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Activation of Protein Kinase C by 1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine Carboxylic Acid Methyl Ester (Bay K 8644), a Calcium Channel Agonist, in Alveolar Type II Cells

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ABSTRACT. A role for calcium channels in the regulation of surfactant secretion is suggested by the observation that endothelin-1-stimulated surfactant secretion is inhibited by calcium channel blockers. 1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester (Bay K 8644), a dihydropyridine derivative, stimulates voltage-dependent and non-voltage-dependent calcium channels in a number of cell types. This study demonstrates that Bay K 8644 increased phosphatidylcholine (PC) secretion in isolated lung epithelial type II cells in a time- and concentration-dependent manner with an EC_{50} of 100 ± 8 nM (mean ± SEM, N = 6). The secretagogue effect of Bay K 8644 was independently decreased in the absence of external calcium, or in the presence of nifedipine, a calcium channel antagonist, or inhibitors of protein kinase C (PKC). Bay K 8644 increased intracellular calcium from 130 ± 8 to 230 ± 14 nM (N = 6, $P < 0.05$), an effect that was blocked by nifedipine. Bay K 8644 also increased the membrane-associated PKC activity in a concentration-dependent manner. In the membranes from Bay K 8644-stimulated cells, the increase in calcium-dependent PKC was greater than that in the calcium-independent PKC, suggesting preferential translocation of calcium-dependent PKC to the membranes. We suggest that both elevated calcium and activation of PKC are required for calcium agonist Bay K 8644-induced surfactant secretion in type II cells. BIOCHEM PHARMACOL 53;9:1307–1313, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. phosphatidylcholine secretion; diacylglycerol; protein kinase C translocation; protein kinase C isozymes; intracellular calcium

Lung epithelial type II cells synthesize and secrete pulmonary surfactant, a phospholipid-rich material that prevents alveolar collapse during end-expiration. The regulation of surfactant PC† secretion in type II cells is mediated via multiple pathways [1]. Various receptors, including β-adrenergic, purinergic, vasopressin, histamine, and endothelin, are involved in the stimulation of lung surfactant PC secretion [1–8]. Agonists for β-adrenergic and histamine receptors stimulate surfactant secretion by increasing cell cyclic AMP (cAMP) and activating cAMP-dependent protein kinase [9, 10], while vasopressin stimulates phospholipid hydrolysis and increases the formation of DAG and inositol phosphates [8]. ATP, an agonist for P_1- and P_2-purinoceptor, activates multiple intracellular pathways to increase cell cAMP, DAG, $[Ca^{2+}]_i$, and PC secretion [11, 12]. Furthermore, direct activation of PKC and increased $[Ca^{2+}]_i$, from either internal or external sources, have also been implicated in the regulation of surfactant secretion [9, 13].

Recently, we demonstrated that ET-1, a vasoactive peptide released by endothelial cells, is a novel and potent secretagogue for surfactant PC in type II cells [2]. In this study, ET-1-stimulated surfactant secretion was associated with elevated DAG and could be inhibited by selective antagonists for calcium channels, nifedipine and nitrendipine. These dihydropyridine compounds can bind to specific sites on L-type calcium channels with high affinity and in a stereospecific manner [14, 15]. 1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester (Bay K 8644), another dihydropyridine compound, acts as an agonist for L-type and

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† *Abbreviations:* $[Ca^{2+}]_i$, intracellular calcium; DAG, 1,2-diacylglycerol; DPPC, dipalmitoyl phosphatidylcholine; ET-1, endothelin-1; LDH, lactate dehydrogenase; PC, phosphatidylcholine; PKC, protein kinase C; and PS, phosphatidylserine.

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other calcium channels in a variety of cell types [16–21]. The present study investigated the effect of this agonist on surfactant PC secretion and demonstrated that Bay K 8644, presumably through Ca^{2+}-channels, affects the subcellular distribution of PKC activity. This study also distinguished the effect of Bay K 8644 on Ca^{2+}-dependent and Ca^{2+}-independent PKC activity. Parts of these studies have been presented in abstract form [22].

MATERIALS AND METHODS

[^{3}H-*methyl*]Choline, [γ-^{32}P] ATP, and [^{14}C-*methyl*]DPPC were obtained from the Amersham Corp., Arlington Heights, IL. Elastase was obtained from the Worthington Biochemical Corp., Freehold, NJ. DAG kinase, staurosporine, H-7, and Bay K 8644 were obtained from Calbiochem, La Jolla, CA. PS and cardiolipin were purchased from Avanti Polar Lipids, Pelham, AL. Fura 2-AM was obtained from Molecular Probes, Eugene, OR. Tissue culture plastic dishes were obtained from Costar, Cambridge, MA. Rat serum IgG, DNase I, DAG, sphingosine, phorbol 12-myristate 13-acetate (PMA), and other standard chemicals were obtained from the Sigma Chemical Co., St. Louis, MO. Tissue culture media and phosphocellulose disks were obtained from Gibco BRL, Grand Island, NY. Bacteriological plates (Falcon 1029) were purchased from Becton and Dickinson, Franklin Lakes, NJ.

Isolation of Type II Cells

Alveolar type II cells were isolated from adult rat lungs according to Dobbs *et al.* [23] as described previously [24]. The cells were cultured for 20–22 hr at 37° in 5% CO_2 in air on tissue culture plastic dishes in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2.6 mM L-glutamine, 100 U/mL penicillin, 40 μg/mL gentamycin, and 40 μg/L streptomycin. At the end of this culture period, >95% of attached cells were viable as judged by exclusion of vital dye (erythrosin B) and >90% of these were type II cells as determined by phosphine 3R fluorescence.

PC Secretion Studies

Release of [^{3}H]PC was measured as described previously [24]. Briefly, after 20–22 hr of labeling with [^{3}H-*methyl*]choline, the cells were washed and equilibrated for 30 min in the incubation buffer (Krebs-Ringer-bicarbonate buffer containing 30 mM HEPES, pH 7.4, and 10 mM glucose). At the end of this equilibration period (zero time), Bay K 8644 was added from a stock solution in ethanol. All inhibitory agents were added at the beginning of the equilibration period. Nifedipine and sphingosine were prepared as a stock solution in ethanol, and staurosporine and H-7 were prepared in dimethyl formamide and subsequently diluted with buffer. The vehicle concentration was <0.5% in the incubation medium. Because of the light sensitivity of Bay K 8644 and nifedipine, all culture dishes were covered with aluminum foil during the period of incubation with each of these agents. To measure PC secretion at 0 time, some incubations were terminated at the end of 30 min of equilibration. At the end of each incubation period, medium and cells were collected, and lipids extracted [25] after the addition of [^{14}C]DPPC as a tracer to assess recovery, and egg PC as a carrier lipid. Radioactivity in ^{3}H-labeled lipids of each sample was corrected for losses during lipid extraction. The corrected radioactivity in the medium lipids was then expressed as percentage of that in the medium plus cells. All secretion results were corrected for secretion at zero time.

Determination of PKC Activity

Type II cells (2×10^6) were cultured on 35 mm culture dishes for 20–22 hr. The cells attached to culture dishes were washed four times with MEM, twice with the incubation buffer, and then equilibrated for 30 min in fresh incubation buffer as described for secretion studies. At the end of this equilibration period, vehicle or Bay K 8644 at indicated concentrations was added, and cells were incubated for the next 5 min. The incubation was terminated by rapid aspiration of the medium, and the culture dish was placed on ice.

PKC activity was assayed by phosphorylation of histone III S with [γ-^{32}P]ATP according to Gibco protocol as described previously [26]. The reaction mixture (50 μL) contained 2–5 μg protein, 20 mM $MgCl_2$, 0.5 mM $CaCl_2$, lipid micelles in the active mixture, or 1 mM EGTA and PKC inhibitor in the basal assay mixture. The reaction was started with 20 μg histone and 100 μM [γ-^{32}P]ATP (sp. act. = 1500 cpm/pmol). After incubation at 30° for 5 min, the reaction was terminated by pipetting a 25-μL aliquot on phosphocellulose paper disks. The paper disks were washed twice in 5% phosphoric acid and twice in water and counted for radioactivity. The PKC activity was expressed as the difference between active and basal assays and calculated as picomoles of ^{32}P incorporated based on the specific activity of ATP. Calcium-independent PKC was measured in the absence of any added calcium and in the presence of lipids and 1 mM EGTA. Calcium-dependent activity was calculated by subtracting calcium-independent activity from the total activity.

DAG Assay

In experiments designed to measure DAG levels, cells were washed and equilibrated in the incubation buffer for 30 min. Vehicle or Bay K 8644 at indicated concentrations was then added, and incubation continued for 30 sec. At the end of this incubation period, the medium was rapidly aspirated, and 1 mL of chloroform:methanol (1:2, v/v) was added to the tissue culture plate. To avoid delays with the processing of a batch of tissue culture dishes, each culture dish was handled separately. Lipids were extracted [25], and

DAG in the lipid extract was measured after phosphorylation to phosphatidic acid using [γ-^{32}P]ATP and DAG kinase followed by separation of [^{32}P]phosphatidic acid by TLC [27] as described previously [12, 24].

$[Ca^{2+}]_i$ Measurement

$[Ca^{2+}]_i$ in type II cells was measured fluorometrically as described by Gerboth *et al.* [28] using Fura 2/AM. After overnight culture on plastic dishes, cells were scraped in buffer containing (in mM) HEPES, 6; NaCl, 140; K^+, 5; Ca^{2+}, 1; Mg^{2+}, 2; glucose, 5) and incubated with Fura 2/AM (2.5 μM) for 30 min. Cells were washed twice and suspended in incubation buffer. In some experiments, the cell suspensions were incubated at 37° for 30 min with nifedipine prior to the addition of Bay K 8644.

$[Ca^{2+}]_i$ levels were measured from the changes in fluorescence ratio of Fura-2 (em = 510; exc = 340 and 380 nm) in a fluorescence spectrophotometer (Aminco Bowman Series 2, SLM Instrument, Urbana, IL). After obtaining the basal fluorescence ratio (resting $[Ca^{2+}]_i$), Bay K 8644 (1 μM) was added and the change in fluorescence ratio was determined. The maximum and minimum fluorescence were obtained after sequential addition of 60 μM digitonin and 20 mM EGTA. Autofluorescence of cultured cells was subtracted from the values obtained with dye-loaded cells. $[Ca^{2+}]_i$ was calculated according to the equation $[Ca^{2+}]_i = K_d(S_f/S_b)\{R - R_{min}/R_{max} - R\}$, where S_f and S_b are the fluorescence values for free and bound forms of fura-2, respectively, and R_{min} and R_{max} are the 340/380 ratios of fully free (obtained in the presence of 20 mM EGTA) and fully bound fura-2 (obtained in the presence of digitonin), respectively. The dissociation constant (K_d) for fura-2 was taken to be 224 nM [29].

Protein was measured using protein dye binding reagent (Bio-Rad Laboratories, Richmond, VA) according to the micro method of Bradford [30] with bovine γ-globulin as the standard. Phospholipid phosphorus was measured according to Marinetti [31] as described previously [32]. Phospholipid mass was calculated by multiplying phospholipid phosphorus by 25.

All secretion experiments were conducted in duplicate. Results from duplicate observations were averaged to yield single data points. Results were evaluated for statistical significance ($P < 0.05$) by Student's *t*-test for paired observations, or by one-way analysis of variance followed by Tukey's post-hoc test for comparison between any two groups.

RESULTS

The secretion of PC increased in a near linear fashion for up to 2 hr in the absence or presence of 1 μM Bay K 8644 (Fig. 1). After a 2-hr incubation, the secretion was 1.05 ± 0.19% (mean ± SEM, N = 4) in the absence and 2.80 ± 0.34% (N = 4, $P < 0.05$) in the presence of 1 μM Bay K 8644. The mean increase over the control secretion was

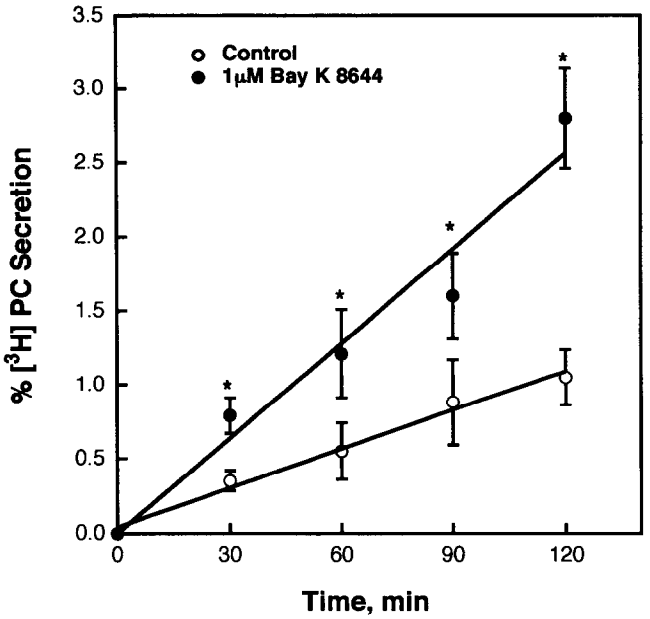

FIG. 1. Time course of PC secretion in type II cells in response to Bay K 8644. Type II cells, labeled with [^{3}H-*methyl*]choline, were equilibrated for 30 min in fresh medium, vehicle, or 1 μM Bay K 8644 was added, and the incubation continued for the indicated periods of time. The percent of total cellular [^{3}H] PC secreted into medium was then measured. Results are means ± SEM of experiments with four different cell preparations. Key: (*) $P < 0.05$ when compared with controls.

166%. The secretion of PC also increased with increasing concentration of Bay K 8644 (Fig. 2) with an estimated EC_{50} of 100 ± 8 nM (mean ± SEM, N = 6).

The secretagogue effect of Bay K 8644 was not due to cellular toxicity as assessed by release of LDH activity in the medium, or vital dye (erythrocin B) exclusion by attached cells. During the 2-hr incubation, the LDH release was 1.0 ± 0.2 and 0.8 ± 0.1% (N = 6, $P > 0.05$), in the absence or presence of 1 μM Bay K 8644, respectively, and 97 ± 0.4 and 97 ± 0.8% (N = 5, $P > 0.05$) of cells excluded erythrosin B, respectively. Thus, two independent parameters indicated that the secretagogue effect of Bay K 8644 was not due to toxicity to type II cells.

Bay K 8644 rapidly increased $[Ca^{2+}]_i$ in type II cells, which reached a maximum within 1 min (data not shown). The mean increase of 77% above the base line could be inhibited by preincubation of cells with 10 μM nifedipine (Table 1). The secretagogue effect of Bay K 8644 was also inhibited by nifedipine. These results suggest that stimulation of surfactant secretion with Bay K 8644 requires influx of calcium. In another set of experiments, Bay K 8644 did not stimulate PC secretion in the absence of external calcium (Table 2). The basal secretion in calcium-free medium was higher than that in medium containing calcium, as described previously by other investigators [9]. This increase in secretion in calcium-free medium was unrelated to calcium channel activity since it could not be blocked with nifedipine in 2 hr (data not shown).

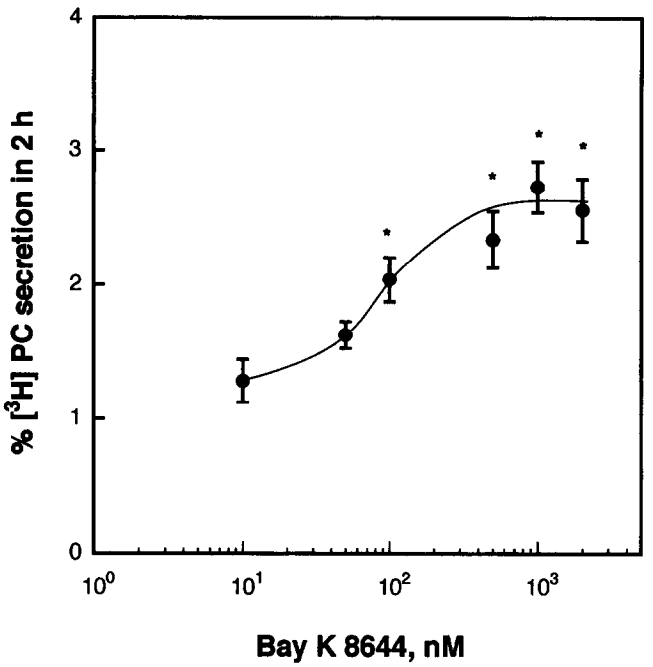

FIG. 2. Effect of Bay K 8644 concentration on PC secretion in type II cells. PC secretion during 2 hr was measured in the absence or presence of the indicated concentrations of Bay K 8644. Results are means ± SEM of experiments with six different cell preparations. Key: (*) $P < 0.05$ when compared with control PC secretion that was 0.92 ± 0.13% in 2 hr.

In our previous studies with ET-1, we had shown that calcium channel activation was associated with increased DAG content in type II cells [2]. Since in these previous studies we had suggested that activation of Ca^{2+} channel occurred via ET-1 receptor, we measured DAG content in type II cells after treatment with Bay K 8644, a direct activator of Ca^{2+} channel. A 30-sec treatment with 1 μM Bay K 8644 increased the DAG content of type II cells by 60% over the basal level (control, 48.6 ± 1.3; Bay K 8644, 74.8 ± 2.5 nmol/mg phospholipid, N = 4, $P < 0.05$). The increase in DAG content was concentration dependent, reaching near maximum at 0.5 μM Bay K 8644 (Fig. 3). The estimated EC_{50} was 38 ± 10 nM (N = 4).

Next, we investigated the effects of Bay K 8644 on PKC activity in the cytosol and membrane fractions of type II cells (Table 3). As reported previously [13, 26, 33], most of the PKC activity in control cells was present in the cytosol fraction (Table 3). Bay K 8644 increased the PKC activity in the membrane fraction by 179% and decreased it in the cytosol fraction by 56% (Table 3). As a result, the membrane-associated PKC increased from 5 to 18% of the total PKC activity during treatment with Bay K 8644. This redistribution of PKC activity by Bay K 8644 suggests translocation of cytosolic PKC to the membranes. The translocation of PKC was dependent on the concentration of Bay K 8644. The membrane-associated activity increased steadily between 50 nM and 1 μM Bay K 8644 (Fig. 4, inset). We also measured the effect of Bay K 8644 on calcium-dependent and calcium-independent PKC activity in type II cells (Table 4). In the cytosol and membrane fractions of

TABLE 1. Nifedipine inhibition of Bay K 8644-stimulated surfactant secretion and elevation in intracellular Ca^{2+} in type II cells

	% [3H]PC secretion in 2 hr	$[Ca^{2+}]_i$, (nM)
Control	0.72 ± 0.08	130 ± 8
Bay K, 1 μM	3.18 ± 0.48*	230 ± 14*
+Nifedipine, 10 μM	1.49 ± 0.32	143 ± 11

Results are means ± SEM of five experiments when measuring [3H]PC secretion and of six experiments when measuring $[Ca^{2+}]_i$.
* $P < 0.05$ vs the corresponding control.

TABLE 2. Calcium dependence of Bay K 8644 stimulation of PC secretion in type II cells

	% [3H]PC secretion in 2 hr		
	Calcium		
	1.3 mM	0 mM	*P* value
Control	0.96 ± 0.11	2.24 ± 0.23	<0.05
Bay K 8644, 1 μM	2.91 ± 0.23	2.82 ± 0.21	>0.05
P value	<0.05	>0.05	

Results are means ± SEM of experiments in seven different cell preparations. The incubation buffer in 0 calcium contained no added calcium.

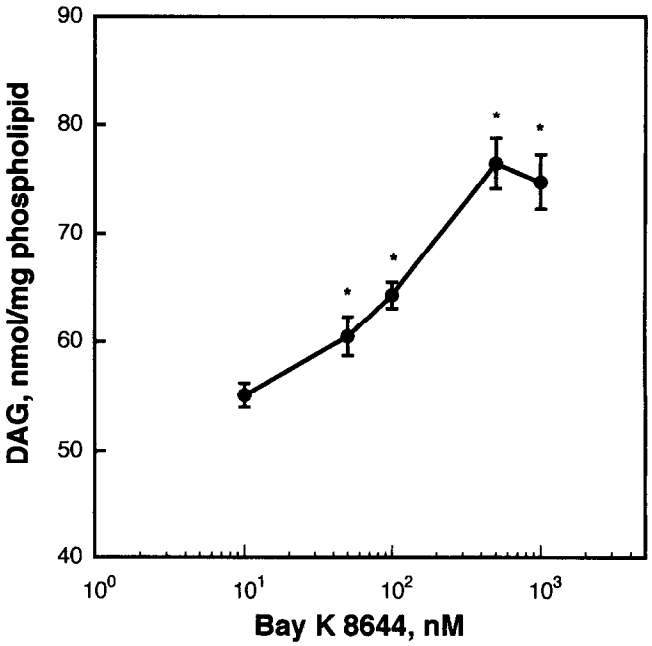

FIG. 3. Effect of Bay K 8644 concentration on DAG content in type II cells. Type II cells were treated for 30 sec with the indicated concentrations of Bay K 8644. Results are means ± SEM of four experiments. The DAG content in control cells was 48.6 ± 1.3 nmol/mg of phospholipid. Key: (*) $P < 0.05$ when compared with controls.

TABLE 3. Effect of Bay K 8644 on PKC activity in cytosol and membrane fractions of type II cells

	PKC activity (pmol P incorporated/ min/mg protein)		% of Total activity in the membranes
	Cytosol	Membranes	
None	975 ± 70	73 ± 6	5.1 ± 0.5
Bay K 8644	627 ± 80*	204 ± 31*	18.3 ± 2.1*

Results are from cells treated without or with 1 μM Bay K 8644 for 5 min and are means ± SEM of experiments in seven different cell preparations.
* $P < 0.05$ vs control.

control cells, the calcium-dependent PKC activity accounted for 43 and 56%, respectively, of total PKC activity in each of these fractions. In cells treated with Bay K 8644, both types of PKC activity decreased to a similar extent in the cytosol fraction. In the membrane fraction of treated cells, however, the calcium-dependent activity increased (256%) more than the calcium-independent (75%) activity. Consequently, almost 71% of the total membrane-associated PKC activity was calcium dependent in treated cells. These results suggest that Bay K 8644 treatment of type II cells preferentially activates calcium-dependent PKC. This was further supported by the greater slope for the calcium-dependent than for the calcium-independent PKC activity in the membrane fraction when the concentration dependence of Bay K 8644 on PKC activity was evaluated (Fig. 4).

Next, we evaluated the role of PKC in stimulation of PC secretion with Bay K 8644 by following secretion in the presence of PKC inhibitors: H-7, staurosporine, and sphingosine. Each substance was present during the equilibration period of 30 min before the addition of Bay K 8644. Each of the PKC inhibitors decreased the Bay K 8644-stimulated secretion (Table 5). The stimulated secretion was inhibited completely with staurosporine (100 nM) and sphingosine (10 μM) and by 70% with 10 μM H-7.

DISCUSSION

An increase in cell calcium is associated with an elevated secretory response in a variety of cells including lung type II cells [9, 34, 35]. Previous investigators have utilized calcium ionophores to demonstrate a correlation between cell calcium and surfactant secretion. Recently, we demonstrated that ET-1 increases surfactant secretion via a calcium-dependent pathway [2]. In the previous study, ET-1 was also shown to increase cellular DAG mass, which suggested activation of PKC by calcium-dependent mechanisms. The present study demonstrated that direct activation of calcium channels (in contrast to activation via ET-1 receptor) with Bay K 8644, a selective agonist for calcium channels in many systems [17, 18, 36], increases PKC activity in the membrane fraction of type II cells. The present study also provides evidence that stimulation with Bay K 8644 induces preferential association of calcium-dependent PKC with the membranes.

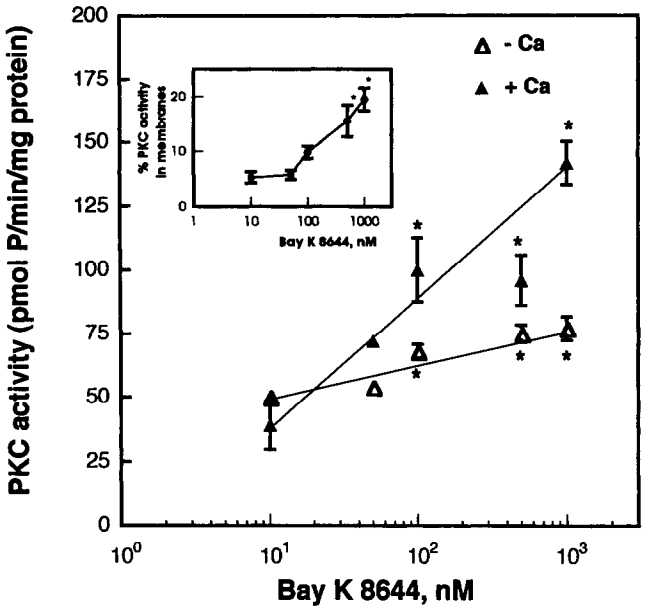

FIG. 4. Concentration dependence of the effect of Bay K 8644 on membrane-associated PKC activity. Cells were incubated for 5 min with the indicated concentrations of Bay K 8644. Calcium-dependent and calcium-independent PKC activity was measured in the membrane fractions. Results are means ± SEM of experiments with four different cell preparations. The calcium-dependent and calcium-independent PKC activities in membrane fractions of control cells were 23 ± 0.58 and 36 ± 2.08 pmol/min/mg protein, respectively. (Inset) Percent of total PKC activity in the membrane fraction as a function of Bay K 8644 concentration. The membrane fraction of control cells contained 3.3 ± 1.3% of the total PKC activity. Key: (*) $P < 0.05$ when compared with corresponding activity in the membrane fraction of control cells.

Bay K 8644 binds to calcium channels and facilitates calcium influx by increasing the mean open time of calcium channels [16, 36, 37]. Our results on $[Ca^{2+}]_i$ also support a similar action of Bay K 8644 in type II cells. The loss of the secretagogue effect of Bay K 8644 with nifedipine, or in the

TABLE 4. Effect of Bay K 8644 on calcium-dependent and calcium-independent PKC activity in cytosol and membranes of type II cells

	PKC activity (pmol P/min/mg protein)			
	Cytosol		Membranes	
Addition	- Calcium	+ Calcium	- Calcium	+ Calcium
None	552 ± 17	423 ± 56	33 ± 2	41 ± 8
Bay K 8644	348 ± 36*	279 ± 46*	58 ± 4*	146 ± 34*

Results are means ± SEM of experiments in seven different cell preparations. Cells were incubated for 5 min in the absence or presence of 1 μM Bay K 8644.
* $P < 0.05$ vs corresponding control.

TABLE 5. Effect of PKC inhibitors on calcium channel agonist-stimulated PC secretion in type II cells

	% [^{3}H] PC secretion in 2 hr
Control	0.83 ± 0.05
Bay K 8644, 1 μM	3.69 ± 0.65*
+ Staurosporine, 100 nM	0.79 ± 0.08
+ H-7, 10 μM	1.79 ± 0.32†
+ Sphingosine, 10 μM	0.98 ± 0.31

Results are means ± SEM of paired experiments in three cell preparations.
* $P < 0.05$ vs all other groups.
† $P < 0.05$ vs control or Bay K 8644.

absence of external calcium, suggests that calcium influx is the stimulus for the secretagogue effect of Bay K 8644. Although incubation of cells in the absence of external calcium can lower the resting $[Ca^{2+}]_i$ [35] and blunt the secretagogue effect of Bay K 8644, Ca^{2+} influx would also be blocked in the presence of nifedipine, which blocks calcium elevation by enhancing closure intervals of calcium channels [17].

Our study is the first demonstration of PKC activation by a calcium agonist in type II cells. Previous studies have implicated PKC activation in the regulation of diverse cellular functions including lung surfactant secretion [38, 39]. Stimulation of surfactant secretion by various secretagogues that rapidly increase cellular DAG, by phorbol esters that mimic DAG effect, or inhibition of stimulated surfactant secretion by various PKC inhibitors [4, 6–8, 12, 13, 24, 40] suggests that PKC activation plays a role in the regulation of surfactant secretion. Various members of the PKC family that phosphorylate serine/threonine residues in substrate proteins in a lipid-dependent manner can be grouped into two broad categories of calcium-dependent and calcium-independent enzymes [39]. As previously observed with ATP, the calcium-agonist Bay K 8644 also stimulated the calcium-dependent membrane PKC activity to a greater extent than the calcium-independent activity (Table 4). Thus, the calcium-dependent PKC appears to play a major role in the stimulation of surfactant secretion.

In summary, this is the first study to demonstrate that the calcium agonist Bay K 8644 increases cell $[Ca^{2+}]_i$ membrane-associated calcium-dependent PKC activity and stimulates surfactant secretion in a time- and concentration-dependent manner in alveolar type II cells. The activation of PKC, in conjunction with calcium elevation, is apparently required for stimulation of surfactant secretion.

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