells pretreated with SMS (10^{-6} M) for 24 hr. The time course of forskolin-stimulated total cAMP accumulation is shown in Fig. 3. The increase in concentration of cAMP was linear from 0.5 min up to at least 5 min. [There was a initial lag in forskolin stimulation, lasting about 0.5 min, which has been noted previously (19).] The initial synthetic rate (slope of the time course from 0.5 to 4.0 min) was significantly higher (about 57%) in SMS-pretreated cells (14.22 ± 1.02 pmol of cAMP/10^6 cells/min) than in untreated cells (9.08 ± 0.84 pmol of cAMP/10^6 cells/min) ($p < 0.001$, 12 experiments). Total cAMP accumulation was higher in SMS-pretreated cells at longer times (up to 180 min). When we measured the extracellular cAMP as described in Methods, we found that a large percentage of the total cAMP is located in the extracellular medium after 30 min of forskolin stimulation. A time course for total, extracellular, and calculated intracellular cAMP is illustrated in Fig. 4. The proportion of extracellular cAMP increases continuously until it reaches up to 90% of the

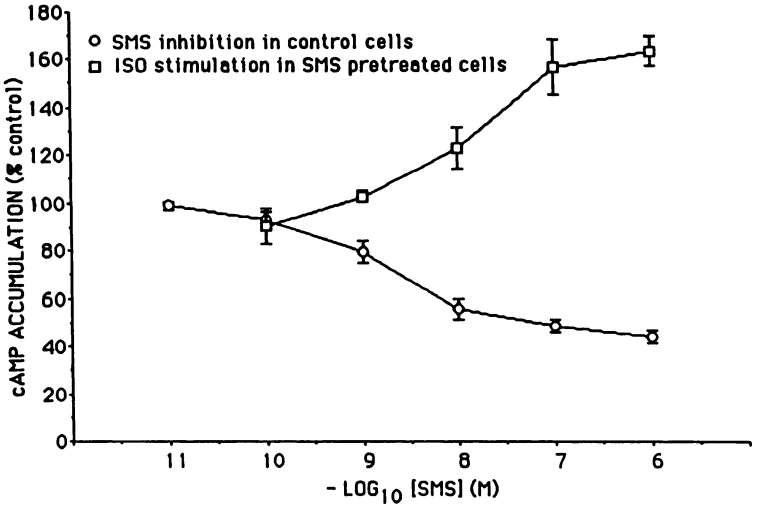

Fig. 1. Dose-response curves for SMS-mediated inhibition of cAMP accumulation (○) and SMS-induced enhancement of isoproterenol (*ISO*)-stimulated cAMP accumulation (□) in S49 wild-type cells. Acute inhibition of cAMP accumulation by SMS was measured in cells stimulated by isoproterenol in the presence of varying concentrations of SMS for 10 min in the presence of phosphodiesterase inhibitors (○). These data are expressed as a percentage of cAMP accumulation in the absence of SMS and represent the mean ± standard error of five experiments. Enhanced cAMP accumulation induced by chronic treatment with SMS was assessed by comparing control cells and cells pretreated with varying concentrations of SMS for 24 hr (□); after both groups of cells were washed, the cells were stimulated with isoproterenol (10^{-6} M) alone for 40 min. Total cAMP accumulation was measured and the percentage of control response in the SMS-pretreated cells was calculated. These data are the mean ± standard error of three to seven experiments.

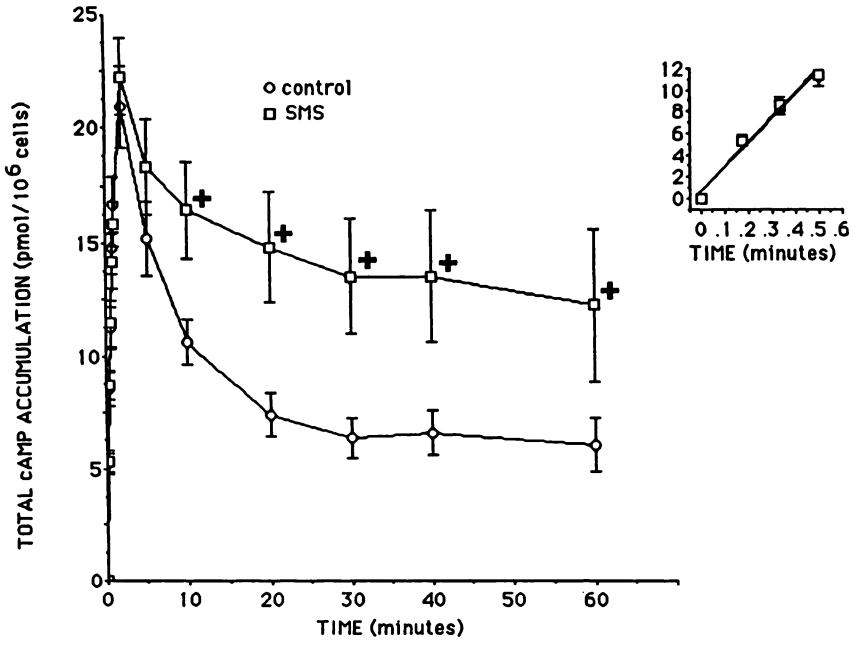

Fig. 2. Time courses of isoproterenol-stimulated total cAMP (both intracellular and extracellular) accumulation in S49 wild-type cells, either untreated (○) or pretreated with 10^{-6} M SMS for 24 hr (□). After pretreatment, cells were washed free of drug and stimulated with 10^{-6} M isoproterenol for various times. Data *points* are the means ± standard error of nine experiments. Points at which the SMS pretreatment resulted in significantly higher isoproterenol-stimulated cAMP accumulation are indicated (+). *Inset*, the early time course (0 to 30 sec) used to estimate the initial synthetic rates, which were similar in the two groups of cells.

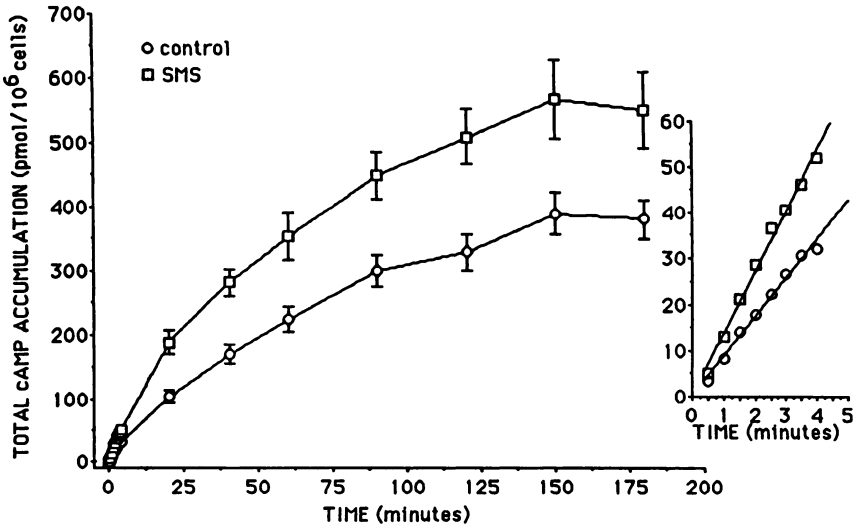

Fig. 3. Time courses of forskolin-stimulated total cAMP (both intracellular and extracellular) accumulation in S49 wild-type cells, either untreated (○) or pretreated with 10^{-6} M SMS for 24 hr (□). After pretreatment, cells were washed free of SMS and stimulated with 10^{-4} M forskolin for various times. Data *points* are the means ± standard error of 12 experiments. *Inset*, the early time courses (0.5 to 4.0 min) used to estimate the initial synthetic rates, which were increased in the SMS-pretreated cells.

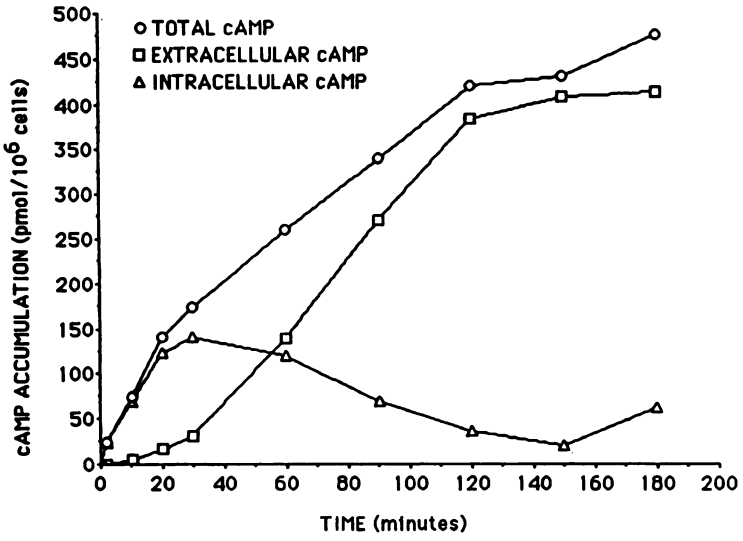

Fig. 4. Time course of forskolin-stimulated cAMP accumulation in S49 wild-type cells, total (○), extracellular (□), and intracellular (△) cAMP. In this representative experiment, extracellular cAMP was determined as described in Methods and intracellular cAMP was calculated as the difference between total and extracellular cAMP. This experiment was repeated eight times with similar results.

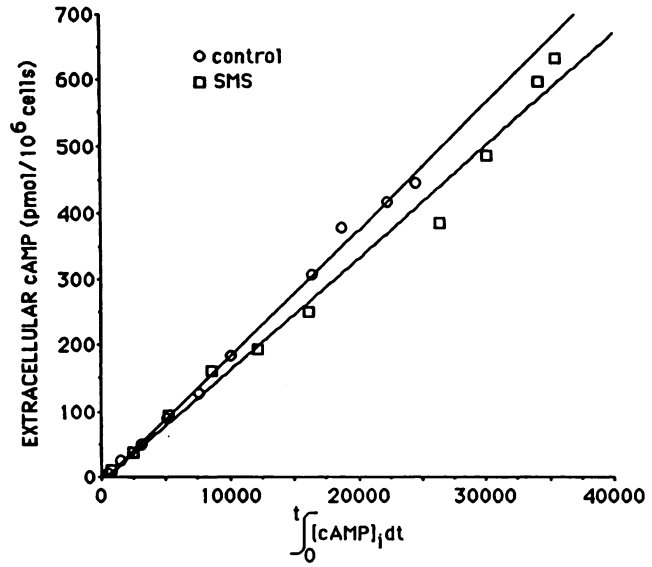

Fig. 5. Egress of cAMP from S49 wild-type cells after forskolin stimulation of both control (○) and SMS-pretreated cells (□). In this representative experiment, extracellular and intracellular cAMP were determined as described in Methods. The area under the time course curve for intracellular cAMP accumulation ($_0\int^t$[cAMP]$_i dt$) was calculated by numerical integration. A straight-line relationship for extracellular cAMP ([cAMP]$_e$) versus $_0\int^t$[cAMP]$_i dt$ indicates a first order process for egress, defined by an egress constant k_e (the slope of the line). This experiment was repeated eight times with similar results.

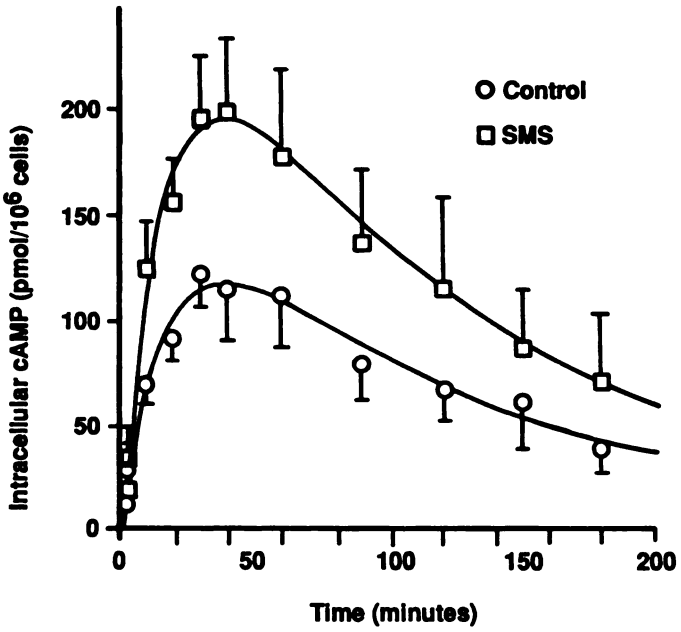

Fig. 6. Time course of forskolin-stimulated intracellular cAMP accumulation in S49 wild-type cells, either controls (○) or pretreated with 10^{-6} M SMS for 24 hr (□). After pretreatment, cells were washed free of drug and stimulated with 10^{-4} M forskolin for various times. The early time points (0.5 to 3.0 min) used to estimate synthetic rates are based on total cAMP at these times. At longer times, intracellular cAMP was calculated from the total and extracellular cAMP. Data *points* are the means ± standard errors of eight experiments. The *curves* were generated by computer modeling to a mathematical expression for cAMP dynamics (see Methods).

total cAMP accumulation by 120 to 180 min. The intracellular cAMP reaches a peak at about 30 min after forskolin stimulation and then declines to about one third of the peak value after 120 min. The presence of a peak in the time course suggests desensitization of forskolin stimulation.

Because our data indicate that egress of cAMP from S49 cells may be a large component of elimination of cAMP from the cells (the other component being degradation), we measured the rate of egress of cAMP from control and SMS-pretreated cells. The comparison of transport of cAMP out of the two groups of cells is shown in Fig. 5. The egress constant k_e was not significantly different in control cells (0.0306 ± 0.0068 min^{-1}) when compared with SMS-pretreated cells (0.0250 ± 0.0049 min^{-1}). Therefore, a difference in the rate of egress does not appear to be a component of the SMS-induced increase in total cAMP accumulation.

The dynamics of intracellular cAMP accumulation in S49 wild-type cells after forskolin stimulation are pictured in Fig. 6. In this series of eight experiments, control and SMS-pretreated cells were stimulated with forskolin as before and an extra aliquot of cells was taken for measurement of extracellular cAMP, in order to calculate the intracellular cAMP as described in Methods. The mean initial synthetic rate from individual

experiments was again significantly higher (about 62%) for SMS-pretreated cells (14.02 ± 1.23 pmol of cAMP/10^6 cells/min) than for control cells (8.66 ± 1.21 pmol of cAMP/10^6 cells/min). A peak of intracellular cAMP accumulation for both groups occurred at 30 to 40 min after forskolin addition, followed by a decline to about one third of peak values by 180 min. These time courses of intracellular cAMP accumulation were fit to a mathematical expression for changes in cAMP concentration with time (see Methods). Estimates of three parameters (the initial synthetic rate, the rate constant for desensitization, and the rate constant for elimination) were made for both control and SMS-pretreated cells (Table 1). The computer-derived estimates of the initial synthetic rates agree well with the initial slopes of the time course curves calculated by linear regression. Computer-derived estimates for both the desensitization rate and the elimination rate were similar in control and SMS-pretreated cells. By subtracting the mean values for egress rate constants, which were calculated from individual experiments, from the computer estimates for elimination rate constants, we obtained estimates for degradation rate constants. Because the mean egress rate constants are similar, the calculated degradation rate constants are also similar for control and SMS-pretreated cells. The preceding assessments obtained from the mean data of eight experiments were not altered when the individual experiments were individually modeled and the resulting parameters averaged (not shown).

The preceding results demonstrated that the initial rate of forskolin-stimulated cAMP accumulation was increased by treatment of wild-type S49 cells with SMS. We next examined the effect of pretreating S49 mutants deficient in cAMP-dependent protein kinase with SMS for 24 hr. In some preliminary experiments, we compared the ability of SMS to acutely inhibit isoproterenol-stimulated cAMP accumulation in wild-type S49 cells with that in two independently selected S49 mutants that are deficient in protein kinase. Although there were some differences in the EC_{50} and maximal effect of SMS amongst these three groups of cells, SMS both potently and efficaciously inhibited cAMP accumulation in the kin$^-$ mutants 24.6.1 and 24.4.6 (Table 2).

Forskolin-stimulated total cAMP accumulation was measured in wild-type cells and each kin$^-$ mutant in the presence or absence of a 24-hr preincubation with SMS. The time courses for kin$^-$ mutant 24.6.1 are shown in Fig. 7. Control and SMS-pretreated kin$^-$ cells showed nearly identical time courses for forskolin-stimulated cAMP accumulation, unlike the difference exhibited by wild-type cells (Fig. 3). Estimates of the initial synthetic rates from paired experiments with each kin$^-$ mutant are presented in Table 3. Kin$^-$ mutant 24.6.1 had a slightly lower synthetic rate than wild-type, and kin$^-$ mutant 24.4.6 had a higher synthetic rate than wild-type cells. Neither kin$^-$ mutant exhibited an increased synthetic rate after SMS pretreatment, unlike the significant increase observed in wild-type cells in these paired experiments.

Discussion

This study provides evidence that SMS, which acutely inhibits adenylate cyclase activity in S49 cells, causes an enhanced sensitivity of the enzyme in wild-type cells after chronic treatment and withdrawal. As an assessment of the adenylate cyclase supersensitivity, we have relied on the increased forskolin-stimulated cAMP synthetic rate. Thus, S49 cells can be added to the growing list of different types of cells that exhibit an adaptive supersensitivity of adenylate cyclase after chronic treatment with inhibitory agonists (1).

Previous studies from other laboratories initially provided evidence for somatostatin inhibition of adenylate cyclase in S49 wild-type cells and cyc$^-$ and H21 mutants (12, 13). We have found similar inhibition of isoproterenol-stimulated cAMP accumulation in wild-type S49 cells by somatostatin and the somatostatin analog SMS. In these studies, we have used SMS for chronic treatments, to take advantage of the increased stability of the analog (14). Chronic SMS treatment of wild-type cells induced an enhanced response to isoproterenol-stimulated cAMP accumulation, and the degree of enhancement was dose-dependent. However, the dose response for SMS-mediated induction of supersensitivity was to the right of that for SMS inhibition of cAMP accumulation (Fig. 1). The apparent lower potency for the effect of chronic treatment could reflect some degradation of the SMS during the 24-hr pretreatment in culture or suggest that the relationship between suppression of cAMP accumulation by SMS and the development of enhanced cAMP response to forskolin is not a linear one. Alternatively, the difference in dose-response curves for inhibition and induction of supersensitivity could suggest that some response other than inhibition of cAMP accumulation per se is involved in the development of enhanced responsiveness.

Although chronic SMS treatment of wild-type S49 cells induced an increase in isoproterenol-stimulated cAMP accumulation at times subsequent to the peak accumulation, we did not detect an increase in initial synthetic rate (Fig. 2). This

TABLE 1

Initial synthetic rates and rate constants for desensitization and elimination (degradation plus egress) for control and SMS-pretreated S49 wild-type cells after forskolin stimulation

Data are from the eight forskolin-stimulated cAMP time course experiments summarized in Fig. 6. V_{S0} (pmol of cAMP/10^6 cells/min) is the computer-derived initial synthetic rate. K_{desens} (min^{-1}) is the computer-derived desensitization rate constant. K_{elim} (min^{-1}) is the computer-derived elimination rate constant. K_e (min^{-1}) is the egress rate constant, calculated as the mean of individual experiments. K_d (min^{-1}) is the degradation rate constant, calculated as the difference between K_{elim} and K_e. Computer-derived values are best estimates and their standard errors for the mathematical model solved by the MKMODEL program (see Methods). Other data are means ± standard error. The computer-derived estimates of V_{S0} agree well with both the initial slopes of the time course curves (control, 10.21; SMS-pretreated, 15.86) and the means of the initial synthetic rates calculated from individual experiments (control, 8.66 ± 1.21; SMS-pretreated, 14.02 ± 1.23).

	V_{S0}	K_{desens}	K_{elim}	K_e	K_d
	pmol of cAMP/10^6 cells/min		*min^{-1}*		
Control	9.97 ± 0.57	0.00790 ± 0.00084	0.0633 ± 0.0066	0.0306 ± 0.0068	0.0327 ± 0.0134
SMS-pretreated	16.36 ± 0.67[a]	0.00808 ± 0.00056	0.0624 ± 0.0045	0.0250 ± 0.0049	0.0374 ± 0.0094

[a] Differs from control, $p < 0.001$

TABLE 2

SMS inhibition of isoproterenol-stimulated cAMP accumulation in S49 wild-type and two kin⁻ mutant cells

Cells were stimulated with isoproterenol (10^{-6} M) for 10 min in the presence of phosphodiesterase inhibitors and from 10^{-11} to 10^{-6} M SMS as described in Methods. Data are means ± standard errors of five experiments.

	EC_{50}	Inhibition at 10^{-6} M SMS
	M	%
S49 wild-type	$2.06 \pm 0.94 \times 10^{-9}$	56.1 ± 2.7
kin⁻ mutant 24.6.1	$7.98 \pm 0.85 \times 10^{-9}$	44.6 ± 2.6
kin⁻ mutant 24.4.6	$1.23 \pm 0.71 \times 10^{-9}$	41.5 ± 3.8

contrasts with the increased initial synthetic rate observed for forskolin stimulation. The initial rate of change in isoproterenol-stimulated cAMP concentration with time (the initial synthetic rate) appeared linear for at most 30 sec. Because some decrement in rate was occasionally apparent even during this short time interval, the actual initial rate is undoubtedly difficult to determine. This finding is consistent with a very rapid desensitization of the β-adrenergic receptor-mediated response. After agonist stimulation, the half-life for disappearance of β receptors from the cell surface of S49 cells has been estimated to be approximately 1 min (24). Also, the β receptor has been shown to be maximally phosphorylated, a likely component of the desensitization process, within 1 min of agonist addition (25). Therefore, we feel that our estimate of similar isoproterenol-stimulated initial synthetic rates in control and SMS-pretreated cells does not rule out some actual difference that cannot be detected by the method used. Despite the apparent lack of difference in initial synthetic rate after isoproterenol stimulation of SMS-pretreated cells, there was a significant increase in accumulation of cAMP at times subsequent to the peak (Fig. 2). Such a difference could be due to a decreased elimination of cAMP in the SMS-pretreated cells or a difference in the rate of change of cAMP synthesis as a function of time.

There was a peak and subsequent fall in forskolin-stimulated intracellular cAMP concentration. Such a time course for cAMP accumulation is also indicative of desensitization (22, 26). One previous study suggested that there was minimal desensitization of forskolin stimulation in S49 cells, at least over a 30-min period, which was the longest time studied (19). Another report provided evidence for a slowly developing peak and fall in cAMP accumulation after forskolin treatment of S49 cells (27). Because the decline in cAMP concentration occurred only after a prolonged period (2 hr) and was prevented by a phosphodiesterase inhibitor, those investigators suggested that the refractoriness may have been due to induction of phosphodiesterase activity (27). Our time course data obtained under different experimental conditions (at a higher forskolin concentration, in the absence of serum, and using a different method for determining intracellular cAMP) suggest a more rapid and extensive desensitization of forskolin stimulation.

Egress of cAMP from the cell into the extracellular medium, although well documented in some other cells (23), has been relatively unappreciated in S49 cells. Studies on elimination of cAMP from S49 cells have for the most part combined degradation and egress as a single factor in defining elimination or turnover (20, 22, 26, 28). The assumption appears to have been that egress is a minor component of elimination. Only in a low phosphodiesterase S49 mutant has egress been noted to be a substantial component of elimination (20). In our experiments, the rate of egress was linearly related to the intracellular concentration of cAMP. This relationship suggested a first-order process for egress of cAMP from S49 cells, as has been observed for other cells (23). The rate constant for egress was similar in control and SMS-pretreated cells. Therefore, this component of the dynamics of does not appear to contribute to the difference in accumulation of cAMP in response to forskolin stimulation in the SMS-pretreated cells. Furthermore, the proportion of extracellular cAMP approached 90% of the total cAMP after 2 hr of forskolin stimulation. A similarly high proportion was observed after 1 hr of isoproterenol stimulation (data not shown). In addition, in comparing our mean values for egress rate constants from individual experiments with the computer estimates for elimination rate constants (Table 1), there is an indication that egress is a significant component (up to about 50%) of elimination.

We have attempted to model the forskolin-stimulated intracellular cAMP time course to a function incorporating the initial synthetic rate and rate constants for desensitization, degradation, and egress. The computer-generated curves for the mean data of forskolin-stimulated intracellular cAMP accumulation are displayed in Fig. 6. These curves fit the experi-

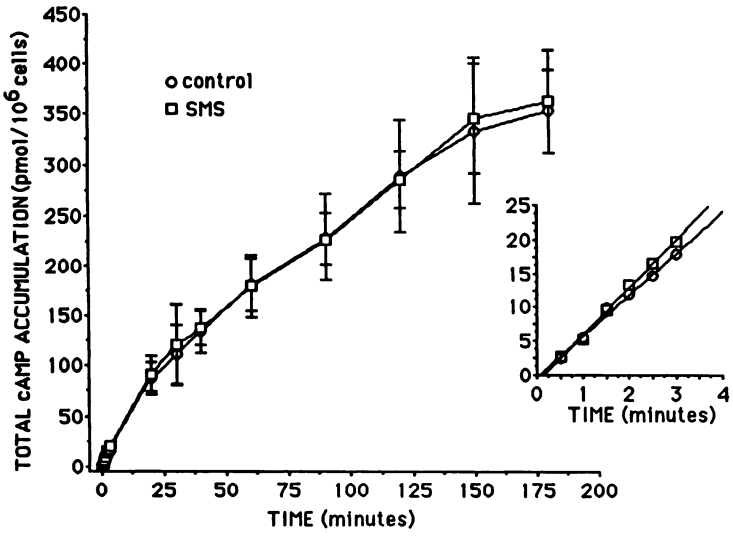

Fig. 7. Time courses of forskolin-stimulated total cAMP accumulation in kin⁻ mutant 24.6.1, either untreated (○) or pretreated with 10^{-6} M SMS for 24 hr (□). Experiments with kin⁻ cells were performed in parallel with additional studies in wild-type cells (Fig. 3). Data *points* are the means ± standard errors SEM of six experiments. *Inset*, the early time courses (0.5 to 3.0 min) used to estimate the initial synthetic rates.

TABLE 3

Forskolin-stimulated initial cAMP synthetic rates in S49 wild-type and two kin⁻ mutant cells

Cells were either untreated controls or pretreated with SMS for 24 hr. Initial synthetic rates (pmol of cAMP/10^6 cells/min) were estimated from the slopes of the early time courses (0.5 min to 3.0 or 4.0 min) of total cAMP accumulation after forskolin (10^{-4} M) stimulation. Data are means ± standard errors. Statistical comparisons were by the paired *t* test.

	n[a]	Initial Synthetic Rate		Significance
		Control	SMS-pretreated	
S49 wild-type	7	8.89 ± 1.60	13.44 ± 1.81	$p < 0.001$
kin⁻ mutant 24.6.1	7	5.97 ± 1.30	6.57 ± 1.57	NS[b]
S49 wild-type	6	8.52 ± 0.38	14.18 ± 0.97	$p < 0.005$
kin⁻ mutant 24.4.6	6	14.77 ± 1.63	16.36 ± 0.86	NS

[a] Number of experiments.
[b] NS, not significant.

mental data quite well. Thus, the data can be modeled to a simple mathematical expression for cAMP dynamics, based on the given assumptions. The apparent good fit, however, does not preclude other possible models that may be more complex, for example incorporating a term for resensitization or allowing for variation in the rate "constants" as a function of time. Nevertheless, the mathematical modeling has enabled a general conclusion that the difference in forskolin-stimulated cAMP time courses between control and SMS-pretreated wild-type S49 cells can be explained largely on the basis of a difference in initial synthetic rates.

In experiments with two independently isolated S49 mutants lacking cAMP-dependent protein kinase activity, chronic SMS treatment and withdrawal did not result in an increase in forskolin-stimulated cAMP synthetic rate. Unlike wild-type S49 cells, the kin⁻ mutants are insensitive to increases in cAMP concentration, whether from hormone stimulation or treatment with cAMP analogs, as revealed by an absence in cAMP-mediated cytotoxicity or phosphorylation of protein substrates (9, 10, 29). Presumably, the kin⁻ mutants would also be insensitive to any decrease in cAMP concentration. In wild-type S49 cells, increased cAMP, resulting from either stimulatory hormone treatment or direct treatment with cAMP analogs, has been correlated with cytolysis, phosphorylation of specific protein substrates, and other protein modifications (9, 10, 29). The effect of adenylate cyclase inhibition by somatostatin on specific phosphorylations has apparently not been studied (10). Treatment with this inhibitory hormone could lower basal cAMP levels and possibly thereby decrease the level of phosphorylation of some specific protein substrates in wild-type cells. Alternatively, changes in cAMP-dependent protein kinase activity could lead to alterations in gene expression in S49 cells treated with SMS. This framework represents perhaps the most straightforward hypothetical mechanism whereby treatment with inhibitory agonists triggers the development of an enhanced adenylate cyclase response. Our results with the kin⁻ mutants lend support to this hypothesis. Because the kin⁻ mutants should be insensitive to any changes in cAMP concentration, treatment with agonists that inhibit adenylate cyclase should have no effect on any other response that is dependent solely on the resultant decrease in cAMP concentration. Accordingly, both kin⁻ mutants were found to be unresponsive to chronic SMS treatment with respect to the subsequent adenylate cyclase response. Therefore, our data from kin⁻ mutants would suggest that the supersensitive adenylate cyclase response in wild-type S49 cells is dependent on the decreased cAMP concentration resulting from treatment with inhibitory drugs.

What other possibilities might account for the lack of SMS-induced adenylate cyclase supersensitivity in kin⁻ S49 cells? An alternative hypothesis invokes some other response to somatostatin, unrelated to inhibition of adenylate cyclase and decreases in cAMP, but still ultimately involving cAMP-dependent protein kinase. Under this scenario, kin⁻ mutants would be insensitive to this hypothetical somatostatin response because of their deficiency in protein kinase activity. In certain other cells, somatostatin and various inhibitors of adenylate cyclase have in fact been shown to have effects other than cyclase inhibition (5–8). However, there is no known relationship between these effects and cAMP-dependent protein kinase activity. Alternatively, both kin⁻ mutations could have additional mutations unrelated to the deficiency in cAMP-dependent protein kinase activity. In other words, it is possible that the kin⁻ mutants could be unresponsive to chronic treatment with SMS independently of their kinase deficit. The fact that an additional deficiency could randomly occur during the cAMP-mediated selection process for kinase deficiency is evident from the 24.4.6 kin⁻ mutant. This mutant has an additional deficiency in cAMP phosphodiesterase activity (9, 11, 17), but this is not relevant to the adenylate cyclase supersensitivity. However, it seems unlikely that both independently isolated mutants would have the same randomly occurring deficits.

In contrast to the difference between S49 wild-type and kin⁻ cells with respect to forskolin-stimulated cAMP accumulation after chronic treatment with a somatostatin analog, a recent report has provided evidence that the kin⁻ mutant is similar to wild-type in an acute somatostatin response. Both wild-type and kin⁻ cells exhibit a translocation of the β-adrenergic receptor kinase from the cytosol to the plasma membrane after a 20-min incubation with somatostatin, similar to the translocation caused by isoproterenol (30). The somatostatin-induced translocation is associated with desensitization of somatostatin inhibition of adenylate cyclase activity (30). Because the translocation and desensitization occur in kin⁻ cells, these phenomena are apparently not involved in the induction of adenylate cyclase supersensitivity after chronic SMS treatment and withdrawal in wild-type S49 cells. These findings with kin⁻ cells also suggest that at least an acute phase of desensitization can occur without a subsequent withdrawal response. It is interesting to note that opiate-mediated desensitization is not necessarily correlated with the adenylate cyclase withdrawal response in NG 108-15 cells (31) and that opiate tolerance and dependence both in isolated tissues and *in vivo* may not necessarily reflect a common adaptive change (32).

In summary, the lack of adenylate cyclase supersensitivity in kin⁻ S49 cells pretreated with SMS provides evidence for the importance of cAMP-dependent protein kinase in the induction of this response in wild-type S49 cells. This observation suggests that the inhibition of adenylate cyclase and the consequent lowering of cellular cAMP concentration may be the crucial signal for induction of the change in adenylate cyclase activity, although other hypothetical mechanisms may also be invoked.

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