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Effect of Staurosporine on the Long-term Secretion of Catecholamines Induced by Various Secretagogues in Cultured Bovine Adrenal Medullary Chromaffin Cells

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Long-term treatment of cultured bovine adrenal medullary chromaffin (BAMC) cells with arachidonic acid (100 μM), angiotesnin II (100 nM), prostaglandin E_2 (PGE_2; 10 μM), veratridine (2 μM) or KCl (55 mM) for 24 hrs increased both norepinephrine and epinephrine levels in the supernatant. Pretreatment with staurosporine (10 nM), a protein kinase C (PKC) inhibitor, completely blocked increases of norepinephrine and epinephrine secretion induced by arachidonic acid, angiotensin II, PGE_2, veratridine or KCl. In addition, K252a, another PKC inhibitor whose structure is similar to that of staurosporine, effectively attenuated both norepinephrine and epinephrine secretion induced by arachidonic acid. However, K252a did not affect the catecholamine secretion induced by angiotensin II, PGE_2, veratridine or KCl. Our results suggest that staurosporine may inhibit long-term catecholamine secretion induced by various secretagogues in a mechanism other than inhibiting PKC signaling. Furthermore, long-term secretion of catecholamines induced by arachidonic acid may be dependent on PKC pathway.

Key Words: Bovine adrenal medullary chromaffin cells, Staurosporine, K252a, Protein kinase C, Catecholamine secretion

INTRODUCTION

Many lines of evidence have demonstrated that protein kinase C (PKC) plays a crucial role in signal transduction in various organs and cell types. Staurosporine, an alkaloid isolated from culture broth of *streptomyces staurosporeus* (Omura et al, 1977), and K252a, an alkaloid isolated from culture broth of Nocardia species, are potent inhibitors of PKC (Kase et al, 1986; Tamaoki et al, 1986). Staurosporine and K252a are structurally similar compounds; staurosporine is 30 times more potent than K252a as an inhibitor of PKC activity.

Staurosporine and K252a inhibit protein kinase activity by competing for the ATP binding site of PKC, cyclic nucleotide-dependent protein kinases, tyrosine kinases, and myosin light chain kinase (Kase et al, 1986; Tamaoki et al, 1986; Nakano et al, 1987; Ozaki et al, 1987; Nakanishi et al, 1988). Davis et al (1989) have reported that staurosporine and K252a are highly selective for PKC over cyclic AMP-dependent protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase.

The bovine adrenal medullary chromaffin (BAMC) cell synthesizes and contains catecholamines such as norepinephrine and epinephrine (for review, see Livett et al, 1983). Regulation of catecholamine secretion and catecholamine gene expression in BAMC cells has been studied extensively (Livett et al, 1983; Bittner & Holz, 1990; Criado et al, 1997; Hwang et al, 1997). It is well known that the secretion of neurotransmitter or hormones is often accompanied by an increase in transcription in order to replenish

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the lost neurotransmitter or hormone. For example, the cyclic AMP-regulated pathway is involved in protein kinase A and in the activation of tyrosine hydroxylase (TH) and in the induction of gene expression of the three catecholamine-synthesizing enzymes, TH, dopamine beta-hydroxylase, and phenylethanolamine *N*-methyltransferase, resulting in changes in intracellular catecholamine levels available for consequent neurohormonal activities (Hwang et al, 1997). Increased transcription of the catecholamine gene can also stimulate secretion (Bittner & Holz, 1990).

PKC plays an important role in catecholamine secretion in BAMC cells. This hypothesis is supported by the finding that stimulation of BAMC cells with phorbol ester, 4 beta-phorbol 12-myristate acetate (TPA), increases catecholamine secretion (Brocklehurst & Pollard, 1985) and the increase of catecholamine secretion induced by other PKC activators was effectively blocked by PKC inhibitors (Loneragan et al, 1996; Marley & Thomson, 1996; Cox & Parsons, 1997). Previous studies have demonstrated that short- and long-term stimulation of BAMC cells with nicotine or angiotensin II causes translocalization of PKC from the cytosolic to the membrane fraction (Touminen et al, 1991, 1992). The primary goal of the present study was to compare the effects of staurosporine and K252a on long-term secretion of catecholamines induced by various secretagogues in BAMC cells.

METHODS

Materials

Arachidonic acid, angiotensin II, prostaglandin E_2, veratridine, KCl, Percoll, and Dulbecco's modified Eagle medium (DMEM) were all purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Fetal calf serum was purchased from Life Technologies (GIBCO BRL Research Products, Grand Islsand, NY, U.S.A.). Collagenase type B and leupeptin are products of Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Triton X-100 was purchased from Research Products International (Mount Prospect, IL, U.S.A.).

Cell culture

Primary cultures of BAMC cells were, with minor modifications, prepared according to the method of Wilson & Viveros (1981). In brief, bovine adrenal glands were retrogradely perfused with collagenase type B (3×15 min, 37°C) and medullae were dissected and digested further in a conical flask (20 min, 37°C). Then, the dispersed BAMC cells were isolated by centrifugation through a Percoll gradient. After subsequent washings, the cells were mixed into DMEM/F12 medium containing 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (45 μg/ml). The cells were plated onto 30-mm wells (5×10^6 cells/well; for catecholamine assay). Two days after plating, the medium was changed to serum-free medium. The cells were kept in an incubator with humidified air/5% CO_2 atmosphere at 37°C for 3~5 days after plating. The drug treatments were initiated after cells were in serum-free medium for 1~3 days.

Catecholamine assay

The level of secreted catecholamine from BAMC cells stimulated by various secretagogues was evaluated according to the previous method (Tank et al, 1985). The norepinephrine and epinephrine content of the culture media and cells was determined by high performance liquid chromatography (HPLC) with electrochemical detection.

Statistics

Arithmetic means, standard errors of means, and standard deviations were calculated. One-way analysis of variance was used to test the overall statistical significance. Comparison between three or more groups was done using Fisher's least significant difference test. Student's *t* test was used to compare two treatments. A $P<0.05$ was considered statistically significant.

RESULTS

Effect of arachidonic acid on the secretions of norepinephrine and epinephrine in BAMC cells

The level of basal secretion of both norepinephrine and epinephrine was 0.6 and 4.3 pmoles/10 μl, respectively. Treatment of BAMC cells with arachidonic acid (100 μM) for 24 hrs increased norepine-

phrine level by 9.0 pmoles/10 μl (about 15-fold compared with control) (Fig. 1a). Additionally, the same dose of arachidonic acid for 24 hrs increased epinephrine level by 41 pmoles/10 μl (about 9.5-fold compared with control) (Fig. 1b). The increased level of both norepinephrine and epinephrine secretion induced by arachidonic acid was completely blocked (0.9 and 9.4 pmoles/10 μl, respectively, $P<0.05$) by pretreatment with staurosporine (10 nM). Moreover, another PKC inhibitor, K252a almost abolished increase of arachidonic acid-induced norepinephrine secretion (2.8 pmoles/10 μl, $P<0.05$) and partially attenuated epinephrine secretion (19 pmoles/10 μl, $P<0.05$) induced by arachidonic acid (Fig. 1).

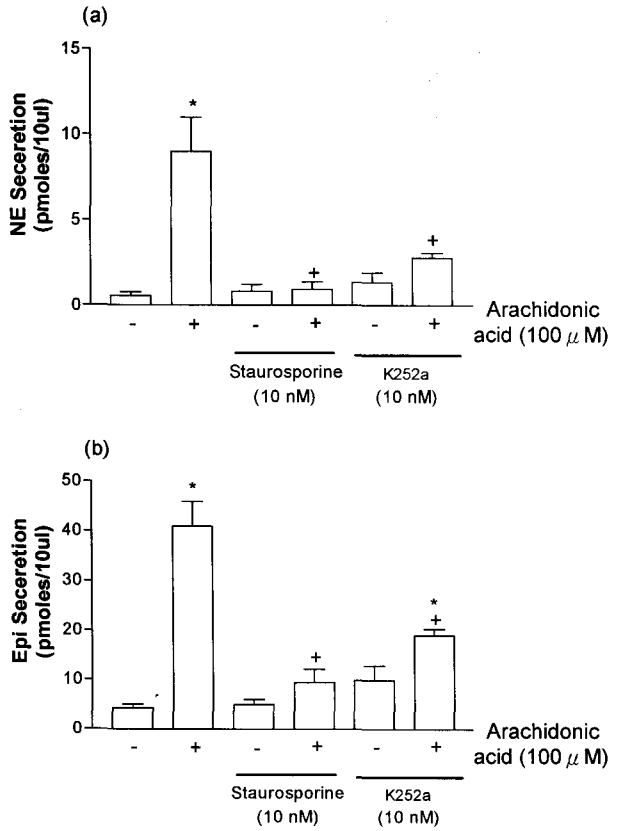

Fig. 1. Effects of staurosporine and K252a on norepinephrine (NE; a) or epinephrine (Epi; b) levels induced by arachidonic acid in the bovine adrenal medullary chromaffin (BAMC) cells. BAMC cells (5×10^6 cells/well) were pretreated 10 nM of staurosporine or K252a for 15 min and then were incubated at 37°C with 100 μM arachidonic acid for 24 hrs. Standard error mean for each bar ranged from 0.5 to 4.0% (n=3 independent experiments with triplicate samples for each experiment). $^*P<0.05$ compared with group of control, $^\dagger P<0.05$ compared with group of treated with arachidonic acid only.

Effect of angiotensin II on the secretions of norepinephrine and epinephrine in BAMC cells

Treatment of BAMC cells with angiotensin II (100 nM) for 24 hrs increased norepinephrine level by 8.1 pmoles/10 μl (about 13.5-fold compared with control) (Fig. 2a). Additionally, the same dose of angiotesnin II for 24 hrs increased epinephrine level by 35.6 pmoles/10 μl (about 8.3-fold compared with control) (Fig. 2b). The increased level of both norepinephrine and epinephrine secretion induced by angiotensin II was completely blocked (0.9 and 9.5 pmoles/10 μl, respectively, $P<0.05$) by pretreatment with staurosporine (10 nM) and K252a (10 nM).

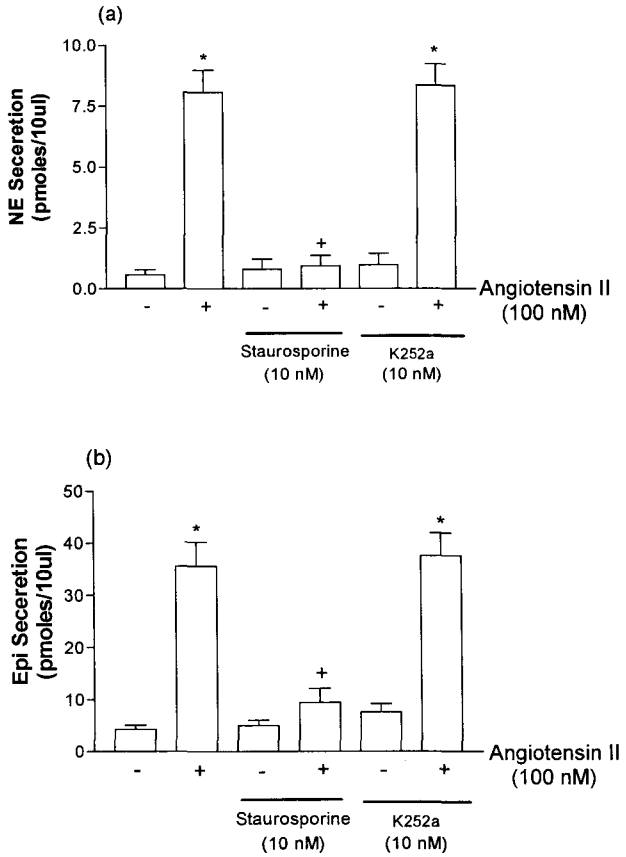

Fig. 2. Effects of staurosporine and K252a on norepinephrine (NE; a) or epinephrine (Epi; b) levels induced by angiotensin II in the bovine adrenal medullary chromaffin (BAMC) cells. BAMC cells (5×10^6 cells/well) were pretreated 10 nM of staurosporine or K252a for 15 min and then were incubated at 37°C with 100 nM angiotensin II for 24 hrs. Standard error mean for each bar ranged from 0.5 to 4.0% (n=3 independent experiments with triplicate samples for each experiment). $^*P<0.05$ compared with group of control, $^\dagger P<0.05$ compared with group of treated with angiotensin II only.

sporine (10 nM). However, K252a did not affect the catecholamines secretion induced by angiotensin II (Fig. 2).

Effect of prostaglandin$_2$ (PGE_2) on the secretions of norepinephrine and epinephrine in BAMC cells

Treatment of BAMC cells with PGE_2 (10 μM) for 24 hrs increased norepinephrine level by 9.3 pmoles/10 μl (about 15.5-fold compared with control) (Fig. 3a). Additionally, the same dose of PGE_2 for 24 hrs increased epinephrine level by 71 pmoles/10 μl (about 16.5-fold compared with control) (Fig. 3b). The increased level of both norepinephrine and epinephrine secretion induced by PGE_2 was completely blocked (0.8 and 9.3 pmoles/10 μl, respectively, $P<0.05$) by pretreatment with staurosporine (10 nM). However, K252a did not affect the catecholamines secretion induced by PGE_2 (Fig 3).

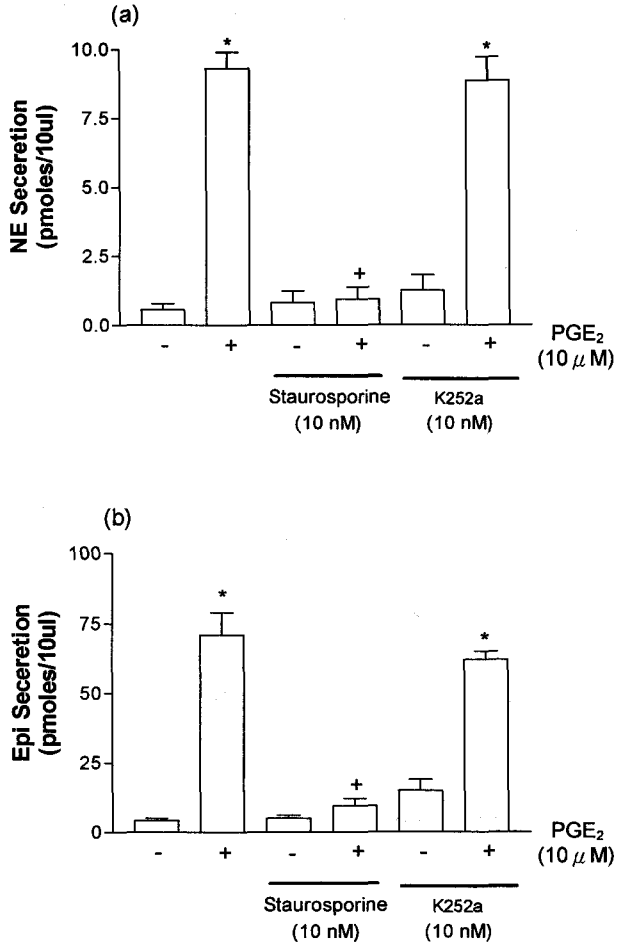

Fig. 3. Effects of staurosporine and K252a on norepinephrine (NE; a) or epinephrine (Epi; b) levels induced by prostaglandin E_2 (PGE_2) in the bovine adrenal medullary chromaffin (BAMC) cells. BAMC cells (5×10^6 cells/well) were pretreated 10 nM of staurosporine or K252a for 15 min and then were incubated at 37°C with 10 μM PGE_2 for 24 hrs. Standard error mean for each bar ranged from 0.5 to 4.0% (n=3 independent experiments with triplicate samples for each experiment). $^*P<0.05$ compared with group of control, $^\dagger P<0.05$ compared with group of treated with PGE_2 only.

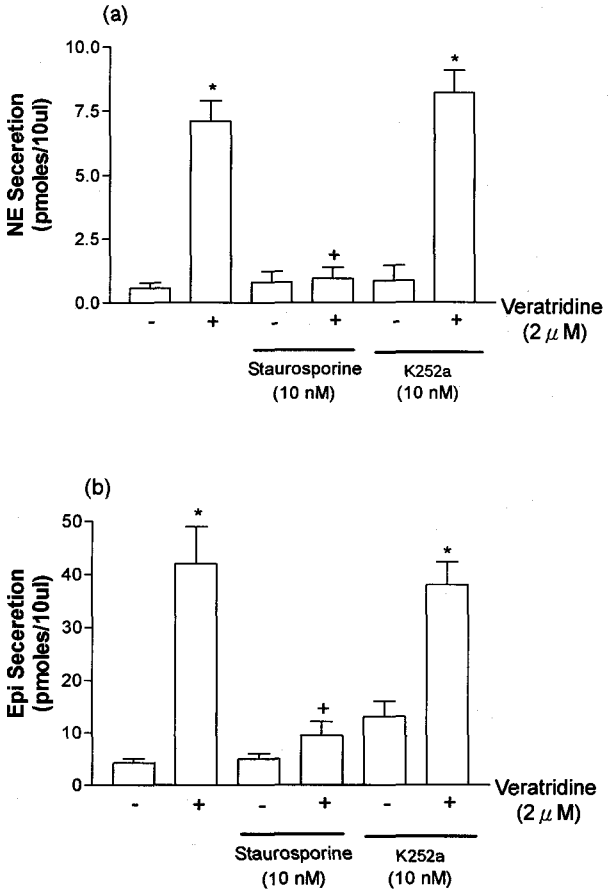

Fig. 4. Effects of staurosporine and K252a on norepinephrine (NE; a) or epinephrine (Epi; b) levels induced by veratridine in the bovine adrenal medullary chromaffin (BAMC) cells. BAMC cells (5×10^6 cells/well) were pretreated 10 nM of staurosporine or K252a for 15 min and then were incubated at 37°C with 2 μM veratridine for 24 hrs. Standard error mean for each bar ranged from 0.5 to 4.0% (n=3 independent experiments with triplicate samples for each experiment). $^*P<0.05$ compared with group of control, $^\dagger P<0.05$ compared with group of treated with veratridine only.

Effect of veratridine on the secretions of norepinephrine and epinephrine in BAMC cells

Treatment of BAMC cells with veratridine (2 μM) for 24 hrs increased norepinephrine level by 7.1 pmoles/10 μl (about 11.8-fold compared with control) (Fig. 4a). Additionally, the same dose of veratridine for 24 hrs increased epinephrine level by 42 pmoles/10 μl (about 9.8-fold compared with control) (Fig. 4b). The increased level of both norepinephrine and epinephrine secretion induced by veratridine was completely blocked (1.0 and 9.4 pmoles/10 μl, respectively, $P<0.05$) by pretreatment with staurosporine (10 nM). However, K252a did not affect the catecholamines secretion induced by veratridine (Fig. 4).

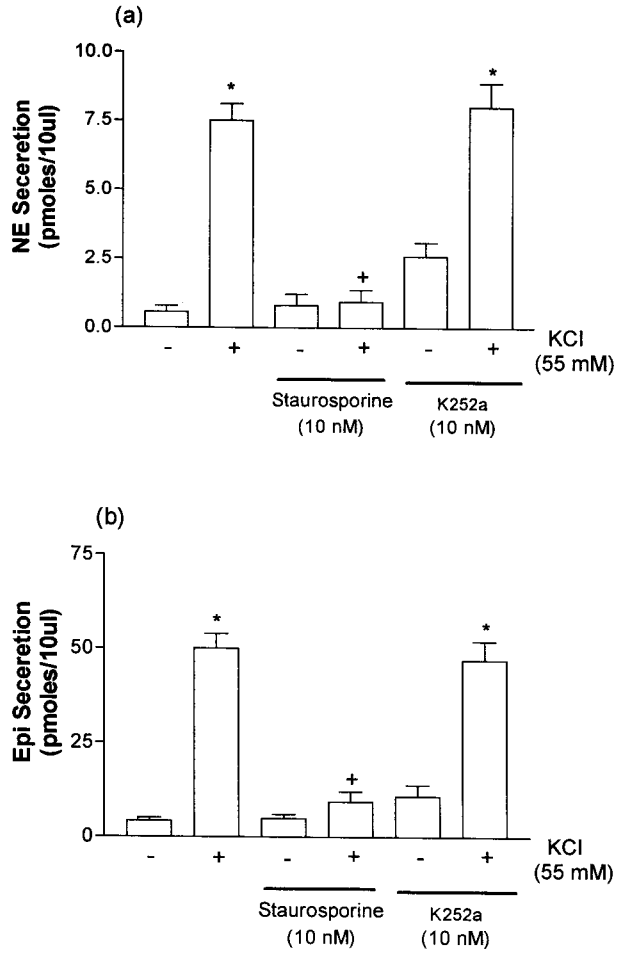

Fig. 5. Effects of staurosporine and K252a on norepinephrine (NE; a) or epinephrine (Epi; b) levels induced by KCl in the bovine adrenal medullary chromaffin (BAMC) cells. BAMC cells (5×10^6 cells/well) were pretreated 10 nM of staurosporine or K252a for 15 min and then were incubated at 37°C with 55 mM KCl for 24 hrs. Standard error mean for each bar ranged from 0.5 to 4.0% (n=3 independent experiments with triplicate samples for each experiment). $^*P<0.05$ compared with group of control, $^\dagger P<0.05$ compared with group of treated with KCl only.

Effect of KCl on the secretions of norepinephrine and epinephrine in BAMC cells

Treatment of BAMC cells with KCl (55 mM) for 24 hrs increased norepinephrine level by 7.5 pmoles/10 μl (about 12.5-fold compared with control) (Fig. 5a). Additionally, the same dose of KCl for 24 hrs increased epinephrine level by 50 pmoles/10 μl (about 11.6-fold compared with control) (Fig. 5b). The increased level of both norepinephrine and epinephrine secretion induced by KCl was completely blocked (0.8 and 9.4 pmoles/10 μl, respectively, $P<0.05$) by pretreatment with staurosporine (10 nM). However, K252a did not affect the catecholamines secretion induced by KCl (Fig. 5).

DISCUSSION

In the present study, we found that long-term treatment (24 hrs) of cultured BAMC cells with various secretagogues, such as arachidonic acid, angiotensin II, prostaglandin E_2, veratridine or KCl for 24 hrs increased both norepinephrine and epinephrine levels in the supernatant. Pretreatment with staurosporine (10 nM), a PKC inhibitor, completely blocked increases of norepinephrine and epinephrine secretion induced by arachidonic acid, angiotensin II, PGE_2, veratridine or KCl.

Staurosporine has inhibitory effects on the secretion of certain chemicals from various cell types in addition to BAMC cells (Dan-Cohen & Naor, 1990; White et al, 1990; Lim et al, 2000). However, K252a, another PKC inhibitor whose structure is similar to that of staurosporine, did not affect the catecholamine secretion induced by angiotensin II, PGE_2, veratridine or KCl. Although in BAMC cells there is a suggestion which PKC is not essential for Ca^{2+}-dependent catecholamine secretion, but acts as modulator (Tachikawa et al, 1990), Maurer et al (1995) have reported that staurosporine reduces catecholamine release by interfering with Ca^{2+} homeostasis, which may be involved in staurosporine-sensitive phosphoprotein (s) in BAMC cells. They also have

demonstrated that selective Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor, KN-62 significantly inhibited catecholamine release and $^{45}Ca^{2+}$ uptake (Maurer et al, 1996). Taken together with the present findings, an inhibitory effect of staurosporine on the secretion of catecholamines induced by various secretogogues appears to be mediated by other protein kinase rather than PKC. In fact, staurosporine often has considered as non-selective inhibitor affecting many kinases and inhibition of neurotransmitter release occurs only at concentration in excess of that required to decrease the effect of phorbol esters in some cases (Daschmann et al, 1988; Regg & Burgess, 1989; Schchtele et al, 1989). However, it is necessary to be ruled out another mechanism like involvement of cytoskeletal alterations. We have previously observed that staurosporine (10^{-8} M) induces the outgrowth of short processes from BAMC cells (Suh et al, 1994). Also, the intracellular concentration of [Met^5]-enkephalin (ME), proenkephalin A and its mRNA level was significantly increased in staurosporine-treated cells. In addition, pretreatment of cells with staurosporine effectively inhibited the long-term stimulatory effects of other secretagogues on ME secretion. In contrast to the results with staurosporine, the structurally similar compound, K252a (10^{-8} M) did not show these effects, although applied different parameter, such as ME secretion (Suh et al, 1994). It also has been reported that neurite-like growth was induced by higher concentration (2.5×10^{-8} M) of staurosporine (Demeneix et al, 1990). In addition to BAMC cells, several lines of evidence demonstrated that staurosporine increases the outgrowth of neurites in other types of cells, such as PC12, PC12h, human neuroblastoma, and normal rat chromaffin cells (Hashimoto & Hagino, 1989; Heikkila et al, 1989; Felipo et al, 1990; Shea & Beermann, 1991; Jalava et al, 1992). In neuronal hybrid cell line, PC12EN, staurosporine, but not K252a, induced neurite outgrowth (Rasouly et al, 1996). Tischler et al (1990), using PC12 cells, have suggested that some components of signal transduction pathway, possibly involving phosphorylation of proteins, are involved in the growth of short processes.

In addition, K252a as well as staurosporine effectively attenuated both norepinephrine and epinephrine secretion induced by arachidonic acid. Wakade et al (1991) have reported that phosphlipase C is involved in receptor-mediated activation of PKC, and phospholipase D is involved in depolarization activation of PKC. In some types of neurons, such as hippocampal glutamatergic neuron, it has been suggested that arachidonic acid plays an important role as an activator of PKC in synergy with diacylglycerol, which may indicate the enhanced transmitter release in the presence of arachidonic acid (Herrero et al, 1992; McGahon & Lynch, 1994). Thus, our results strongly suggest that arachidonic acid-induced long-term secretion of catecholamines may be dependent on the PKC pathway in the BAMC cells.

The present study showed that structurally similar compounds and widely used as PKC inhibitors, staurosporine and K252a, have a different effect on catecholamine secretion induced by angiotensin II, PGE_2, veratridine or KCl in the BAMC cells.

An previous study for endogenous PKC-like kinase activity of Sf9 cells have showed that staurosporine most efficiently inhibited kinase activity of the PKC subtypes, whereas K252a was at least 10 times less effective and had different sensitivities to PKC subtypes (Geiges et al, 1997). It also has been reported that the modulation of human basophil histamine release by PKC inhibitors may differs with secretagogue and with inhibitor (Bergstrand et al, 1992). That is, various purported PKC inhibitors on leukocyte histamine release triggered by different stimuli. They have proposed that various secretagogues trigger human leukocyte histamine release through in part separate pathways probably involving different kinase activities (PKC isozymes). These earlier studies imply that other possible mechanism like different sensitivity to PKC isozymes may exist between staurosporine and K252a, although they are structurally similar PKC inhibitors. Thus, it is remained to be elucidated that which subtypes are involved and differentiated their sensitivities from several PKC inhibitors in the catecholamine secretion induced by various secretagogues in the BAMC cells.

In summary, our results suggest that staurosporine may inhibit long-term catecholamine secretion induced by various secretagogues in a mechanism other than inhibiting PKC signaling only. Furthermore, two structurally similar PKC inhibitors, staurosporine and K252a, may have different sensitivities to PKC subtypes in the catecholamine secretion. Finally, long-term secretion of catecholamines induced by arachidonic acid may be dependent on PKC pathway.

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