Korean J. Toxicol.
Vol. 9, No. 2, pp. 187-194, 1993

EFFECT OF PERTUSSIS TOXIN ON THE SECRETION OF [MET^5]-ENKEPHALIN AND EXPRESSION OF PROENKEPHALIN A mRNA INDUCED BY NICOTINE, ANGIOTENSIN II, AND PHORBOL MYRISTATE ACETATE IN BOVINE ADRENAL MEDULLARY CHROMAFFIN CELLS

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(Received July 5, 1993)
(Accepted July 28, 1993)

ABSTRACT: *[Met^5]-Enkephalin (ME) secretion and the expression of proenkephalin A (proENK) mRNA were studied following long-term exposure of bovine adrenal medullary chromaffin (BAMC) cells to pertussis toxin. Prolonged (24 hr) stimulation of BAMC cells with pertussis toxin increased the secretion of ME as well as proENK gene expression. BAMC cells were also incubated with pertussis toxin in the presence or absence of other secretagogues such as nicotine, angiotensin II and phorbol myristate acetate. Nicotine, angiotensin II, and phorbol myristate acetate treated alone increased both ME secretion and proENK mRNA levels. Pertussis toxin synergistically increased the secretion of ME and proENK mRNA levels induced by nicotine and angiotensin II but had only an additive effect with phorbol myristate acetate on the secretion of ME and the level of proENK mRNA. Our results suggest that a pertussis toxin-sensitive G-proteins are involved in the long-term regulation of ME secretion as well as proENK mRNA. Pertussis toxin appears to have a synergistic effect on nicotine- and angiotensin II-but not phorbol myristate acetate-induced responses.*

Key Words: *Adrenal Medulla, Angiotensin II, G-proteins, Gene regulation, Nicotine, Opioid peptides, Petussis toxin, PMA.*

INTRODUCTION

Exposure of bovine adrenal medullary chromaffin (BAMC) cells to pertussis toxin (or islet-activating protein), resulted in ADP-ribosylation of the three α-subunits of the 41, 40 and 39 k Da G proteins in BAMC cells. In addition, ADP-ribosylation of pertussis toxin sensitive G-proteins by pertussis toxin increased the basal release

of catecholamines in a delayed manner and potentiated the catcholamine release induced by several secretagogues such as acetylcholine, nicotine, histamine, and KCl in BAMC cells. Several studies have suggested that pertussis toxin exerts its effect by a step distal to Ca^{2+} mobilization. This contention was supported by evidence that pertussis toxin does not increase intracellular calcium levels or inositol triphosphate formation (Sasakawa *et al.*, 1988). Furthermore, pertussis toxin does not affect either $^{45}Ca^{2+}$ uptake or the level of cyclic AMP (Tanaka *et al.*, 1990).

[Met^5]-Enkephalin (ME), an opioid pentapeptide, is co-stored and co-released with catecholamines in the adrenal medulla *in vivo* as well as *in vitro* (Viveros *wt al.*, 1979; Livett, 1981). Although the physiological function of ME in the adrenal medulla is not fully understood, the regulation of ME secretion and the expression of the proenkephalin A (proENK) gene by several secretagogues and second messengers were extensively studied. For example, nicotine, angiotensin II, arachidonic acid, and PGE_2 increase both ME secretion and proENK mRNA levels (Suh *et al.*, 1992a, 1992b, 1992c, 1993; Mar *et al.*, 1992; Stachowiak *et al.*, 1991; Wan *et al.*, 1990). 12-0-tetradecanoylphorbol-13-acetate (TPA) or 8-bromo-cyclic AMP increases ME release as well as proENK mRNA expression (Kley, 1988; Quach *et al.*, 1984; Mar *et al.*, 1992). In addition, calcium and calmodulin are involved in ME secretion and proENK gene expression (Suh *et al.*, 1992c, 1993; Kley *et al.*, 1986, 1987a and 1987b; Stachowiak *et al.*, 1991).

We have previously reported that long-term exposure of BAMC cells to pertussis toxin increases both ME secretion and proENK mRNA levels (Suh *et al.*, 1992c). The present study was, then, designed to investigate the effect of pertussis toxin on ME secretion and the expression of proENK mRNA incuced by various secretagogues such as nicotine, angiotensin II, and phorbol myristate acetate.

MATERIAL AND METHODS

Cell Culture

Primary cultures of BAMC cells were prepared according to the method of Wilson (1987). Briefly, bovine adrenal glands were retrogradely perfused with collagenase (3×15 min, 37℃) and medullae were dissected and further digested in a trypsinizing flask (20 min, 37℃). The BAMC cells were isolated by Renografin gradient centrifugation. The isolated cells were mixed into DMEM/F12 medium containing 10% fetal calf serum and plated on 30 mm wells (5×10^6 cells/well) in 5 m*l* of medium; and the cells were incubated in humidified air/5% CO_2-atmosphere at 37℃. Two days after plating, the cells were incubated with serum free medium for 24 hr before the start of the experiment.

Radioimmunoassay

The level of ME-like immunoreactivity was determined by radioimmunoassay as described (Hong *et al.*, 1978). The medium was boiled for 10 min. The radioimmunoassay for ME was carried out in duplicate. Iodinated ME (10,000 cpm) was incubated overnight at 4℃ with various concentrations of unlabelled ME, sample medium or cellular extract, and rabbit anti-ME serum, in a final volume of 0.5 m*l*. Bound ME was separated from free ME by incubation with 0.2 m*l* of a charcoal slurry containing 1.35% ovine serum albumin in RIA solution for 20 min followed

by centrifugation at 4000×g for 10 min. Five hundred μl of the supernatant were counted for radioactivity. The antiserum had the following cross-reactivities toward opioid peptides: ME, 100%; [Met-O^5]-enkephalin, 80%; [Leu^5]-enkephalin, <1%; [Met^5]-enkephalin-Arg^6-Phe^7, <0.4%; dynorphin (1-8), <0.06%; β-endorphin, <0.2%.

Isolation of RNA and mRNA Assay

Total RNA was extracted from BAMC cells 24 hr after drug treatment in a single step with a guanidium isothiocyanate/phenol/chloroform gradient procedure and the relative abundance of proENK mRNA was assayed by Northern blot analysis as described (Chomczynski and Sacchi, 1987). Ten μg of RNA were denatured and electrophoresed on a 1.2% agarose gel and transferred to nylon hybond-N hybridization filter sheet (Amersham, Arlington Heights, IL). After baking for 2 hr at 80℃, transfer membranes were prehybridized in a buffer at 42℃ for at least 4 hr. The [γ-^{32}P]-ATP radiolabelled proENK probe (5'-GCC GAG CGC CAG CAG CCA AGT GCA GAG TCC CAG GAA CCG CGC-3') was added (specific activity of 1×10^7 cpm/ml) and the membrane was incubated overnight (>14 hrs) at 42℃. Following hybridization, the membranes were washed three times in 2×SSC (sodium chloride sodium citrate) containing 0.1% sodium dodecyl sulfate at 42℃ for 20 min. The membranes were then dried and exposed to Kodak XAR-5 film at −70℃ for 1~3 days. The intensities of the hybridized bands were quantified by scanning densitometry with a Gilford densitometer. After hybridization with the proENK probe, membranes were rehybridized using a radiolabeled actin probe (5'-AGG ATG CCT CTC TTG CTC TGG GCC TCG TCG CCC AC -3') as an internal standard.

Experimental Procedure

All the expriments were performed 24 hr after the medium was changed to a serum free medium to reduce variations between separate cell preparations. In the first group, various concentrations of pertussis toxin (100 ng/ml) alone were administered for 24 hr. In the second group, the cells were incubated with pertussis toxin plus either nicotine, angiotensin II, or phorbol myristate acetate or PGE2. The cells were harvested 24 hr after drug treatment.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to test for overall statistical significance. Comparisons the secretion of ME between groups were made using Fisher's least significant difference test (Miller, 1966).

Materials

Pertussis toxin were purchased from Research Biochemical Inc. (Natick, MA). Nicotine, angiotensin II, and phorbol myristate acetate and PGE2 were purchased from Sigma Chemical Company (St. Louis, MO).

RESULTS

Effect of Pertussis Toxin on Secretion of ME and proENK mRNA

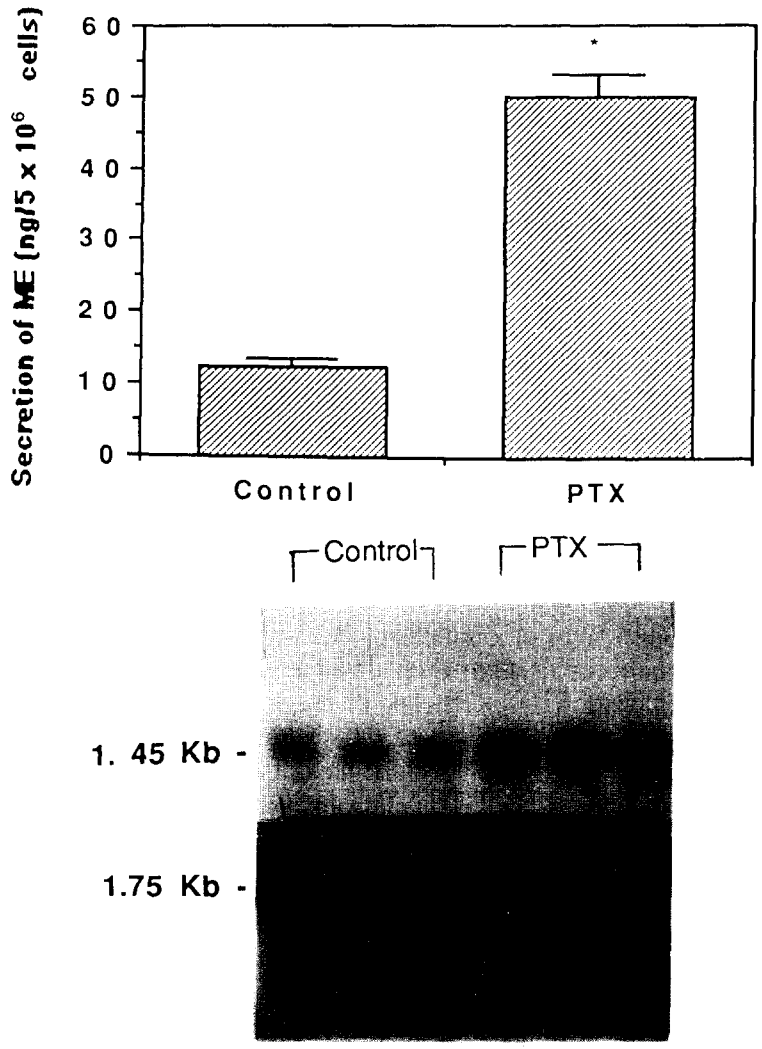

Figure 1. Pertussis toxin (PTX) increases ME secretion (a) and proENK mRNA (b) level in BAMC cells. BAMC cells (5×10^6 cells/well) were treated with pertussis toxin (100 ng/ml) for 24 hr. ME level was measured by radioimmunoassay. Total RNA was prepared using a guanidinium thiocyanate/phenol/chloroform gradient method. Ten μg of total RNA were used for the determination of proENK mRNA (1.45 Kb) levels with a Northern blot method. β-Actin mRNA (1.75 Kb) was used as an internal standard. The vertical bars indicate the S.E.M. (*, $p<0.01$ compared to control).

To determine if pertussis toxin increases the secretion of ME and proENK mRNA level, the BAMC cells were treated with pertussis toxin (100 ng/ml) for 24 hr. Long-term stimulation of BAMC cells with pertussis toxin caused a pronounced ME release and proENK mRNA level as shown in Figure 1a and b.

Effects of Pertussis Toxin on the Secretion of ME and proENK mRNA level induced by nicotine, angiotensin II, and phorbol myristate acetate.

To assess if there is any interaction between pertussis toxin and various secretagoues in the secretion of ME and expression of proENK mRNA, pertussis toxin

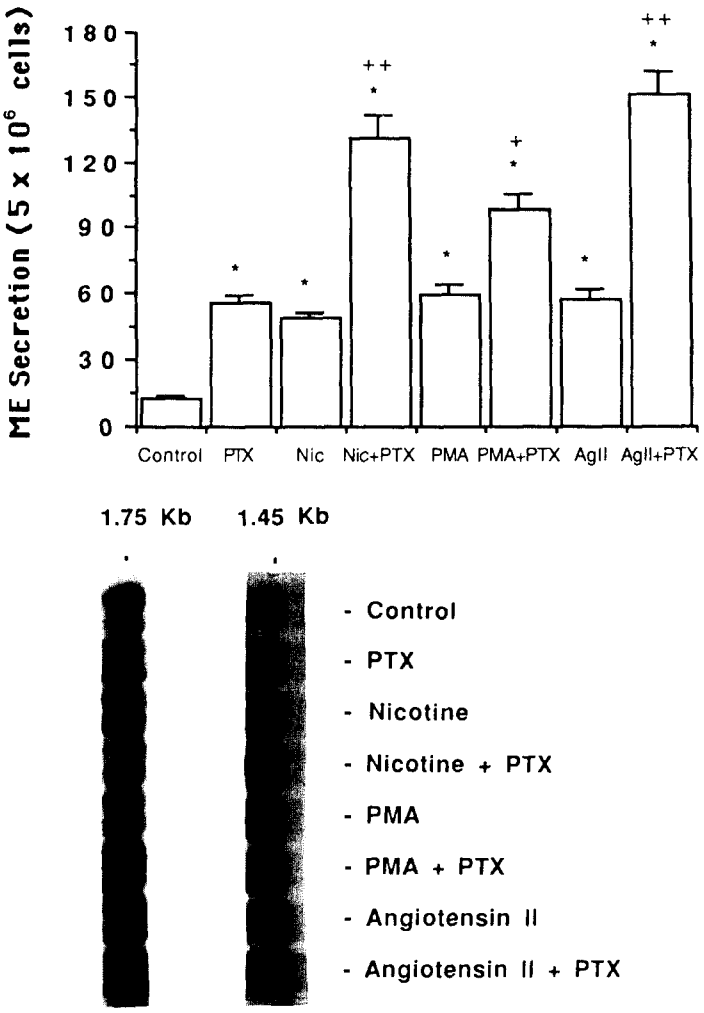

Figure 2. Effect of pertussis toxin (PTX) on ME secretion (a) and proENK mRNA (b) levels induced by nicotine (Nic), angiotensin II (AgII), and phorbol myristate acetate (PMA). The BAMC cells (5×10^6 cells) were incubated with pertussis toxin (100 ng/ml) plus nicotine (10 μM), angiotensin II (100 nM), or phorbol myristate acetate (1 μM) for 24 hr. The vertical bars indicate the S.E.M. (*$p < 0.01$; compared to control; +, $p < 0.05$; + +, $p < 0.01$ compared to either nicotine, PMA or angiotensin II group).

was coincubated with either nicotine, angiotensin II, or phorbol myristate acetate for 24 hr. Nicotine, angiotensin II, and phorbol myristate alone increased ME secretion as well as the expression of proENK mRNA (Figure 2a and b). The treatment of BAMC cells with pertussis toxin plus either nicotine or angiotensin II showed a synergistic interaction in the secretion of ME and proENK mRNA level (Figure 2a). However, the treatment of BAMC cells with pertussis toxin plus phorbol myristate acetate showed only an additive effect for the expression of proENK mRNA and secretion of ME (Figure 2a and b).

DISCUSSION

This paper demonstrates that pertussis toxin-sensitive G-proteins may have a tonic inhibitory effect on the secretion of ME in BAMC cells since inactivation of pertussis toxin-sensitive G-proteins caused a delayed, but enhanced, the secretion of ME. This observation is in agreement with our previous study that the long-term (24 hr) treatment of BAMC cells with pertussis toxin caused a pronounced increase in ME secretion as well as proENK mRNA level (Suh *et al.*, 1992c), further suggesting that pertussis toxin sensitive G-proteins play an important role in the regulation of the long-term secretion of ME and proENK gene expression in BAMC cells.

Increases of secretion of ME induced by pertussis toxin is probably not due to its toxic effect since the concentration of pertussis toxin used in the present study does not cause the release of endogenous lactate dehydrogenase, indicating that the cells remained intact during incubation with pertussis toxin (Brocklehurst and Pollard, 1988).

In the present study, the interaction of pertussis toxin with different secretagogues was studied. Our results suggest that pertussis toxin showed a selective synergistic interaction for both the increased secretion of ME and the enhanced expression of proENK mRNA induced by various secretagogues. This contention was supported by the finding that long-term exposure of BAMC cells with nicotine, angiotensin II, and phorbol myristate acetate increased both ME secretion and proENK mRNA level. These results are in line with our previous findings (Suh *et al.*, 1992a, 1992b, 1992c, 1993). We found that, in a current study, pertussis toxin synergistically increased both ME secretion and proENK mRNA level induced by nicotine and angiotensin II. However, pertussis toxin increased both ME secretion and proENK mRNA level induced by phorbol myristate acetate only in an additive manner. Although ME secretion secretion cannot be compared directly with catecholamine secretion, our results are in agreement with previous studies which reported that pertussis toxin alone causes the release of catecholamine and the secretion of catecholamines induced by nicotine was ehanced by pertussis toxin in BAMC cells (Brocklehurst and Pollard, 1988; Sasakawa *et al.*, 1988; Tanaka *et al.*, 1987). However, the detailed mechanims involved in a selective synergistic interaction between pertussis toxin and various secretagogues are not known. The signal transduction interrelationship among pertussis toxin-sensitive G proteins, ME secretion and proENK gene expression remains to be determined.

ACKNOWLEDGEMENTS

This work was supported in part by the Research Grant from Hallym College of Medicine Endowment Fund.

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