0026-895X/91/0399-08$03.00/0

MOLECULAR PHARMACOLOGY, 39:399-406

Serum-Induced Sensitization of Cyclic AMP Accumulation in 1321N1 Human Astrocytoma Cells

ROBERT A. JOHNSON,[1] LYNNETT J. ARNESON-ROTERT, JOANNE M. HOFFMAN, and MYRON L. TOEWS

Department of Pharmacology, University of Missouri-Columbia, Columbia, Missouri 65212 (R.A.J., M.L.T.), and Department of Pharmacology, University of Nebraska Medical Center, Omaha, Nebraska 68198-6260 (L.J.A.-R., J.M.H., M.L.T.)

Received July 26, 1990; Accepted December 21, 1990

SUMMARY

Exposure of 1321N1 human astrocytoma cells to fresh medium containing fetal bovine serum induced a marked increase in the subsequent ability of isoproterenol and forskolin to stimulate cAMP accumulation in intact cells, compared with cells exposed to fresh medium without serum. This "sensitization" of cAMP accumulation by serum was dose dependent, occurred rapidly, was maintained in the continuing presence of serum, and reversed rapidly upon removal of serum. Preliminary characterization of the sensitizing factor(s) in serum has been performed, but the factor(s) remain to be identified. Sensitization appeared to result from an increase in maximal response and not from changes in the potency of isoproterenol or forskolin. The protein kinase C inhibitor staurosporine inhibited serum-induced sensitization. Furthermore, down-regulation of protein kinase C almost completely eliminated the subsequent ability of serum to induce sensitization, indicating involvement of protein kinase C in the serum effect. Pretreatment of cells with pertussis toxin also markedly reduced subsequent sensitization induced by serum, suggesting involvement of a pertussis toxin-sensitive guanine nucleotide-binding protein in the pathway for serum-induced sensitization. The rate of cAMP degradation was not changed in sensitized cells, but some increase in adenylyl cyclase activity was retained in broken cell preparations from sensitized cells, suggesting increased synthesis of cAMP by adenylyl cyclase as the mechanism for sensitization.

Many hormones, neurotransmitters, and other cellular regulators, as well as many related drugs, mediate their effects on cells by altering the intracellular concentrations of the "second messenger" cAMP. Most of these agents exert their effects by activating cell surface receptors coupled to either stimulation or inhibition of the enzyme adenylyl cyclase, thus changing the rate of synthesis of cAMP (1, 2). Some agents, most notably forskolin, bypass cell surface receptors to activate directly the adenylyl cyclase catalytic unit or its interaction with the guanine nucleotide-binding proteins associated with adenylyl cyclase (3). In a few cases, changes in intracellular cAMP concentration are mediated by changes in the rate of degradation of cAMP by phosphodiesterases rather than by changes in the rate of synthesis by the adenylyl cyclase system (4–6).

The ability of the adenylyl cyclase system to respond to these various regulators is subject to longer-term adaptive modulation as well. The most well characterized of these adaptive changes is the decrease in responsiveness, referred to as desensitization, that generally occurs during the course of prolonged or repeated exposure to agents that stimulate adenylyl cyclase (7, 8). On the other hand, adaptive increases in adenylyl cyclase activity have been observed following pretreatment with agents that inhibit adenylyl cyclase, and these may represent a mechanism for desensitization of inhibitory responses (9–11).

Many recent reports have provided evidence that the activity of the adenylyl cyclase system can also be modulated by exposure of cells to agents that do not themselves directly interact with the adenylyl cyclase system. In particular, phorbol esters and other direct activators of protein kinase C (12) have been shown to alter the adenylyl cyclase system, with both increases and decreases in activity occurring in different cells and tissues (Ref. 13 and references therein). These effects have been variously attributed to changes in receptors, guanine nucleotide-binding proteins, adenylyl cyclase activity, or phosphodiesterase activity. Similar effects have been observed following pretreatment of cells with agonists acting at receptors coupled to polyphosphoinositide hydrolysis, which leads indirectly to activation of protein kinase C by formation of endogenous diacylglycerol (Ref. 13 and references therein).

In a previous study (13), we showed that activation of protein kinase C, either directly with phorbol esters or indirectly with agonists acting at receptors coupled to polyphosphoinositide

This work was supported in part by National Institutes of Health Research Grant GM34500 and by Research Career Development Award HL01593 to M.L.T.
[1]Current address: Department of Physiology, University of Missouri-Columbia, Columbia, MO 65212.

ABBREVIATIONS: PMA, phorbol, 12-myristate,-13-acetate; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; G_i, inhibitory guanine nucleotide-binding protein.

hydrolysis, led to an increase in the subsequent stimulation of cAMP accumulation in intact 1321N1 human astrocytoma cells. We refer to these phenomena as "sensitization." During the course of these studies, we observed markedly greater stimulation of cAMP accumulation in cells pretreated in serum-containing medium than in cells pretreated in serum-free medium. The present studies characterize and investigate the mechanism of this serum-induced sensitization phenomenon.

Experimental Procedures

Materials. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): PMA, carbachol, isoproterenol, IBMX, prostaglandin E_1, and trypsin. Forskolin was purchased from Calbiochem (San Diego, CA), heparin from Elkins-Sinn (Cherry Hill, NJ), pertussis toxin from List Biologicals Laboratories, Inc. (Campbell, CA), staurosporine from Kyowa Hakko USA (New York, NY), and [^{3}H] adenine from ICN Radiochemicals (Irvine, CA). Cholera toxin was a gift from R. Finkelstein (University of Missouri, Columbia, MO). Cell culture medium and trypsin were from GIBCO (Grand Island, NY). Fetal and newborn bovine sera were from GIBCO; horse serum was from Hyclone (Logan, UT).

Cell culture. 1321N1 human astrocytoma cells were grown in monolayer culture at 37°, in a humidified atmosphere containing 8% CO_2. Growth medium was low glucose DMEM supplemented with 5% fetal bovine serum. Confluent monolayers were removed from tissue culture flasks with 0.05% trypsin and plated at a density of 20,000 cells/cm^2 in plastic tissue culture dishes. Experiments were performed on cells 4 or 5 days after plating, when they were just confluent.

Assays of intracellular cAMP accumulation. cAMP accumulation in intact cells grown on 35-mm dishes was determined by the method of Shimizu *et al.* (14), as previously described (13). Growth medium was aspirated and the cell sheets were washed once with 2 ml of DMEM buffered to pH 7.4 with 20 mM HEPES (DMEM-HEPES). Except for the time course experiments shown in Figs. 1 and 2, cells were then incubated for 1 hr at 37° in 1 ml of DMEM-HEPES containing 2 μCi of [^{3}H]adenine. Serum and/or pretreatment drugs, or the appropriate vehicle, were included during this prelabeling step. Prelabeling medium and pretreatment drugs were aspirated, and the cells were washed and then incubated for 2 min at 37° in DMEM-HEPES, in the absence or presence of various agents to stimulate cAMP accumulation. This stimulation medium was then aspirated, and 1 ml of 5% trichloroacetic acid was added to stop further reactions

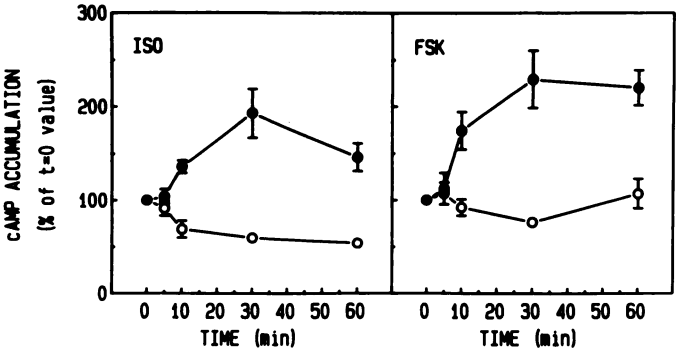

Fig. 1. Time courses for serum-induced changes in intact cell cAMP accumulation. Cells were washed free of growth medium and then incubated for the indicated times in DMEM-HEPES in the absence (○) or presence (●) of 5% fetal bovine serum. Cells were then washed, and cAMP accumulation stimulated by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was measured in 2-min assays. Data are expressed as the percentage of activity in untreated control cells and are the means ± standard errors from three experiments, each performed in triplicate. The percentage of conversion values in control cells (100% values in figure) were 0.29 ± 0.06 for isoproterenol and 0.14 ± 0.03 for forskolin.

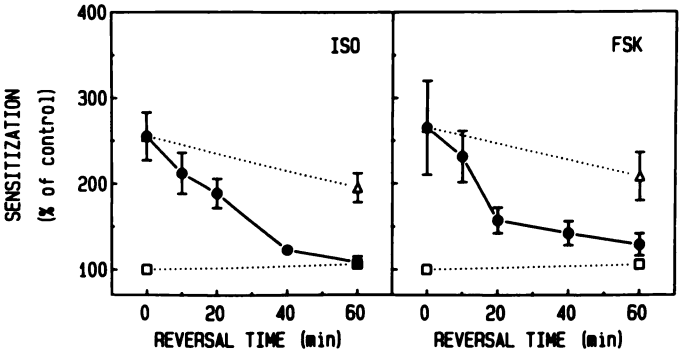

Fig. 2. Time courses for reversal of serum-induced sensitization. Cells were incubated for 60 min in DMEM-HEPES in the absence (□) or presence (●, △) of 5% fetal bovine serum. Cells were then washed and incubated for the indicated times in the absence (□, ●) or presence (△) of serum. Finally, cells were again washed, and cAMP accumulation stimulated by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was measured in 2-min assays. Data are expressed as the percentage of activity in control cells (incubated for 60 min in the absence of serum) and are the means ± standard errors from three experiments, each performed in triplicate. The percentage of conversion values in control cells (100% values in figure) were 0.30 ± 0.10 for isoproterenol and 0.35 ± 0.18 for forskolin.

and to extract cellular nucleotides. For the time course experiments, cells were pretreated in the absence or presence of serum for various times in DMEM-HEPES, as described above, but labeling of cells with [^{3}H]adenine was for only 2 min during the stimulation with isoproterenol or forskolin, as previously described (15). In all experiments, [^{3}H] cAMP and [^{3}H]ATP in the trichloroacetic acid extracts were then separated by sequential chromatography over Dowex and alumina columns, essentially as described (4). Radioactivity in the column eluates was determined by liquid scintillation counting in 10 ml of BudgetSolve. Accumulation of [^{3}H]cAMP was then expressed as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP ([^{3}H]cAMP/([^{3}H] cAMP + [^{3}H]ATP)). Incorporation of ^{3}H into the ATP fraction was typically 500,000 cpm/dish, and this value was not markedly altered by any of the pretreatments.

Assays of adenylyl cyclase activity in broken cell preparations. Cells grown on 100-mm dishes were incubated for 60 min under various conditions to induce sensitization, as described above. Cells were then washed once with 5 ml of ice-cold hypotonic lysis buffer (1 mM HEPES, pH 7.4, 1 mM EDTA) and incubated for 20 min on ice in an additional 5 ml of lysis buffer. Lysis buffer was aspirated, and cells were scraped from the dishes with a rubber policeman. The lysates were then homogenized for 3 sec with a Tissumizer (Tekmar, Inc., Cincinnati, OH), at setting 90.

Adenylyl cyclase activity in the lysates was assayed by the method of Salomon *et al.* (16), essentially as modified by Clark *et al.* (17). The assay solution consisted of 60 mM HEPES (pH 7.7), 0.5 mM EDTA, 1.8 mM $MgCl_2$ (low [Mg^{2+}]) or 5.4 mM $MgCl_2$ (high [Mg^{2+}]), 8 mM creatine phosphate, 16 units/ml creatine phosphokinase, 10 μM GTP, 0.12 mM IBMX, 0.2 mM ATP, approximately 0.3 μCi of [α-^{32}P]ATP, and 40 μl of cell lysate, in a final volume of 100 μl. Incubation was for 30 min at 37°. The assays were terminated by addition of 1 ml of 5% trichloroacetic acid containing 0.1 mM cAMP and approximately 30,000 cpm/ml [^{3}H]cAMP, to normalize for recovery. Nucleotides were separated by sequential chromatography over Dowex and alumina columns, as described above. Radioactivity in the column eluates was determined by liquid scintillation counting in 10 ml of BudgetSolve. Recovery of [^{32}P]cAMP was normalized to recovery of [^{3}H]cAMP in the same sample. Protein concentration in the lysates was determined by the method of Lowry *et al.* (18).

Data anaylsis. Computerized curve-fitting of dose-response curves was performed by nonlinear regression using GraphPAD (GraphPAD Software, San Diego, CA). Statistical analysis of the significance of

changes induced by the various treatments was by analysis of variance, using the Number Cruncher Statistical System (NCSS, Kaysville, UT).

Results

Characterization of serum-induced sensitization. During the course of our studies of carbachol- and PMA-induced sensitization of cAMP accumulation, we observed that stimulation of cAMP accumulation was consistently higher in cells pretreated in their original growth medium containing serum than in cells where the pretreatment was carried out in fresh medium without serum. Furthermore, the fold sensitization induced by pretreatment with carbachol or with PMA was markedly lower in cells pretreated in growth medium containing serum, because of the higher value in control cells. Therefore, our previous studies (13) of carbachol- and PMA-induced sensitization used pretreatment in DMEM-HEPES in the absence of serum, to increase the magnitude of the observed sensitization. In the present studies, we have further investigated the mechanism of the apparent sensitization of cAMP accumulation induced by serum.

The time courses for the serum-induced changes in sensitivity of the cAMP system were investigated (Fig. 1). When original growth medium containing serum was removed from cells and replaced with DMEM-HEPES without serum, cAMP accumulation stimulated by isoproterenol exhibited a time-dependent decrease to a stable level about 50% lower than that in cells maintained in growth medium with serum. In contrast, when original growth medium was replaced by DMEM-HEPES containing fresh serum, there was a marked increase in isoproterenol-stimulated cAMP accumulation to levels 2- to 3-fold higher than those in cells incubated in DMEM-HEPES without serum. Stimulation of cAMP accumulation by forskolin was relatively constant following the switch from original growth medium with serum to DMEM-HEPES without serum. Replacement of growth medium with DMEM-HEPES containing fresh serum led to sensitization of forskolin stimulation to levels 2- to 3-fold higher than those in cells incubated in fresh medium without serum. For both isoproterenol stimulation and forskolin stimulation, there was little effect following 5 min of pretreatment, half-maximal sensitization occurred between 10 and 30 min of pretreatment, and the increase in activity was retained in the continuing presence of serum for times up to 4 hr (the longest time tested; data not shown). For both isoproterenol and forskolin stimulation, sensitization was essentially completely reversible upon removal of serum, with a half-time of approximately 30 min in both cases (Fig. 2).

The serum concentration dependence of sensitization was investigated (Fig. 3). Half-maximal sensitization of both isoproterenol and forskolin stimulation occurred with approximately 1% fetal bovine serum. Newborn bovine serum and horse serum also induced sensitization, with horse serum apparently exhibiting somewhat greater effects. Bovine serum albumin, at concentrations similar to the total protein concentration in the various dilutions of serum, did not induce sensitization, suggesting that sensitization is not simply a nonspecific protein-induced phenomenon. Several different lots of serum have been used during the course of these studies, and similar extents of sensitization have been observed with each.

Although the identity of the factor(s) in serum responsible for sensitization has not been established, preliminary studies on the nature of the factor(s) have been performed (Table 1).

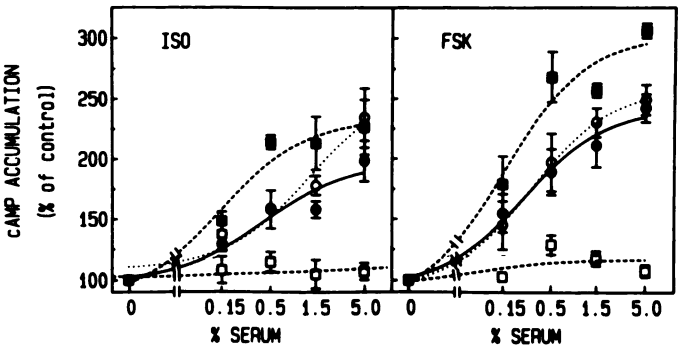

Fig. 3. Concentration and serum source dependence of sensitization. Cells were incubated for 60 min in DMEM-HEPES containing the indicated concentrations of fetal bovine serum (●——●), newborn bovine serum (○· · · · ·○), or horse serum (■– – –■) or equivalent protein concentrations (100% serum equals 50 mg/ml protein) of bovine serum albumin (□– – –□). Cells were then washed, and cAMP accumulation stimulated by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was measured in 2-min assays. Data are expressed as the percentage of the activity observed in control cells and are the means ± standard errors from three experiments, each performed in triplicate. The percentage of conversion values in control cells (100% values in figure) were 0.48 ± 0.03 for isoproterenol and 0.20 ± 0.03 for forskolin.

TABLE 1

Characterization of the sensitizing activity in serum

Cells were incubated for 60 min in the absence or presence of the indicated pretreatment agents (all at 5%) and then washed. cAMP accumulation stimulated by 10 μM isoproterenol and by 30 μM forskolin was then measured in 2-min assays. Because the absolute values for fold sensitization induced by control serum were variable from experiment to experiment, values for sensitization by the various pretreatments are presented as the percentage of the sensitization induced by control serum in the same experiment. Values are the means ± standard errors from at least four experiments.

Pretreatment (60 min)	Sensitization	
	Isoproterenol	Forskolin
	% of effect with control serum	
Fetal bovine serum		
Untreated	100	100
Dialyzed[a]	99 ± 8	152 ± 24
Heat-denatured (56°, 30 min)	87 ± 19	73 ± 12
Boiled (100°, 1–2 min)	75 ± 14	55 ± 6
Trypsin-treated[b]	90 ± 15	122 ± 14
Adult bovine serum	123 ± 20	111 ± 11
Adult bovine plasma[c]	60 ± 2	69 ± 13
Adult bovine serum + heparin[c]	77 ± 9	81 ± 15
Fetal bovine serum + heparin[c]	49 ± 2	59 ± 9

[a] Dialyzed against 20 mM HEPES, pH 7.4, for 72 hr at 4°, using 12–14-kDa cutoff Spectra/Por membrane (Fisher Scientific).
[b] Incubated with 1 mg/ml trypsin for 30 min at 37°.
[c] Heparin was present at 0.25 units/ml final concentration.

Extensive dialysis did not remove the sensitizing activity from serum. The activity was relatively stable to standard heat denaturation (30 min at 56°) and was quite stable even to brief boiling. The activity was not destroyed by treatment with trypsin. Sensitizing activity was present in both adult bovine serum and plasma obtained from animals at slaughter, although the activity in plasma was somewhat lower than that in serum. This lower activity can apparently be accounted for by an inhibitory effect of the heparin that is present in plasma, because addition of heparin to either fetal or adult serum reduced sensitization to values similar to those seen with plasma.

To determine whether the factor(s) in serum might induce sensitization through well characterized neurotransmitter receptors, the effects on sensitization of a variety of neurotrans-

mitter receptor antagonists were tested (data not shown). The effect of serum was not blocked by atropine or by diphenhydramine, suggesting that the effect is not mediated by activation of muscarinic or histaminergic receptors, respectively, which have been shown previously to induce sensitization in these cells (13). Serum-induced sensitization also was not prevented by phentolamine, propranolol, *cis*-flupenthixol, or methiothepin, suggesting the lack of involvement of α- and β-adrenergic, dopaminergic, and serotoninergic receptors, respectively.

cAMP accumulation in sensitized cells. The specificity of serum-induced sensitization was investigated by comparing the effects of serum on stimulation of cAMP accumulation by various stimuli (Table 2). Although basal activity in these cells is low and difficult to measure accurately, there appeared to be a small but statistically significant decrease in basal activity following serum pretreatment. The decrease in basal activity observed in this group of experiments was somewhat greater than in most; the average decrease in basal activity from 16 additional experiments, including those in Figs. 5–7 (basal values not shown), was only 16 ± 5%. Stimulation by isoproterenol was increased by 1.9-fold and stimulation by forskolin was increased 3.8-fold in serum-pretreated cells. The combination of isoproterenol plus forskolin causes a synergistic increase in cAMP that is much greater than expected based on the effects of either agent alone (3). Pretreatment with serum led to a 1.6-fold increase in the ability of this combination to increase cAMP levels. Treatment of cells with cholera toxin elevates cAMP accumulation by about 8-fold. Interestingly, exposure to serum did not increase the cAMP accumulation in cholera toxin-treated cells. The combination of isoproterenol plus cholera toxin increased cAMP to a similar extent as isoproterenol alone, and serum caused a 2.5-fold increase in this response. The response to cholera toxin plus forskolin was much greater than the responses to either agent alone, and serum increased the response by 1.9-fold, greater than the effect of serum on cholera toxin stimulation but less than its effect on forskolin stimulation. The response to isoproterenol plus forskolin in cholera toxin-pretreated cells was similar to that in cells without cholera toxin, and the effect of serum was also similar, with a 1.3-fold increase in cAMP accumulation. Prostaglandin E_1 also stimulates cAMP accumulation in these cells, and in a separate set of experiments pretreatment with serum led to a 1.55 ± 0.09-fold increase in stimulation by prostaglandin E_1, similar to that for stimulation by isoproterenol in the same set of experiments (1.50 ± 0.11-fold; three experiments).

The effect of serum pretreatment on isoproterenol stimulation was to increase the maximal response, with little or no change in the potency of isoproterenol for stimulating cAMP accumulation (Fig. 4). Whether serum increases the potency or the maximal effect of forskolin could not be determined, because dose-response curves had not begun to plateau even at the highest concentrations that were testable due to limited solubility of forskolin (Fig. 4). Nonetheless, it is clear that serum led to a similar marked increase in stimulation at all concentrations of forskolin tested. Studies of the time courses of cAMP accumulation in control and serum-pretreated cells indicated that sensitization was not due to a change in the kinetics of cAMP accumulation, because similar extents of sensitization were observed at all time points tested, ranging from 1 to 10 min (data not shown).

Mechanisms involved in serum sensitization. Our previous studies of carbachol- and PMA-induced sensitization in these cells indicated that activation of protein kinase C can lead to sensitization of cAMP accumulation. Accordingly, we investigated the possible involvement of protein kinase C in serum-induced sensitization, by two different approaches. The effects of the protein kinase C inhibitor staurosporine (19) on serum-induced sensitization were determined (Fig. 5). The effects on PMA-induced sensitization were also determined, as a control for the effectiveness of staurosporine in inhibiting protein kinase C activity. In these experiments, PMA sensitized isoproterenol stimulation by 2.1 ± 0.1-fold and sensitized forskolin stimulation by 2.8 ± 0.3-fold in the absence of staurosporine. In the presence of staurosporine, PMA sensitized isoproterenol stimulation by only 1.4 ± 0.4-fold (61 ± 5% inhibition) and sensitized forskolin stimulation by only 1.3 ± 0.1-

TABLE 2

Specificity of serum-induced changes in cAMP accumulation stimulated by various agents

Cells were preincubated for 60 min in the absence or presence of 100 ng/ml cholera toxin, pretreated for an additional 60 min in the absence or presence of 5% fetal bovine serum, and then washed. cAMP accumulation was then measured in 2-min assays in the absence (basal) or presence of 10 μM isoproterenol or 30 μM forskolin or both isoproterenol and forskolin. Values for stimulation are presented as fold of basal activity in control cells and are the means ± standard errors from three experiments, each performed in triplicate. The basal value in control cells (1.0 in table) was 0.019 ± 0.006% of conversion.

Stimulator	Fold stimulation of cAMP accumulation		Fold sensitization
	Control	Serum-pretreated	
Basal	1.0	0.6 ± 0.0*	0.6
Isoproterenol	41 ± 8	77 ± 7*	1.9
Forskolin	9.2 ± 1.0	35 ± 9*	3.8
Isoproterenol + forskolin	199 ± 43	312 ± 66*	1.6
Cholera toxin	7.8 ± 1.3	7.7 ± 0.4	1.0
Cholera toxin + isoproterenol	29 ± 4	72 ± 12*	2.5
Cholera toxin + forskolin	85 ± 12	157 ± 17*	1.9
Cholera toxin + isoproterenol + forskolin	222 ± 38	297 ± 41	1.3

* Significantly different from the corresponding value in control cells ($p < 0.05$).

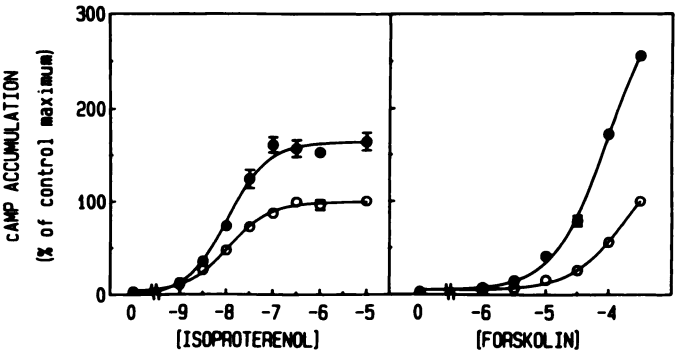

Fig. 4. Isoproterenol and forskolin concentration dependence of cAMP accumulation in control and serum-sensitized cells. Cells were incubated for 60 min in DMEM-HEPES in the absence (○) or presence (●) of 5% fetal bovine serum. Cells were then washed, and cAMP accumulation stimulated by the indicated concentrations of isoproterenol and forskolin was measured in 2-min assays. Data are expressed as the percentage of the maximal activity observed in control cells and are the means ± standard errors from three experiments, each performed in triplicate. The percentage of conversion values in control cells (100% values in figure) were 0.57 ± 0.10 for isoproterenol and 0.48 ± 0.06 for forskolin.

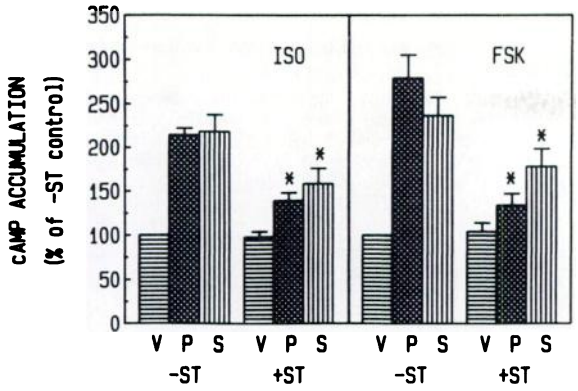

Fig. 5. Effects of staurosporine on sensitization. Cells were incubated for 60 min in DMEM-HEPES in the absence (vehicle) (*V*) or presence of 1 μM PMA (*P*) or 5% fetal bovine serum (*S*), each in the absence (*−ST*) or presence (*+ST*) of 1 μM staurosporine. Cells were then washed, and cAMP accumulation stimulated by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was measured in 2-min assays. Data for cAMP accumulation are presented as the percentage of the value obtained in control cells in the absence of staurosporine and are the means ± standard errors from five to eight experiments, each performed in triplicate. *, Sensitization in the presence of staurosporine was significantly less than that in the absence of staurosporine ($p < 0.05$). The percentage of conversion values in control cells (100% values in figure) were 0.44 ± 0.06 for isoproterenol and 0.32 ± 0.08 for forskolin.

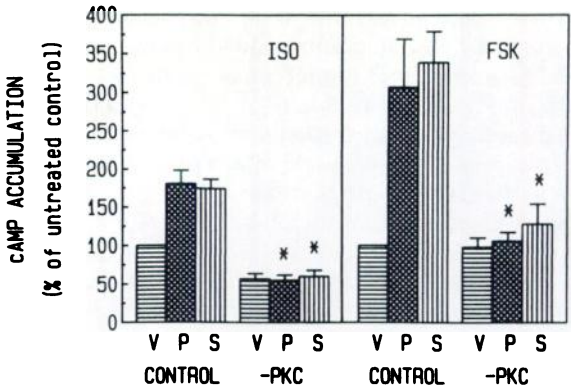

Fig. 6. Effects of protein kinase C down-regulation on sensitization. Cells were incubated overnight (18 hr) in the absence (*CONTROL*) or presence (*−PKC*) of 10 μM PMA to induce down-regulation of protein kinase C. Cells were then washed and incubated for 60 min in DMEM-HEPES in the absence (vehicle) (*V*) or presence of 1 μM PMA (*P*) or 5% fetal bovine serum (*S*). Finally, cells were again washed, and cAMP accumulation stimulated by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was measured in 2-min assays. Data for cAMP accumulation are presented as the percentage of the value obtained in vehicle-pretreated control cells and are the means ± standard errors from four experiments, each performed in triplicate. *, Sensitization in the protein kinase C down-regulated cells was significantly less than in the control cells ($p < 0.05$). The percentage of conversion values in control cells (100% values in figure) were 0.36 ± 0.03 for isoproterenol and 0.15 ± 0.02 for forskolin.

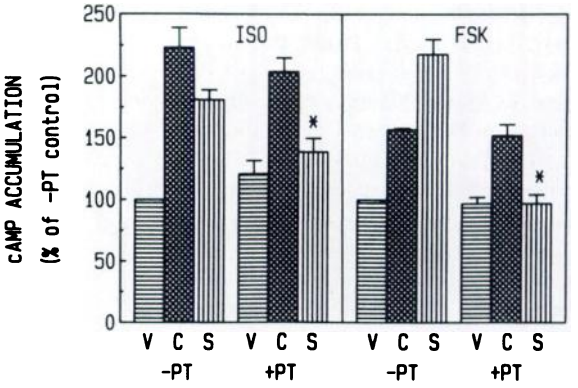

Fig. 7. Effects of pertussis toxin pretreatment of cells on sensitization. Cells were incubated overnight (18 hr) in the absence (*−PT*) or presence (*+PT*) of 100 ng/ml pertussis toxin to inactivate pertussis toxin-sensitive guanine nucleotide-binding proteins. Cells were then washed and incubated for 60 min in DMEM-HEPES in the absence (vehicle) (*V*) or presence of 100 μM carbachol (*C*) or 5% fetal bovine serum (*S*). Finally, cells were again washed, and cAMP accumulation stimulated by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was measured in 2-min assays. Data for cAMP accumulation are presented as the percentage of the value obtained in control cells not pretreated with pertussis toxin and are the means ± standard errors from five experiments, each performed in triplicate. *, Sensitization in the pertussis toxin-pretreated cells was significantly less than in the control cells ($p < 0.05$). The percentage of conversion values in control cells (100% values in figure) were 0.26 ± 0.06 for isoproterenol and 0.16 ± 0.06 for forskolin.

fold (87 ± 7% inhibition). Serum sensitized isoproterenol stimulation by 2.2 ± 0.2-fold and forskolin stimulation by 2.4 ± 0.2-fold in the absence of staurosporine. In the presence of staurosporine, serum sensitized isoproterenol stimulation by only 1.6 ± 0.2-fold (50 ± 9% inhibition) and sensitized forskolin stimulation by only 1.8 ± 0.2-fold (47 ± 10% inhibition). Thus, staurosporine inhibits sensitization induced by PMA and by serum, although the effects were larger for PMA than for serum, particularly in the case of forskolin stimulation. Staurosporine alone did not alter stimulation by either isoproterenol or forskolin in control cells.

As a second approach to investigate the involvement of protein kinase C, cells were incubated for 18 hr in the presence of PMA, conditions previously shown to markedly down-regulate protein kinase C activity (13, 20), before induction of sensitization. Again, studies of PMA-induced sensitization were included to control for the effectiveness of the down-regulation (Fig. 6). As in our previous study (13), down-regulation of protein kinase C decreased the absolute value for isoproterenol-stimulated cAMP accumulation in nonsensitized cells by about 40%, whereas stimulation by forskolin was not affected. In control cells, PMA sensitized isoproterenol stimulation by 1.8 ± 0.2-fold and forskolin stimulation by 3.1 ± 0.6-fold, and serum sensitized isoproterenol stimulation by 1.7 ± 0.1-fold and forskolin stimulation by 3.4 ± 0.4-fold. In down-regulated cells, sensitization by both PMA and serum was essentially completely prevented. In down-regulated cells, the fold sensitization values were as follows: PMA sensitization of isoproterenol stimulation, 1.0 ± 0.0; PMA sensitization of forskolin stimulation, 1.1 ± 0.1; serum sensitization of isoproterenol stimulation, 1.1 ± 0.2; and serum sensitization of forskolin stimulation, 1.3 ± 0.1.

Studies with pertussis toxin were performed to investigate the involvement of a pertussis toxin-sensitive guanine nucleotide-binding protein in serum-induced sensitization (Fig. 7). Cells were incubated with pertussis toxin under conditions previously shown to ADP-ribosylate and inactivate G_i in these cells (21). Effects on carbachol-induced sensitization also were investigated, as a control for nonspecific effects of pertussis toxin. As in our previous study, the effects of pertussis toxin on carbachol-induced sensitization were small and not statistically significant. Pertussis toxin caused some reduction in carbachol-induced sensitization of isoproterenol stimulation (1.74 ± 0.13-fold versus 2.23 ± 0.16-fold), whereas carbachol-induced sensitization of forskolin stimulation was not reduced

(1.60 ± 0.17-fold versus 1.57 ± 0.10-fold). In contrast, pertussis toxin markedly inhibited serum-induced sensitization. Serum-induced sensitization of isoproterenol stimulation was reduced by 76%, from 1.81 ± 0.08-fold to 1.17 ± 0.06-fold. Serum-induced sensitization of forskolin stimulation was completely inhibited, from 2.18 ± 0.12-fold in control cells to 0.99 ± 0.05-fold in pertussis toxin-pretreated cells.

Because phosphodiesterase inhibitors were not routinely included in the cAMP accumulation assays, the possible involvement of changes in phosphodiesterase activity in the mechanism of serum-induced sensitization was investigated. Sensitization of both isoproterenol and forskolin stimulation was still observed even when a maximally effective concentration of IBMX (100 μM) was included during the stimulation phase of the assay (data not shown), suggesting that decreased activity of phosphodiesterase is not the mechanism of serum-induced sensitization. To more directly assess the possible role of decreased degradation/efflux of cAMP as a mechanism for the observed sensitization, time courses of cAMP breakdown were determined, as in previous studies with these cells (4, 9). In control and serum-pretreated cells, cAMP degradation occurred at similar rates, with a half-time of approximately 2 min in both cases (Fig. 8). The rates of cAMP breakdown also were not altered in cells that were sensitized by pretreatment with either carbachol or PMA (data not shown), consistent with the inability of IBMX to prevent sensitization by these agents that was reported previously (13). Thus, changes in phosphodiesterase activity or cAMP efflux do not appear to account for sensitization in these cells.

Assays of adenylyl cyclase enzyme activity in broken cell preparations from control cells and from cells sensitized by pretreatment with carbachol, PMA, and serum also were compared (Table 3). Because the concentration of Mg^{2+} ion present during the adenylyl cyclase assay has been shown to be important for observation of some sensitization and desensitization phenomena (22, 23), assays were performed in the presence of both low and high concentrations of Mg^{2+}. In the more commonly used high [Mg^{2+}] assay conditions, isoproterenol and forskolin stimulated adenylyl cyclase activity of control preparations by about 2- and 10-fold, respectively. None of the pretreatments significantly altered basal activity. Neither carbachol nor PMA pretreatment altered stimulation by isoproterenol or by forskolin. However, pretreatment of cells with serum did significantly increase stimulation by both isoproterenol and by forskolin. In the low [Mg^{2+}] assays, the extent of stimulation was reduced, with isoproterenol and forskolin causing only about 1.5- and 4-fold stimulation, respectively, in control preparations. Pretreatment with carbachol did not significantly alter stimulation by isoproterenol or by forskolin. However, under these assay conditions, pretreatment with either PMA or serum led to significant increases in adenylyl cyclase activity stimulated by both isoproterenol and by forskolin.

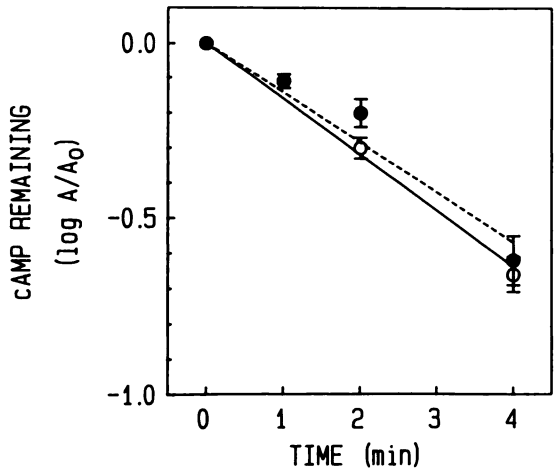

Fig. 8. Effects of serum sensitization on cAMP degradation/efflux. Cells were incubated for 60 min in DMEM-HEPES in the absence (○) or presence of 5% fetal bovine serum (●) and were then washed and incubated for 5 min with DMEM-HEPES containing 10 μM isoproterenol to stimulate cAMP accumulation. This stimulation medium was replaced with DMEM-HEPES containing 10 μM propranolol to block further stimulation of cAMP accumulation, and intracellular cAMP remaining at various times was then determined. Data are presented as the logarithm of the ratio of cAMP remaining at the indicated times (A) divided by the cAMP present at the end of the stimulation period (A_0) and are the means ± standard errors from five experiments, each performed in triplicate. The A_0 values (percentage of conversion) were as follows: control, 0.29 ± 0.13; serum, 0.66 ± 0.22.

TABLE 3

Effects of sensitizing agents on adenylyl cyclase activity in broken cell preparations

Cells were incubated for 60 min in the absence or presence of 100 μM carbachol, 1 μM PMA, or 5% fetal bovine serum. Cells were then washed and lysed, and adenylyl cyclase activity in the lysates was determined in the absence (basal) or presence of 10 μM isoproterenol or 30 μM forskolin. Assays contained either 1.8 mM $MgCl_2$ (low [Mg^{2+}]) or 5.4 mM $MgCl_2$ (high [Mg^{2+}]). Data are presented as averages ± standard errors from five experiments, each performed in triplicate.

Assay	Pretreatment	Adenylyl cyclase activity	Fold sensitization
		pmol/min/mg of protein	
High [Mg^{2+}]			
Basal	Control	11 ± 2	
	Carbachol	12 ± 1	
	PMA	13 ± 2	
	Serum	13 ± 2	
Isoproterenol	Control	22 ± 3	
	Carbachol	23 ± 2	1.07 ± 0.05
	PMA	22 ± 2	1.03 ± 0.05
	Serum	34 ± 3[a]	1.61 ± 0.15
Forskolin	Control	104 ± 7	
	Carbachol	114 ± 7	1.09 ± 0.03
	PMA	123 ± 10	1.19 ± 0.08
	Serum	144 ± 11[b]	1.39 ± 0.10
Low [Mg^{2+}]			
Basal	Control	11 ± 2	
	Carbachol	11 ± 2	
	PMA	13 ± 2	
	Serum	12 ± 2	
Isoproterenol	Control	15 ± 2	
	Carbachol	16 ± 2	1.04 ± 0.05
	PMA	18 ± 2	1.21 ± 0.02
	Serum	23 ± 3[a]	1.56 ± 0.05
Forskolin	Control	36 ± 3	
	Carbachol	38 ± 3	1.05 ± 0.04
	PMA	64 ± 8[b]	1.81 ± 0.20
	Serum	76 ± 8[b]	2.16 ± 0.19

[a] Significantly greater than the corresponding value in control cells ($p < 0.05$).
[b] Significantly greater than the corresponding value in control cells ($p < 0.01$).

Discussion

These results demonstrate a marked effect of serum on the function of the receptor-regulated adenylyl cyclase system of 1321N1 human astrocytoma cells. Pretreatment of cells in medium containing serum increased subsequent stimulation of intracellular cAMP accumulation by isoproterenol and by prostaglandin E_1, agents acting through cell surface receptors coupled to adenylyl cyclase activation. Stimulation by forskolin, which is thought to directly activate the catalytic unit of adenylyl cyclase (3), also was increased following serum pretreatment. The effect occurred rapidly upon exposure to serum, was maintained in the continuing presence of serum, but reversed rapidly and completely following removal of serum.

Preliminary characterization of the serum effect suggests that a novel factor in serum may be responsible. The activity is apparently associated with a relatively large molecule, because it was not lost upon dialysis. However, the activity was quite stable to heating and to trypsin proteolysis. Sensitizing activity was present in both serum and plasma, and heparin inhibited the activity of the serum factor. A potential explanation for these findings is that sensitization is mediated by a small molecule associated with albumin or other plasma proteins; this possibility is being further investigated.

The mechanism(s) by which serum induces this sensitization of cAMP accumulation was investigated. Because previous studies (13) had shown that sensitization of cAMP accumulation in these cells could be induced by activation of protein kinase C, either directly with phorbol esters or indirectly by activation of receptors coupled to polyphosphoinositide hydrolysis and generation of endogenous diacylglycerol, the involvement of protein kinase C in serum-induced sensitization was investigated. Down-regulation of protein kinase C blocked the ability of the protein kinase C activator PMA to induce sensitization, indicating that down-regulation of protein kinase C had occurred. Sensitization induced by serum also was prevented by protein kinase C down-regulation, suggesting a requirement for protein kinase C in the serum effect as well. The protein kinase C inhibitor staurosporine inhibited PMA-induced sensitization, although it was less effective than protein kinase C down-regulation. Sensitization induced by serum was also inhibited by staurosporine, but the extent of inhibition was somewhat less than the effect on PMA-induced sensitization. Together, these results provide evidence for the involvement of protein kinase C in serum-induced sensitization, but they leave open the possibility of additional mechanisms as well.

The nature of the serum-induced modification in the cAMP pathway was investigated. A decrease in phosphodiesterase activity could lead to the observed increase in cAMP accumulation. However, this is apparently not the site of modification, because sensitization was observed in the presence of a maximally effective concentration of the phosphodiesterase inhibitor IBMX. Direct assays of the rates of cAMP degradation/efflux in control and sensitized cells also indicated that changes in degradation and/or efflux are not the mechanism of sensitization. Thus, it seems likely that sensitization results from an increase in cAMP synthesis rather than a decrease in the rate of cAMP degradation. Further evidence for serum-induced changes in adenylyl cyclase activity comes from the assays of enzyme activity in broken cell preparations. Pretreatment of cells with serum increased adenylyl cyclase enzyme activity, particularly if assays were performed in the presence of low [Mg^{2+}]. Thus, the sensitized state can be at least partially retained following cell lysis, suggesting that a stable (covalent) modification may be involved. However, the increase in broken cell adenylyl cyclase activity was less than the increase in intact cell cAMP accumulation. As reported previously, carbachol and PMA also induce sensitization of intact cell cAMP accumulation (13), but sensitization by these agents is much less well retained in adenylyl cyclase assays with broken cell preparations. It seems likely that mechanisms unique to the intact cell may be largely responsible for the sensitization induced by carbachol and by PMA and may also contribute to some extent to sensitization induced by serum.

To test for the possible involvement of changes in the inhibitory guanine nucleotide-binding protein G_i as the mechanism of sensitization, cells were treated with pertussis toxin to inactivate the function of G_i. As in our previous study, pertussis toxin pretreatment of control cells did not mimic sensitization (i.e., did not increase cAMP accumulation), suggesting that decreased inhibition of adenylyl cyclase by G_i is not the mechanism of sensitization in these cells. However, pertussis toxin pretreatment almost completely eliminated serum-induced sensitization. This suggests that serum activates a signal transduction pathway involving a pertussis toxin-sensitive guanine nucleotide-binding protein to induce sensitization. The inhibition of sensitization by pertussis toxin is not due to a nonspecific effect on sensitization mechanisms or on adenylyl cyclase activity, because sensitization induced by carbachol was not blocked by pertussis toxin. Carbachol has been shown previously to activate polyphosphoinositide hydrolysis (24) and to induce sensitization (13) in these cells through a pathway that is not pertussis toxin sensitive.

The increase in stimulation by isoproterenol and by prostaglandin E_1 could result from changes in their specific receptors or from changes in more distal components of the pathway. The effect of serum on isoproterenol stimulation was to increase the maximal response, with little or no change in the potency of isoproterenol, suggesting that interaction of isoproterenol with the β-adrenergic receptor is not altered but, rather, the ability of adenylyl cyclase to respond to receptor activation is increased. The fact that stimulation by forskolin also was sensitized provides further evidence that serum induces modifications at points distal to these receptors, because forskolin is thought to activate the catalytic unit directly or to alter interaction of guanine nucleotide-binding proteins with the catalytic unit (3). A modification of the activity of the adenylyl cyclase catalytic unit seems most likely, although the failure of serum to sensitize the stimulatory effect of cholera toxin remains unexplained.

The effects of PMA in these experiments were somewhat different from those in two previous studies from our laboratory. In the current studies PMA consistently induced sensitization of both isoproterenol- and forskolin-stimulated cAMP accumulation in intact cells, whereas in our previous study (13) isoproterenol stimulation was generally unchanged. Furthermore, in the current studies PMA did not induce the desensitization of isoproterenol-stimulated adenylyl cyclase activity in broken cell preparations that was observed in another earlier study (25). As discussed previously (13), PMA apparently can induce two distinct modifications of isoproterenol stimulation in these cells, namely, a desensitization of β-adrenergic receptor

function and a sensitization of adenylyl cyclase function. In the previous studies both effects occurred together and, thus, no consistent change in isoproterenol stimulation was observed in intact cells. For reasons that have not been investigated, the PMA-induced desensitization of β-adrenergic receptors did not occur in the present studies and, thus, sensitization of isoproterenol stimulation in intact cells was observed.

A few previous studies have reported decreases in cAMP accumulation due to growth of cells in the presence of serum (26–28). In contrast, the sensitization studied here occurs very rapidly upon exposure of cells to serum and reverses rapidly following serum removal. One previous study reported a phenomenon that may be related to the sensitization studied here, namely, that an unidentified component of blood plasma could increase hormone-stimulated adenylyl cyclase activity in intact human mononuclear leukocytes (29). In a preliminary screening of other cells, we did not observe serum-induced sensitization in DDT_1 MF-2 hamster smooth muscle cells, OK opossum kidney epithelial cells, HT29 human colonic epithelial cells, or bovine bronchial epithelial cells.[2] Sensitization by serum was observed in another glial cell line, C62B rat glioma cells, and a preliminary characterization of sensitization in these cells has been presented (30). Sensitization in C62B cells also is pertussis toxin sensitive, but it is apparently not mediated by protein kinase C and does involve changes in phosphodiesterase activity. Thus, multiple mechanisms for serum-induced sensitization appear to exist. More recently, we have also observed serum-induced sensitization in human lymphoma cells and in rat osteosarcoma cells.[2] Although sensitization by serum does not occur in all cells, it appears to be relatively widespread and, therefore, may be of considerable significance, not only in terms of growth of cultured cells but also presumably physiologically. Identification of the factor(s) responsible and more detailed studies of the specific molecular modifications involved may reveal important new information regarding cellular regulation.

In summary, serum induces sensitization of cAMP accumulation in intact 1321N1 cells, most likely due to an increase in the activity of the catalytic unit of adenylyl cyclase. The mechanism for the serum effect appears to involve both protein kinase C and a pertussis toxin-sensitive guanine nucleotide-binding protein. The identity of the serum factor involved and the details of the signal transduction pathway by which sensitization is brought about are currently under investigation.

Acknowledgments

The authors thank Dr. D. Romberger and S. Liewer for their expert assistance with some of the experiments in this manuscript.

[2]Unpublished results.

References

1. Levitzki, A. Regulation of hormone-sensitive adenylate cyclase. *Trends Pharmacol. Sci.* **8**:299–303 (1987).
2. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649 (1987).
3. Seamon, K. B., and J. W. Daly. Forskolin: its biological and chemical properties. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20**:1–150 (1986).
4. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated activation of phosphodiesterase. *Mol. Pharmacol.* **22**:310–319 (1982).
5. Miot, F., C. Erneux, J. N. Wells, and J. E. Dumont. The effects of alkylated xanthines on cyclic AMP accumulation in dog thyroid slices exposed to carbamylcholine. *Mol. Pharmacol.* **25**:261–266 (1984).
6. Buxton, I. L., and L. L. Brunton. Action of the cardiac α_1 receptor: activation of cyclic AMP degradation. *J. Biol. Chem.* **260**:6733–6737 (1985).
7. Sibley, D. R., and R. J. Lefkowitz. Biochemical mechanisms of β-adrenergic receptor regulation. *ISI Atlas Sci. Pharmacol.* **2**:66–70 (1988).
8. Clark, R. B. Desensitization of hormonal stimuli coupled to regulation of cyclic AMP levels. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20**:151–209 (1986).
9. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated control of cyclic AMP metabolism: agonist-induced changes in nucleotide synthesis and degradation. *Mol. Pharmacol.* **23**:384–392 (1983).
10. Jones, S. B., M. L. Toews, J. T. Turner, and D. B. Bylund. α_2-Adrenergic receptor-mediated sensitization of forskolin-stimulated cyclic AMP production. *Proc. Natl. Acad. Sci. USA* **84**:1294–1298 (1987).
11. Thomas, J. M., and B. B. Hoffman. Adenylate cyclase supersensitivity: a general means of cellular adaptation to inhibitory agonist? *Trends Pharmacol. Sci.* **8**:308–311 (1987).
12. Kikkawa, U., and Y. Nishizuka. The role of protein kinase C in transmembrane signalling. *Annu. Rev. Cell Biol.* **2**:149–178 (1986).
13. Johnson, R. A., and M. L. Toews. Protein kinase C activators sensitize cyclic AMP accumulation by intact 1321N1 human astrocytoma cells. *Mol. Pharmacol.* **37**:296–303 (1990).
14. Shimizu, H., J. W. Daly, and C. R. Creveling. A radioisotopic method for measuring the formation of adenosine 3′,5′-cyclic monophosphate in incubated slices of brain. *J. Neurochem.* **16**:1609–1619 (1969).
15. Toews, M. L., and J. P. Perkins. Agonist-induced changes in β-adrenergic receptors on intact cells. *J. Biol. Chem.* **259**:2227–2235 (1984).
16. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
17. Clark, R. B., M. W. Kunkel, J. Friedman, T. Goka, and J. A. Johnson. Activation of cAMP-dependent protein kinase is required for heterologous desensitization of adenylyl cyclase in S49 wild-type lymphoma cells. *Proc. Natl. Acad. Sci. USA* **85**:1442–1446 (1988).
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
19. Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita. Staurosporine, a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**:397–402 (1986).
20. Blackshear, P. J., D. J. Stumpo, J.-K. Huang, R. A. Nemenoff, and D. H. Spach. Protein kinase C-dependent and -independent pathways of proto-oncogene induction in human astrocytoma cells. *J. Biol. Chem.* **262**:7774–7781 (1987).
21. Hughes, A. R., M. W. Martin, and T. K. Harden. Pertussis toxin differentiates between two mechanisms of attenuation of cyclic AMP accumulation by muscarinic cholinergic receptors. *Proc. Natl. Acad. Sci. USA* **81**:5680–5684 (1984).
22. Johnson, J. A., T. J. Goka, and R. B. Clark. Phorbol ester-induced augmentation and inhibition of epinephrine-stimulated adenylate cyclase in S49 lymphoma cells. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**:199–215 (1986).
23. Clark, R. B., J. Friedman, J. A. Johnson, and M. W. Kunkel. β-Adrenergic receptor desensitization of wild-type but not cyc lymphoma cells unmasked by submillimolar Mg^{2+}. *FASEB J.* **1**:289–297 (1987).
24. Masters, S. B., M. W. Martin, T. K. Harden, and J. H. Brown. Pertussis toxin does not inhibit muscarinic-receptor-mediated phosphoinositide hydrolysis or calcium mobilization. *Biochem. J.* **227**:933–937 (1985).
25. Toews, M. L., M. Liang, and J. P. Perkins. Agonists and phorbol esters desensitize β-adrenergic receptors by different mechanisms. *Mol. Pharmacol.* **32**:737–742 (1987).
26. Dibner, M. D., and P. A. Insel. Serum catecholamines desensitize β-adrenergic receptors of cultured C6 glioma cells. *J. Biol. Chem.* **256**:7343–7346 (1981).
27. Dibner, M. D., and P. A. Insel. Growth of C6 glioma cells in serum-containing medium decreases β-adrenergic receptor number. *J. Cell. Physiol.* **109**:309–315 (1981).
28. Darfler, F. J., R. J. Hughes, and P. A. Insel. Characterization of serum-induced alterations in the cyclic AMP pathway in S49 lymphoma cells. *J. Biol. Chem.* **256**:8422–8428 (1981).
29. Motulsky, H. J., D. Smith, B. I. Terman, and R. D. Feldman. Regulation of hormone-stimulated cyclic AMP accumulation in intact human mononuclear leukocytes by blood plasma. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**:329–343 (1987).
30. Toews, M. L., and L. J. Arneson-Rotert. Serum-induced sensitization of cyclic AMP accumulation in C62B rat glioma cells. *FASEB J.* **4**:A1985 (1990).

Send reprint requests to: Myron L. Toews, Ph.D., Department of Pharmacology, University of Nebraska Medical Center, 600 S. 42nd Street, Omaha, NE 68198-6260.