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Defining the Role of Protein Kinase C in Epinephrine- and Bradykinin-Stimulated Arachidonic Acid Metabolism in Madin-Darby Canine Kidney Cells

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SUMMARY

Madin-Darby canine kidney cells (MDCK) are known to release free arachidonic acid and arachidonic acid metabolites (AA) in response to tumor-promoting phorbol esters, such as tetradecanoyl phorbol-13-acetate, and to agonists active at α_1-adrenergic and bradykinin B_2 receptors. These experiments were conducted to define the role of Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) activation in the stimulation of AA release, in the clonal isolate cell line MDCK-D1, by use of three inhibitors of protein kinase C, sphingosine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), and staurosporine. We found that α_1-adrenergic- and bradykinin-stimulated [^{3}H]AA release can be distinguished by differential dependence on protein kinase C; epinephrine-stimulated release was more dependent on protein kinase C activation than was bradykinin-stimulated release. The inhibition of bradykinin-stimulated AA release by sphingosine (20.2 ± 6.1%) was substantially less than the inhibition observed for tetradecanoyl phorbol-13-acetate- (67.2 ± 5.5%) and epinephrine-stimulated release (50.2 ± 9.2%). These findings were confirmed by results using H-7 and staurosporine. The relative independence of bradykinin-stimulated AA release of protein kinase C was also demonstrated by the inability of phorbol ester-induced down-regulation of protein kinase C to eliminate bradykinin-stimulated AA release. The inhibition of α_1-adrenergic receptor-mediated AA release by sphingosine, H-7, and staurosporine was not due to a change in receptor number or affinity. Analysis of the products comprising [^{3}H]AA release indicated that treatment with sphingosine did not change the composition of the released AA (34–48% prostaglandin E_2, 17–27% free arachidonic acid, and 25–51% unidentified metabolites). These results indicate that two different types of hormone receptors in the same cell type can promote AA release by mechanisms that differ in their dependence on protein kinase C. The protein kinase C-dependent mechanism may represent protein kinase C-mediated activation of phospholipase A_2.

Hormones (1), growth factors (2), and tumor-promoting phorbol esters (3) stimulate deacylation of arachidonic acid from membrane lipids, making it available for synthesis of cyclooxygenase and lipoxygenase products. The precise mechanisms by which this deacylation occurs is not clearly understood. Previous work in our laboratory has involved use of a cloned epithelial cell line, the MDCK-D1 cell, to show that in addition to stimulation of AA release, α_1-adrenergic and bradykinin B_2 receptors also couple to PI hydrolysis (4, 5). Similar results have been described in other cell types (6, 7). Hormone-mediated PI hydrolysis and phosphatidylcholine hydrolysis (8) can result in production of diacylglycerol. Hormone-mediated PI hydrolysis can also release inositol 1,4,5-trisphosphate, which facilitates calcium mobilization from intracellular storage sites (9), and inositol 1,3,4,5-tetrakisphosphate, which may stimulate calcium entry through plasma membrane calcium channels (10). All of these processes can facilitate activation of the Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C). The possibility of a role for protein kinase C in mediating AA release has been suggested by evidence that tumor-promoting phorbol esters, which activate protein kinase C *in vivo* and *in vitro* (11), can stimulate AA release in several types of cells, including MDCK cells (12, 13), pinealocytes (14), neutrophils (15), platelets (16), and rat aorta (17).

In these experiments, we set out to define the role of activation of protein kinase C in the stimulation of AA release by agonists active at bradykinin and α_1-adrenergic receptors. We have used sphingosine (18–20), H-7 (21), and staurosporine (22), three inhibitors of protein kinase C, to block receptor-stimulated events that are hypothesized to be mediated by activation of protein kinase C. Based on this approach, we

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ABBREVIATIONS: AA, free arachidonic acid and arachidonic acid metabolites; PI, phosphoinositide; DiC_8, dioctanoylglycerol; PLA_2, phospholipase A_2; PGE_2, prostaglandin E_2; TPA, 12-tetradecanoyl phorbol-13-acetate; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazine-*N*-2-ethanesulfonate; MDCK-D1, a cloned Madin-Darby canine kidney cell (26); IM, incubation medium.

demonstrate that there appears to be more than one mechanism for hormone receptor-mediated AA release in these cells and that these mechanisms can be distinguished in part by differential dependence on protein kinase C.

Experimental Procedures

Materials. [^{3}H]Arachidonic acid was from Amersham or New England Nuclear. [^{3}H]Prazosin, [^{3}H]adenine, and the PGE_2 radioimmunoassay kit were from New England Nuclear. DiC_8 was a gift from Dr. P. M. Conn (University of Iowa) or from Avanti Polar Lipids. TPA, 4-α-phorbol, epinephrine, bradykinin, sphingosine, ceramides, and H-7 were all from Sigma. Staurosporine was from Calbiochem. All other materials were from standard sources.

Cell culture. The methods for cell culture have been described previously (4, 23). Briefly, MDCK-D1 cells were grown in DMEM with 10% MDCK serum (75% heat-inactivated horse serum, 25% heat-inactivated fetal calf serum), 15 mM HEPES, and 3.7 g/liter sodium bicarbonate, pH 7.4. Subconfluent cells were subcultured every 2–4 days with a trypsin-EDTA solution by dilution into 250-ml flasks. For experiments, culture flasks were treated with trypsin-EDTA solution and diluted into 35-mm dishes and were used after 2–3 days, when they were 50–90% confluent (5–9 × 10^5 cells/dish), conditions previously shown to optimize hormonal responses in these cells (4).

[^{3}H]AA release. An adaptation of a previously described method was used to assay [^{3}H]AA release (4). Briefly, cells in 35-mm dishes were incubated in an atmosphere of 90% air and 10% CO_2 for 3–6 hr with 0.30–0.33 μCi/ml [^{3}H]arachidonic acid in labeling medium (DMEM with 0.5% MDCK or fetal bovine serum , 15 mM HEPES, and 3.7 g/liter sodium bicarbonate, pH 7.4). After labeling, monolayers were washed four times with IM, which consisted of DMEM with 15 mM HEPES and 0.05% bovine serum albumin. Then 1 ml of IM with or without inhibitor was added. Cells treated with sphingosine or 0.1% ethanol in IM for 10 min were then washed another four times and 1 ml of IM containing TPA, epinephrine, bradykinin, or nothing (vehicle) was added. Cells treated with H-7, staurosporine, or IM alone were incubated for 30 or 5 min, respectively, before addition of 100 μl of 10-fold concentrated stock solutions of drugs without further washing. Cells were incubated at 37° for the appropriate period. One milliliter of extracellular medium was aspirated, 4.5 ml of liquid scintillation cocktail was added, and ^{3}H was counted in a liquid scintillation counter with an efficiency of 40%. In experiments where PGE_2 production was measured by radioimmunoassay, aspirated medium was then frozen at −70° for storage before assay. In experiments where arachidonic acid metabolites were separated by thin layer chromatography, the medium was extracted twice with 1-ml volumes of *n*-butanol. The butanol was evaporated under nitrogen and the extract was resuspended in 100–150 μl of butanol. This extract was concentrated, spotted onto coated silica gel G plates, and analyzed as previously described, in a solvent system of petroleum ether/diethyl ether/acetic acid, 70:30:2. Spots were identified by comigration with PGE_2 or arachidonic acid standards that were visualized in iodine vapor (5).

Radioligand binding studies. α_1-Adrenergic receptor number and affinity were assayed by [^{3}H]prazosin binding to intact cells (4, 23). Subconfluent cells (50–90% confluent) in 35-mm dishes were washed four times with IM. For binding isotherms, cells were treated with inhibitors as described above. Then, appropriate concentrations of [^{3}H]prazosin in 1 ml of IM were added to the dish. Samples were incubated for 1 hr at 37° and were stopped by washing three times with IM. Cells were dissolved in 0.5 ml of 5% sodium dodecyl sulfate, and dishes were rinsed with 0.5 ml of water. Samples were counted in a liquid scintillation counter. Triplicates were averaged. Nonspecific binding was determined at each concentration by the addition of 10 μM phentolamine and was subtracted from the total binding to determine specific [^{3}H]prazosin binding. K_d and B_{max} were determined using a nonlinear curve-fitting program. Competitive binding curves were generated by addition of various concentrations of epinephrine together with 0.08 nM [^{3}H]prazosin in 35-mm dishes. Phentolamine (10 μM) was added to determine nonspecific binding (4, 23), as described above.

Data analysis. Measurements in duplicate or triplicate 35-mm culture dishes were averaged. These values were pooled and mean ± standard error was determined for the number of experiments indicated in the figure legends. The standard error is smaller than the size of the points where no error bars are shown. Saturation binding isotherms were fit using Graphpad nonlinear curve-fitting software for calculation of the equilibrium dissociation constant (K_d) and the total number of binding sites (B_{max}).

Results

Additivity of epinephrine- and bradykinin-stimulated AA release. Previous experiments documented that agonists active at α_1-adrenergic receptors and B_2 bradykinin receptors promote prominent increases in release of AA from MDCK-D1 cells that are labeled with [^{3}H]arachidonic acid (4, 5). The current studies were designed to explore whether the responses to these two classes of hormone receptors occurred through similar mechanisms. In initial experiments, we tested whether the α_1-adrenergic agonist epinephrine and bradykinin produced additive stimulation of AA release from [^{3}H]arachidonic acid-labeled cells. AA release stimulated by 1 μM epinephrine, 1 μM bradykinin, or both is shown in Table 1. AA release was almost fully additive (85–90%), whether the agonists were added to separate culture dishes or simultaneously to the same culture dish. In 14 experiments, bradykinin was 1.4 ± 0.1-fold more effective as a stimulant of AA release than was epinephrine. Previous work in this laboratory has shown that TPA produces a greater than additive enhancement of bradykinin-stimulated AA release (5). By contrast TPA blocks epinephrine-stimulated AA release.[1] These results all suggest that epinephrine and bradykinin might be acting to stimulate AA release by different mechanisms.

Role of protein kinase C in TPA-, epinephrine-, and bradykinin-stimulated AA release. We next tested whether the ability of the two agonists to stimulate AA release could be differentiated by sensitivity to inhibitors of protein kinase C. Sphingosine has recently been proposed as an agent that can be used to assess the involvement of protein kinase C in studies with intact cells (18–20). The relative sensitivities of TPA-, epinephrine-, and bradykinin-stimulated AA release to inhibition by sphingosine can be compared by pooling data from the experiments shown in Fig. 1, 2, and 3, (*top*). AA release stimulated by TPA (0.1 μM) at 60 min was inhibited 67.2 ± 5.5% (mean ± SE; 13 experiments). AA release stimulated by epinephrine (1 μM) at 20 min was inhibited 50.2 ± 9.2% (10 experiments), whereas AA release stimulated by bradykinin (1 μM) at 20 min was inhibited by 20.2 ± 6.1% (16 experiments). Ceramides (10 μM) were not inhibitory (data not shown). The inhibition of bradykinin-stimulated AA release differed significantly from the inhibition observed for TPA- and epinephrine-stimulated release ($p < 0.01$) by single classification analysis of variance and the Student-Newman-Keuls test. Thus, TPA-stimulated AA release was most sensitive to inhibition by sphingosine, epinephrine-stimulated AA release was intermediate in sensitivity, and bradykinin-stimulated AA release was relatively insensitive to inhibition by sphingosine.

The time course of AA release from MDCK-D1 cells in the presence and absence of sphingosine is shown in Fig. 1. AA

[1] S. R. Slivka, unpublished observations.

TABLE 1

Additivity of epinephrine and bradykinin-stimulated AA release in MDCK-D1 cells

Dishes (35-mm) of subconfluent MDCK-D1 cells were labeled for 18–24 hr with 0.3–0.5 μCi/ml [^{3}H]arachidonic acid and then stimulated with 1 μM concentrations of either epinephrine, bradykinin, or both for 20 min (three experiments), as described in Experimental Procedures. Results shown are cpm determined in an individual experiment, with each point determined in triplicate. Unstimulated values have been subtracted from each value obtained in the presence of hormone. The numbers in parentheses represent expected values if the responses were additive.

	AA Release
	cpm
Expt. 1	
Bradykinin	2625
Epinephrine	2135
Both	4035 (4760)
Expt. 2	
Bradykinin	2385
Epinephrine	1051
Both	2976 (3336)

release exhibited different kinetics for the three agents used. Release in response to bradykinin was the most rapid and appeared to reach a steady state between 5 and 30 min. By contrast, the epinephrine-stimulated response was somewhat slower and did not reach steady state during a 30-min period. TPA-stimulated release was much slower than that stimulated by either agonist. These kinetics are similar to those reported by others (4, 5, 7).[2] There was no substantial alteration in the time course of AA release stimulated by any of the agents following treatment with sphingosine.

Fig. 2 shows the effect of treatment with 10–15 μM sphingosine on the concentration dependence of AA release stimulated by TPA, epinephrine, and bradykinin. Maximal AA release stimulated by TPA was markedly blunted, although the potency of TPA was not reduced. As was found in the kinetic experiments, the response to epinephrine was reduced to a greater extent by sphingosine than was that to bradykinin. The extent of inhibition by sphingosine of AA release stimulated by epinephrine and bradykinin at 2, 5, and 10 min was the same as that observed at 20 min.

The concentration dependence of inhibition by sphingosine of AA release is shown in Fig. 3 (*top*). Maximal inhibition of 63.0 ± 8.5% was observed at 12 μM sphingosine for TPA-stimulated release. Higher concentrations of sphingosine did not result in further inhibition (data not shown). Maximal inhibition of epinephrine-stimulated release was 93.4 ± 7.8% at a concentration of 10 μM sphingosine. Thus, the concentration dependence for inhibition by sphingosine was similar for TPA- and epinephrine-stimulated AA release.

We conducted additional experiments with two other protein kinase C inhibitors, H-7 (21) and staurosporine (22), to confirm the differential dependence of bradykinin and epinephrine on protein kinase C activation. Fig. 3 (*middle*) shows that treatment of MDCK-D1 cells with H-7 inhibited epinephrine-, bradykinin-, and TPA-stimulated AA release in a concentration-dependent manner (EC_{50}, approximately 15 μM for TPA, 30 μM for epinephrine) with maximal inhibition by 100 μM H-7. Thus, H-7 was able to inhibit TPA- and bradykinin-stimulated AA release to a somewhat greater extent than sphingosine, although higher concentrations of H-7 were required to produce the maximum effect. TPA-stimulated AA release was most sensitive to inhibition by H-7, epinephrine-stimulated AA release was of intermediate sensitivity, and bradykinin-stimulated AA release was relatively insensitive. Fig. 3 (*bottom*) shows that treatment of MDCK-D1 cells with staurosporine inhibited epinephrine-, bradykinin-, and TPA-stimulated AA release in a concentration-dependent manner (EC_{50}, approximately 12 nM for TPA, 6 nM for epinephrine) with maximal inhibition by 100 nM staurosporine. TPA-stimulated AA release was most sensitive to inhibition by staurosporine and epinephrine-stimulated AA release was intermediate in sensitivity, whereas bradykinin-stimulated AA release was largely insensitive.

The large error bars in some of the experiments measuring agonist-stimulated AA release point out the variability in inhibitor efficacy that we have observed. Surface dilution effects of sphingosine have been described, which produce variations in efficacy depending on cell number (18, 19). In addition, we found that sphingosine, but not H-7 or staurosporine, could increase AA release in the absence of any subsequent stimulation, perhaps due to interaction with membrane phospholipids. Previous work in neutrophils (24) has shown that sphingosine can lead to cell permeabilization. Because of these concerns, as well as concerns about staurosporine cytotoxicity, we assessed cell integrity following the sphingosine and staurosporine treatment protocols. No difference was observed between sphingosine- or staurosporine- and vehicle-treated cells in their ability to exclude trypan blue (data not shown). In other experiments, cells were labeled with 0.5 μCi of [^{3}H]adenine to quantify any increased leakiness during the experimental protocol. Although a small increase in [^{3}H]adenine leakage was observed in the cells treated with sphingosine, the increase was ≤25% of that from cells that were permeabilized with saponin, and was consistently less than leakage from cells treated with TPA alone (data not shown). Staurosporine did not increase cell leakiness by this method. We conclude that cell integrity was not compromised by treatment with sphingosine or staurosporine in these experiments.

The results obtained with sphingosine, H-7, and staurosporine, therefore, suggest that bradykinin-stimulated AA release in MDCK-D1 cells is less dependent on activation of protein kinase C than is epinephrine-stimulated release. We reasoned that, if bradykinin is able to stimulate AA release without activation of protein kinase C, bradykinin-stimulated release should not be reduced in cells in which protein kinase C levels are decreased (down-regulated). Experiments were conducted where TPA and bradykinin were used to stimulate AA release in cells that had been treated for 18–24 hr with 10 nM TPA to down-regulate protein kinase C. Consistent with our working hypothesis, TPA-stimulated release was completely eliminated in MDCK-D1 cells that had been treated with TPA for 18–24 hr, whereas bradykinin-stimulated release was unaffected (116 ± 21% of control; three experiments). Epinephrine-stimulated AA release was also eliminated by 18–24-hr treatment of the cells with TPA. However, control experiments showed the [^{3}H] prazosin binding was also markedly blunted in cells incubated for 18–24 hr with TPA, making it difficult to definitively interpret results obtained in this protocol with epinephrine stimulation.

Recent work has shown that sphingosine is also an inhibitor of the Ca^{2+}/calmodulin protein kinase (25). It has also been

[2] The somewhat different kinetics observed here for epinephrine-stimulated release from those reported probably is due to the different protocols used for labeling with [^{3}H]arachidonic acid.

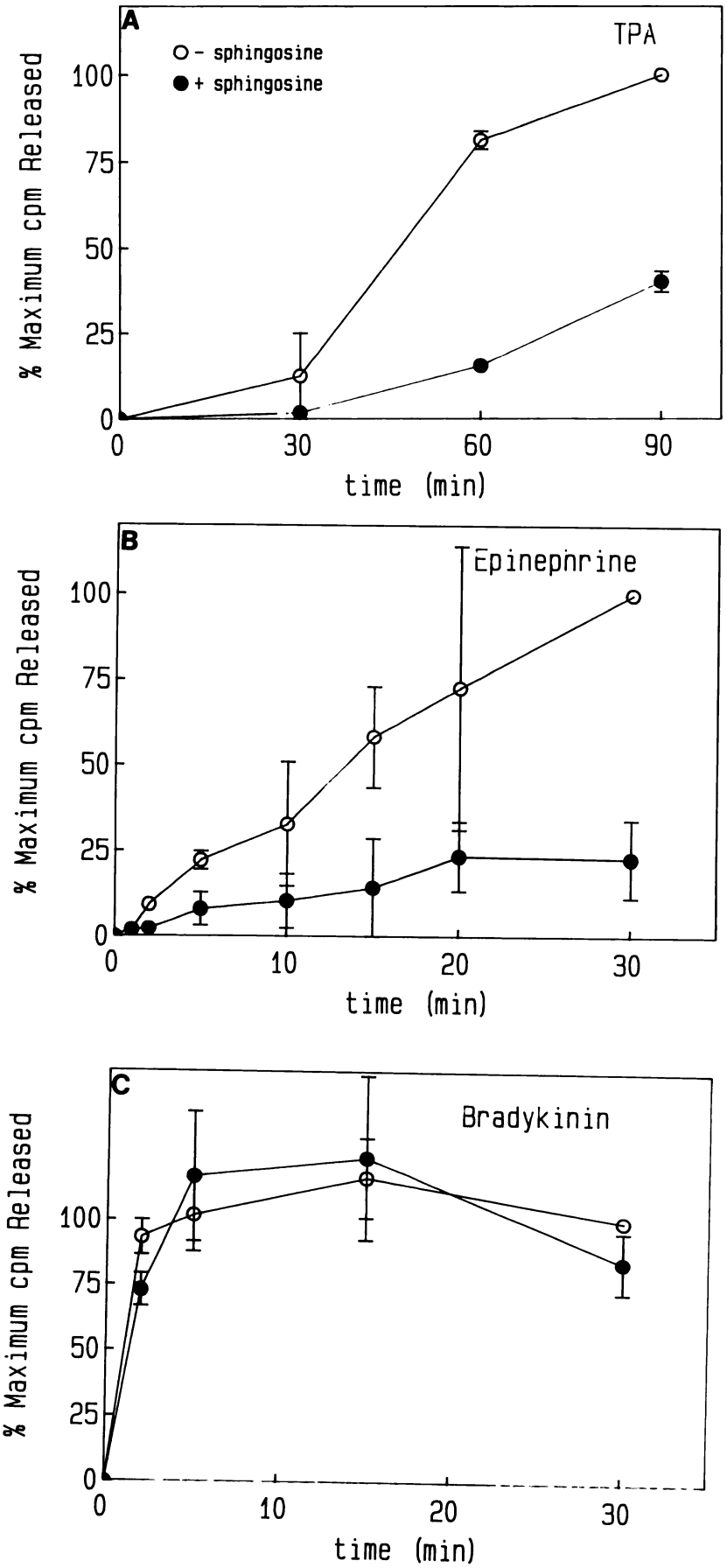

Fig. 1. Time course of TPA-, epinephrine-, and bradykinin-mediated [^{3}H]AA release. Dishes (35-mm) of subconfluent MDCK-D1 cells were labeled for 3–6 hr in 1 ml of labeling medium with 0.3–0.5 μCi/ml [^{3}H]arachidonic acid, incubated with (●) or without (○) 10 μM sphingosine, and stimulated with 0.1 μM TPA (three experiments) (A), 0.1 or 1.0 μM epinephrine (four experiments) (B), or 1.0 μM bradykinin (three experiments) (C), for the time indicated, as described in Experimental Procedures. *Points* shown are mean ± standard error. The mean of unstimulated values at each time point has been subtracted. Unstimulated values in the absence of sphingosine were 551 ± 301 cpm at 20 min and 1345 ± 505 cpm at 60 min and were increased up to 2-fold in the presence of sphingosine. Mean fold stimulation for agents in the absence of sphingosine were 6.7 ± 0.7 for TPA at 60 min, 5.8 ± 3.4 for epinephrine at 30 min, 7.7 ± 0.2 for bradykinin at 2 min, and 3.0 ± 0.2 for bradykinin at 30 min.

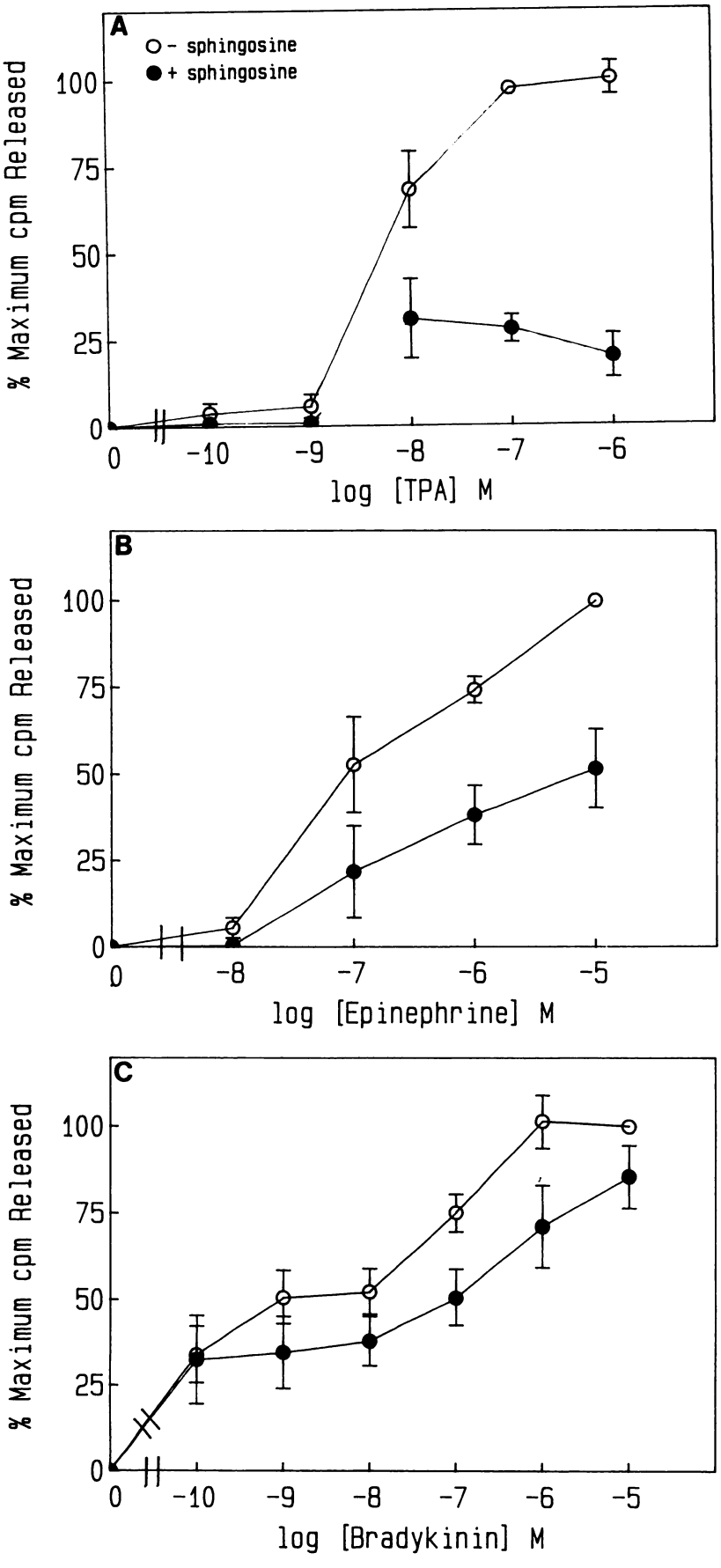

Fig. 2. Concentration dependence of TPA-, epinephrine-, and bradykinin-mediated [^{3}H]AA release. Dishes (35-mm) of subconfluent MDCK-D1 cells were labeled for 3–6 hr in 1 ml of labeling medium with 0.3–0.5 μCi/ml [^{3}H] arachidonic acid, incubated with (●) or without (○) 10 or 15 μM sphingosine, and stimulated for 60 min with TPA (seven experiments) (A), 20 min with epinephrine (five experiments) (B), or 20 min with bradykinin (eight experiments) (C), as described in Experimental Procedures. *Points* shown are mean ± standard error. The mean of unstimulated values has been subtracted from each data point. Values for basal and stimulated cpm released are as indicated in the legend to Fig. 1.

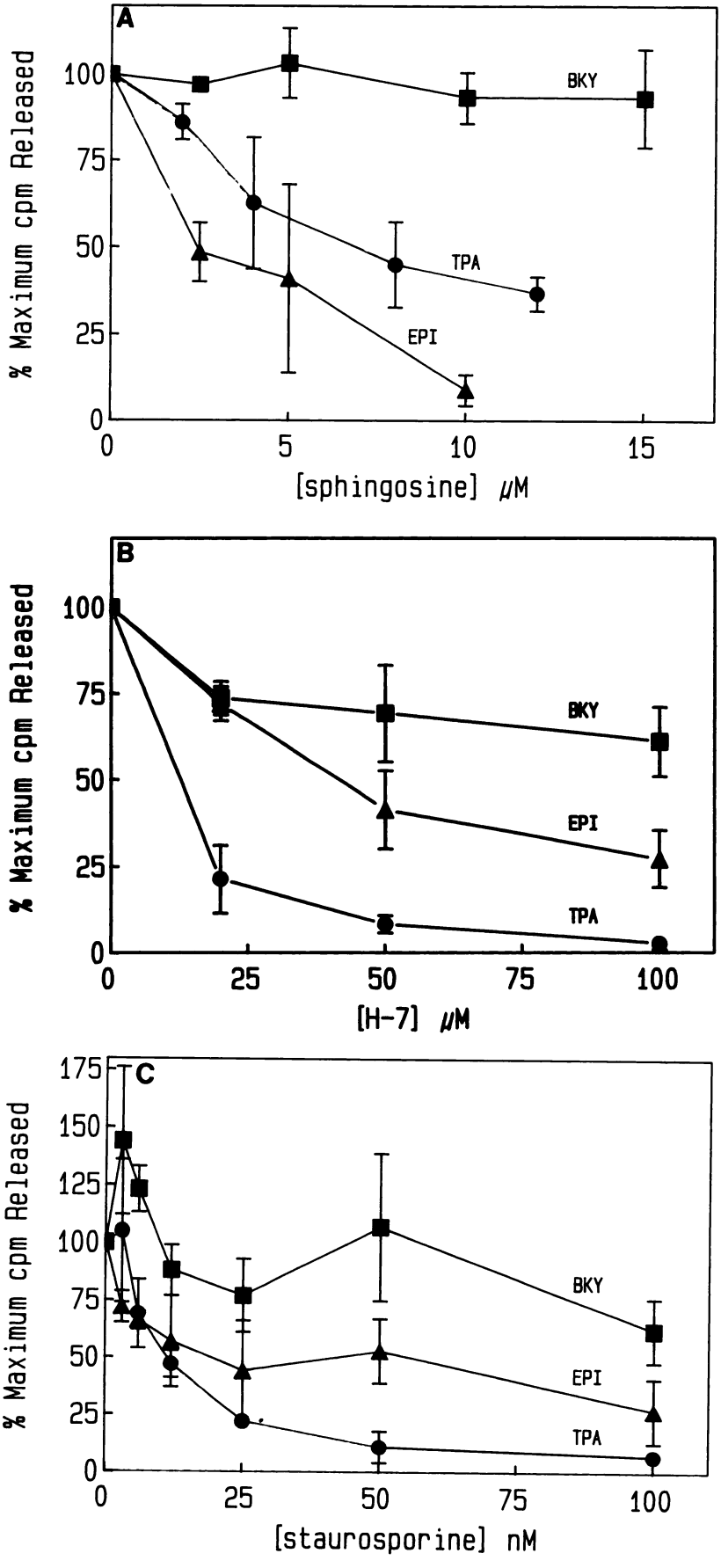

Fig. 3. Concentration dependence of sphingosine, H-7, and staurosporine inhibition of TPA-, epinephrine-, and bradykinin-mediated [^{3}H]AA release. Dishes (35-mm) of subconfluent MDCK-D1 cells were labeled for 3–6 hr in 1 ml of labeling medium with 0.3–0.5 μCi/ml [^{3}H]arachidonic acid and incubated with sphingosine for 10 min, and then stimulated with 0.1 μM TPA for 60 min (three experiments) (●), 1.0 μM epinephrine for 20 min (three experiments) (▲), or 1.0 μM bradykinin for 20 min (five experiments) (■) (A), incubated with H-7 for 30 min and then stimulated with 0.1 μM TPA (●), 1.0 μM epinephrine (▲), or 1.0 μM bradykinin (■) for 40 min (three experiments) (B), or incubated with staurosporine for 5 min and then stimulated with 0.1 μM TPA for 60 min (three experiments) (●), 1.0 μM epinephrine for 20 min (three experiments) (▲), or 1.0 μM bradykinin for 20 min (five experiments) (■) (C), as described in Experimental Procedures. *Points* shown are mean ± standard error. The mean of unstimulated values at each concentration of inhibitor has been subtracted from each data point. *100%* is the stimulated release in wells incubated without inhibitor. Values for basal and stimulated cpm released are as indicated in the legend to Fig. 1.

recently suggested that there may be differences between stimulation of cellular processes by TPA and diacylglycerols (26). We, therefore, compared the abilities of TPA and a synthetic diacylglycerol, DiC_8, to stimulate AA release in MDCK-D1 cells, as well as their sensitivities to inhibition by sphingosine and staurosporine (Fig. 4). Treatment with 0.1 μM TPA for 60 min stimulated AA release 6.4 ± 3.3-fold, whereas treatment with 200 μM DiC_8 for 60 min stimulated AA release 7.6 ± 3.6-fold. Treatment with 0.1 μM 4-α-phorbol for 60 min did not stimulate AA release (data not shown). After incubation with sphingosine or staurosporine, the stimulation by TPA was inhibited by 72 ± 12 or 89 ± 7%, respectively, whereas the stimulation by DiC_8 was inhibited by 75 ± 8 or 67 ± 15%, respectively. The two agents, therefore, appeared to be equivalent in their ability to stimulate AA release and in their sensitivity to sphingosine and staurosporine. This is consistent with the conclusion that both agents are stimulating AA release by activation of protein kinase C and that sphingosine and staurosporine are inhibiting protein kinase C-mediated processes in these experiments.

Composition of [^{3}H]AA. We determined the products comprising the released [^{3}H]AA from cells that had been labeled with [^{3}H]arachidonic acid and then incubated with and without sphingosine in order to investigate the step(s) in arachidonic acid metabolism regulated by protein kinase C (Table 2). The table shows the composition of the AA released in response to TPA and hormonal stimulation, as a percentage of the total ^{3}H cpm released. The composition was 34–48% PGE_2 and 17–27% free arachidonic acid. The remaining metabolites were not identified. The relative proportions of the released AA products were not changed by sphingosine treatment. Because sphingosine produced a prominent decrease in overall AA release stimulated by epinephrine and TPA (Figs. 1–3) but did not change the composition of products, we conclude that availability of free arachidonic acid is regulated by protein kinase C. This regulation may represent an effect of protein kinase C on deacylation from membrane phospholipids and/or reacylation of arachidonic acid to membrane phospholipids.

Effect of protein kinase C inhibitors on α_1-adrenergic receptor binding. Previous work has suggested that protein kinase C may regulate α_1-adrenergic receptors (27). In addition, sphingosine has recently been shown to increase epidermal growth factor receptor number and affinity (28) and to inhibit thyrotropin-releasing hormone binding to its receptors (29), by mechanisms at least partly independent of protein kinase C. In order to establish that the diminution of epinephrine-mediated AA release by sphingosine and H-7 did not result from a decrease in α_1-adrenergic receptor number or affinity, we conducted saturation isotherm and competition binding studies of [^{3}H]prazosin to α_1-receptors on intact MDCK-D1 cells (Fig. 5). Sphingosine and staurosporine had no effect on either receptor number or affinity, whereas H-7 increased the K_d approximately 3-fold. In the competition binding studies, none of the agents decreased the ability of epinephrine to compete for [^{3}H]prazosin sites. Taken together, the data indicate that the changes observed in α_1-mediated AA release with sphingosine, H-7, and staurosporine are not due to effects of the inhibitors on agonist binding to α_1-receptors.

Comparison of [^{3}H]AA release with PGE_2 production. In order to eliminate the possibility that nonequilibrium metabolic labeling with [^{3}H]arachidonic acid results in measurement of [^{3}H]AA release not representative of the actual mass of AA released by cells (30), we sought to confirm our observations regarding the effects of sphingosine by a method independent of metabolic labeling. Therefore, we measured PGE_2 release by radioimmunoassay in experiments where [^{3}H]AA release (Fig. 6) was also measured. We found that TPA- and epinephrine-stimulated PGE_2 release were more sensitive to inhibition by sphingosine than was bradykinin-stimulated release. Furthermore, the measurement of PGE_2 release exhibited agonist concentration dependence very similar to that measured by [^{3}H]AA release for TPA, epinephrine, and bradykinin (data not shown). We conclude that our observations on the differing sensitivities to inhibition by sphingosine of bradykinin- and epinephrine-stimulated [^{3}H]AA release are not a consequence of nonequilibrium metabolic labeling with [^{3}H]arachidonic acid.

Discussion

The current results strongly suggest that there is more than one mechanism for regulation of AA release by hormones in

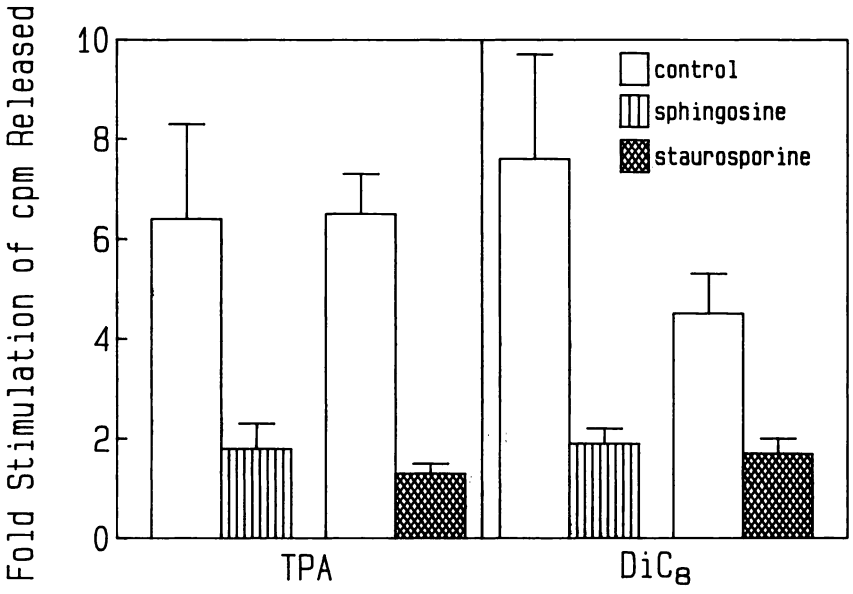

Fig. 4. Stimulation of [^{3}H]AA release by TPA and DiC_8. Dishes (35-mm) of subconfluent MDCK-D1 cells were labeled for 3–6 hr in 1 ml of labeling medium with 0.3–0.5 μCi/ml [^{3}H]arachidonic acid, incubated with 10 μM sphingosine (▥), with 50 nM staurosporine (■), or with appropriate vehicle (□), and stimulated for 60 min with 0.1 μM TPA (11 experiments for sphingosine treatment; 6 experiments for staurosporine treatment) or 200 μM DiC_8 (3 experiments for sphingosine treatment; 3 experiments for staurosporine treatment). *Points* shown are mean ± standard error. Values for unstimulated cpm released are as indicated in the legend to Fig. 1.

TABLE 2

Products comprising [^{3}H]AA release stimulated by TPA, epinephrine, and bradykinin: effect of sphingosine

Dishes (35-mm) of subconfluent MDCK-D1 cells were labeled for 3–6 hr in 1 ml of labeling media with 0.3–0.5 μCi/ml [^{3}H]arachidonic acid, incubated with or without 10 μM sphingosine for 10 min, and stimulated with 0.1 μM TPA for 60 min or 1.0 μM epinephrine or 1.0 μM bradykinin for 20 min, in triplicate. Control wells were incubated with IM alone, in parallel. Medium was aspirated and separated by thin layer chromatography, as described in Experimental Procedures. The mean of unstimulated values has been subtracted from each data point before calculation of percentages. Mean values for unstimulated PGE_2 release (cpm) with vehicle treatment were: 20 min, 255; and 60 min, 652. Mean values for stimulated PGE_2 release (cpm) with vehicle treatment were: TPA, 2532; epinephrine, 992; and bradykinin, 2019. Data shown are mean ± standard error for three experiments performed in triplicate and are reported as the percentage of total products identified as PGE_2, arachidonic acid, or other metabolites.

Treatment	Sphingosine	PGE_2	Arachidonic Acid	Other Metabolites
			% of total	
TPA	–	45.7 ± 6.9	24.3 ± 6.9	30.7 ± 6.1
	+	45.7 ± 5.4	23.7 ± 4.5	29.7 ± 5.0
Epinephrine	–	46.3 ± 8.1	24.3 ± 6.7	28.0 ± 3.2
	+	34.0 ± 7.1	26.3 ± 1.4	39.3 ± 9.7
Bradykinin	–	44.5 ± 7.0	20.0 ± 6.6	35.0 ± 4.6
	+	48.0 ± 4.6	17.7 ± 5.3	38.0 ± 5.8

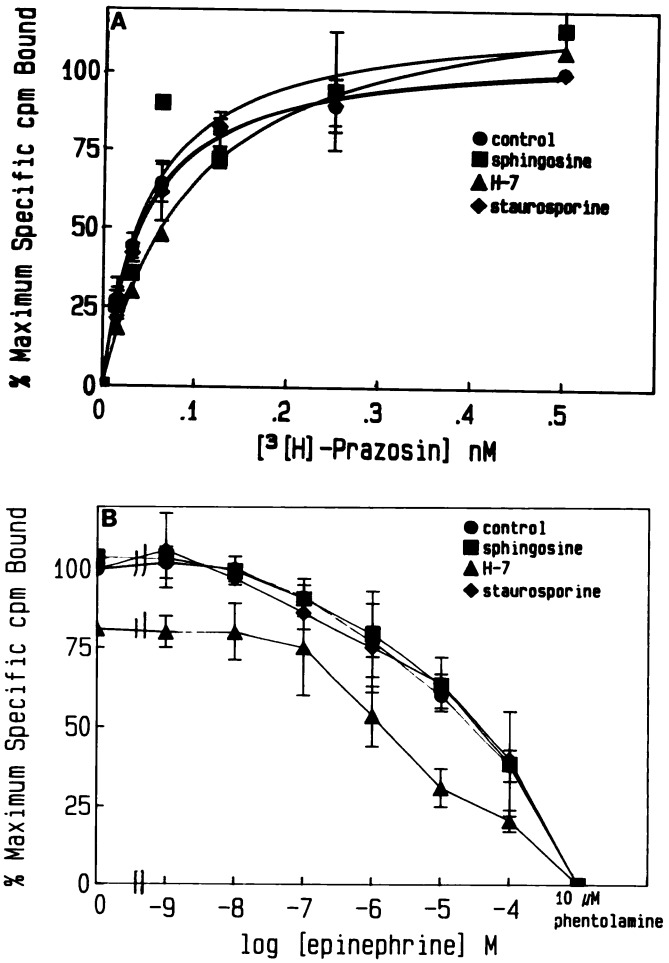

Fig. 5. Effect of incubation with sphingosine, H-7, and staurosporine on α_1-adrenergic receptor binding. Dishes (35-mm) of subconfluent MDCK-D1 cells were incubated with 10 μM sphingosine for 10 min (■), 100 μM H-7 for 30 min (▲), 50 nM staurosporine for 5 min (◆), or vehicle for 10, 30, or 5 min (●), as described in Experimental Procedures, and were then incubated with varying concentrations of [^{3}H]prazosin or [^{3}H]prazosin and 10 μM phentolamine for at least 60 min, washed, and collected, as described in Experimental Procedures, to determine saturation binding (A), or were treated with 0.08 nM [^{3}H]prazosin and the indicated concentration of epinephrine or 10 μM phentolamine for at least 60 min, washed, and collected, as described in Experimental Procedures, to assess competitive binding by the agonist (B). Data shown are mean ± range for two experiments. Scatchard analysis indicated a K_d of 0.055 nM in cells treated with IM alone, 0.059 nM in cells treated with sphingosine, 0.160 nM in cells treated with H-7, and 0.035 nM in cells treated with staurosporine. B_{max} values were 11.9, 14.3, 18.8, and 10.4 fmol/dish, respectively. Nonspecific binding (3.0 fmol/dish at 0.08 nM [^{3}H]prazosin) for each concentration of [^{3}H]prazosin has been subtracted. Nonspecific binding was not altered by treatment with sphingosine, H-7, or staurosporine.

MDCK-D1 cells, which can be distinguished by differential dependence on protein kinase C. α_1-Adrenergic and bradykinin receptors seem to utilize these mechanisms to different extents to stimulate AA release in these cells. The different time courses of bradykinin- and epinephrine-stimulated AA release also suggest that the two hormones act by different mechanisms. Based on the differing sensitivities of bradykinin and epinephrine stimulation to inhibitors of protein kinase C, as well as on the inability to eliminate bradykinin-stimulated AA release by down-regulation of protein kinase C, epinephrine-stimulated release appears to be more dependent on protein kinase C activation than bradykinin-stimulated release. The decreases in epinephrine-mediated release brought about by treatment with sphingosine, H-7, and staurosporine are not attributable to a change in α_1-receptor number or affinity.

The three inhibitors used in these experiments, sphingosine, H-7, and staurosporine, have been described as inhibitors of protein kinase C both *in vitro* and *in vivo*, at concentrations similar to those used here. Sphingosine acts by inhibiting activation of protein kinase C by its cofactors (18). H-7 directly interacts with the enzyme catalytic site (21). Staurosporine may compete with ATP (31). Recent work has shown that sphingosine inhibits the Ca^{2+}/calmodulin protein kinase (25). H-7 is also an inhibitor of the cAMP-dependent protein kinase (21), but our previous studies indicate that the cAMP-dependent protein kinase does not stimulate AA release (4, 32). Staurosporine also inhibits myosin light chain kinase (33). Lack of specificity is a drawback of using these agents to infer precise enzymatic mechanisms. However, the similarity of effects that we observe with all three inhibitors used here suggests that it is their common effect on protein kinase C, rather than their other diverse effects, that is responsible for the results that we have shown. The incomplete inhibition of TPA-mediated release with sphingosine is consistent with previous results indicating that not all of the TPA-stimulated AA release in MDCK cells can be eliminated by treatment with inhibitors of protein kinase C (13). It is possible that some of the TPA-stimulated AA release is occurring by a protein kinase C-independent mechanism. The inability of high concentrations of TPA to overcome the inhibition by sphingosine may also be due to the mechanism of action of sphingosine, which does not appear to be simple competition at the phorbol ester binding site (18). It is not clear why the hormones showed such large variability in their sensitivities to sphingosine and staurosporine. It is possible that this variability might result from differential sensitivities of protein kinase C isozymes to the inhibitors. Overall, we believe that our data support the conclusion that there is more than one mechanism for regulation of AA release by epinephrine and bradykinin, which can be distinguished by differential dependence on protein kinase C. α_1-Adrenergic receptors appear to utilize the protein kinase C-dependent

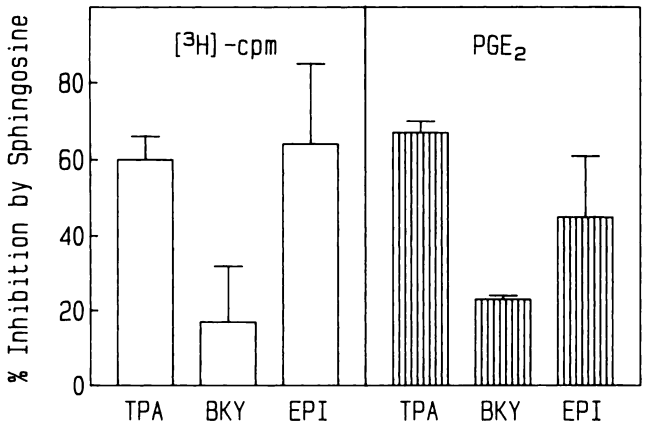

Fig. 6. Comparison of inhibition by sphingosine of PGE_2 release and [^{3}H]AA release. Dishes (35-mm) of subconfluent MDCK-D1 cells were labeled for 3–6 hr in 1 ml of labeling medium with 0.3–0.5 μCi/ml [^{3}H]arachidonic acid, incubated with 10 μM sphingosine or 0.1% ethanol in IM, and then stimulated for 60 min with 0.1 μM TPA or 20 min with 1.0 μM epinephrine or 1.0 μM bradykinin, as described in Experimental Procedures. Control wells incubated with IM alone were incubated in parallel. Aspirated medium was divided into two 500-μl aliquots. One was counted in a liquid scintillation counter and the other was frozen for subsequent analysis by radioimmunoassay, as described in Experimental Procedures. *Bars* shown are per cent inhibition in cells incubated with sphingosine, compared with cells incubated with vehicle alone, of PGE_2 release (▨) or [^{3}H]AA release (□). Mean ± range for two experiments are shown for each panel. The mean of unstimulated values has been subtracted from each data point. Values for control and stimulated cpm released are as indicated in the legend to Fig. 1. Mean values for unstimulated PGE_2 release (pg/500 μl) with vehicle treatment were: 20 min, 18; and 60 min, 98. Mean values for stimulated PGE_2 release (pg/500 μl) with vehicle treatment were: TPA, 1930; epinephrine (*EPI*), 176; and bradykinin (*BKY*), 827.

mechanisms to a greater extent to simulate AA release from MDCK-D1 cells than do bradykinin receptors. This interpretation is consistent with the previous observation that bradykinin-stimulated PGE_2 synthesis does not directly involve protein kinase C in fibroblasts (34).

Protein kinase C may exert its effects on either the deacylation of arachidonic acid from membrane phospholipids, the reacylation of arachidonic acid to membrane phospholipids, a subsequent arachidonic acid-metabolizing step, or some combination thereof. The observation that sphingosine does not change the products comprising released AA favors the idea that the deacylation step and/or the reacylation step are important regulatory sites for protein kinase C. It is generally accepted that deacylation of arachidonic acid from membrane phospholipids is the rate-limiting step for eicosanoid production. Protein kinase C may stimulate AA release by several possible mechanisms, including activation of PLA_2 by phosphorylation (13), regulation of PLA_2 inhibitory proteins such as the lipocortins (35), or regulation of PLA_2 stimulatory proteins (36). Activation of a phospholipase C followed by diacylglycerol/monoacylglycerol lipase activity could also liberate arachidonic acid (37). Other studies indicate that TPA stimulates release of label from the phosphatidylethanolamine pool in MDCK cells (13). Work in neutrophils (15) also suggests that it is the deacylation step that is regulated by protein kinase C.

Previous work in MDCK-D1 cells indicates that it is likely that bradykinin, epinephrine, and TPA all promote deacylation of phospholipids by PLA_2 (4, 5) and that this may be a step at which protein kinase C enhances AA release (10, 12). It is possible that the reduction in released AA observed following treatment with the inhibitors used here is due to a direct inhibitory effect on PLA_2. When this possibility was tested with a PLA_2 isolated from the macrophage-like cell line P388D_1 (38, 39), sphingosine did not inhibit PLA_2 activity.[3] In addition, 50 nM staurosporine had no effect on AA release stimulated by 1 μg/ml mellitin,[4] a potent activator of PLA_2.

Regulation of the reacylation step has recently been demonstrated (40) in macrophages, in which stimulation by diacylglycerol has been shown to inhibit activity of lysophosphatide acyltransferase and to increase prostaglandin production, although these responses were apparently not dependent on protein kinase C. The possibility that protein kinase C can regulate multiple enzymes involved in phospholipid metabolism and eicosanoid formation will require further study. However, the current findings emphasize that hormones that stimulate these pathways can show differential dependence on protein kinase C for producing their responses.

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[3] M. D. Lister and E. A. Dennis, unpublished observations. These experiments were conducted under standard assay conditions (42), with dipalmitoyl-phosphatidylcholine vesicles as substrate in the presence of 4 or 10 mol % sphingosine, relative to phospholipid.

[4] B. A. Weiss, unpublished observations.

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