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Priming effect of vasoactive intestinal peptide on the respiratory burst of neutrophils non-mediated by plasma membrane receptors

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Abstract. Vasoactive intestinal peptide (VIP) primed the respiratory burst of human neutrophils in response to phorbol myristate acetate. Maximal and half-maximal effects were achieved at 10 and 0.5 nM VIP respectively. The absence of plasma membrane receptors to VIP in neutrophils suggests that priming of the respiratory burst should be considered as a side effect of VIP. However, from the above indicated concentration range, the priming of the neutrophil by VIP cannot be considered as a pharmacological effect. The enhancement of the formation of reactive oxygen metabolites by VIP may be important in the pathology of VIP-producing tissues.
Key words. VIP; neutrophils; respiratory burst.

Vasoactive intestinal peptide (VIP), a 28-amino acid neuropeptide that was isolated for the first time from porcine duodenum[1], is considered as a local hormone or a neuromodulator released by VIP-containing nerves[2]. There is increasing evidence that this neuropeptide plays a role in communication between the nervous and immune systems[3–5]. In this context, specific receptors for VIP have been demonstrated in human peripheral blood mononuclear cells[6,7] and human blood monocytes[8,9]. VIP has been shown to stimulate adenylate cyclase in human lymphocyte membranes[10], and the accumulation of cyclic AMP and the activity of cyclic AMP-dependent protein kinase in human peripheral blood mononuclear cells[6]. VIP inhibits the production of reactive oxygen compounds (respiratory burst) in monocytes activated by serum opsonized zymosan[11]. On the other hand, we have recently described the enhancement by VIP of reactive oxygen metabolites production in peripheral blood lymphocytes[12]. In this paper, we demonstrate for the first time the enhancement by VIP of respiratory burst in human neutrophils. The most striking finding was that, in spite of the lack of VIP receptors in neutrophils, the priming effect was obtained at very low VIP concentrations and values far below those expected in nerve endings.

Materials and methods

Synthetic rat VIP (HPLC pure) was from Peninsula Laboratories Europe (Merseyside, UK). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), and PMA (4b-phorbol-12b-myristate-13a-acetate) were from Sigma Chemical Co. (St. Louis, Missouri, USA). Rabbit complement was from Behring (Marburg, Germany). Other reagents were of analytical grade.

Human neutrophils were prepared from freshly venesected blood by dextran sedimentation, ficoll-hypaque separation and hypotonic lysis of remaining erythrocytes[13]. Cells, 99% neutrophils, were resuspended in PBS buffer supplemented with 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$ pH 7.4. Cells were stored on ice and assayed within 2 hours. Cell viability, checked with either 0.25% Trypan blue or 50 μmol/l ethidium bromide, was over 95%.

Human lymphocytes were prepared from freshly venesected blood of healthy donors according to Boyum[14]. Briefly, blood was diluted 1/2 with 0.9% (w/v) NaCl and 6 ml aliquots were layered over 3 ml of lymphocyte isolation solution (Hypaque-Ficoll) and centrifuged (4 °C, 20 min, 600 × *g*) to obtain mononuclear leucocytes which were washed by low speed centrifugation (4 °C, 5 min, 200 × *g*) in 0.9% NaCl. To eliminate monocytes 5 ml of the suspension, 3×10^6 cells/ml, were poured into a Petri dish and incubated at 37 °C for 60 min. Non-adherent cells were collected and washed by low speed centrifugation in 0.9% NaCl. Finally, cells were resuspended in PBS supplemented with 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_3$. The cells were 98% lymphocytes, as identified by light microscopy after Giemsa staining.

Oxygen radical production was analyzed by measuring luminol-dependent chemiluminescence according to Lucas and Solano[13] by means of a Berthold LB 9500 C luminometer, attached to a chart-recorder, modified to enable the injection of reagents via a microsyringe through a light-tight septum. All experiments were carried out at 37 °C. Samples for chemiluminescence determination were prepared by adding aliquots of the cell suspensions to PBS buffer supplemented with 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 μM luminol, 2 units/ml horseradish-peroxidase, and 100 μM sodium azide. Further details are given in the legends to figures. The reduction of ferricytochrome *c*, 50 μM final concentra-

tion, was determined by the increase in the absorbance at 546 nm. The addition of 20 µg/ml superoxide dismutase to the reaction mixture inhibited by 100% the reduction of cytochrome induced by the effectors used in the present work. Appropriate control assays demonstrated no spontaneous changes either in the chemiluminescence reaction or in the reduction of cytochrome *c* by any of the stimuli in the absence of cells. In addition the solvents used did not modify the activity of the cells in the above described tests.

Results and discussion

VIP primed the respiratory burst of neutrophils induced by PMA, leading to an increased chemiluminescence response as determined in neutrophils preincubated for two minutes with VIP and stimulated with 50 nM PMA, the optimum concentration obtained in preliminary experiments (figure 1 shows the actual traces obtained in representative experiments). The dose-dependent response of the priming effect of VIP fitted a sigmoidal shape curve (see fig. 2). The degree of stimulation was analyzed by non-linear regression and we obtained Hill slope values close to 1 and a $K_{0.5}$ of 0.5 nM VIP. Maximal effect was achieved at approximately 10 nM VIP. We compared this response to that of lymphocytes, which are known to express VIP receptors[6,7] by analyzing the chemiluminescence response of these cells under the same experimental conditions, i.e. preincubation with VIP and stimulation by PMA. The pattern of the chemiluminescence response was similar in both lymphocytes and neutrophils (see fig. 2) although in lymphocytes Hill Slope and $K_{0.5}$ values were 1.5 and 0.2 nM, which agree with the data previously reported on the stimulation of adenylate cyclase by VIP in lymphocytes[12].

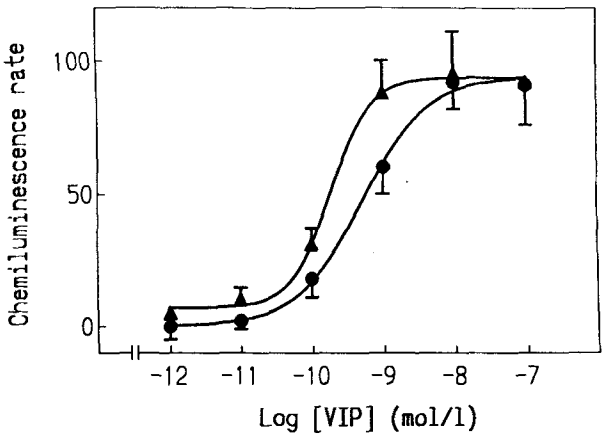

Figure 2. Dose-response of the priming effect of VIP on the respiratory burst following PMA stimulation. Circles refer to neutrophils (5×10^4) and triangles refer to lymphocytes (2×10^5), which were preincubated for 2 min at 37 °C in 1 ml PBS supplemented with 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$ in the absence and in the presence of VIP at the concentrations indicated. Chemiluminescence response was triggered by addition of 50 nM PMA, continuously recorded and the maximal rate obtained from the digital output. The inter-assay coefficient of variation of the chemiluminescence response was close to 30%. The chemiluminescence rate values in response to PMA obtained in 27 preparations of neutrophils was 13581 ± 783 cps (mean ± SEM). Therefore, chemiluminescence values were normalized within each experiment, taking 100% as the maximal rate obtained following PMA stimulation of cells preincubated in the absence of VIP. Results (mean and SEM of five experiments) are given as the increase over 100% of the maximal rate.

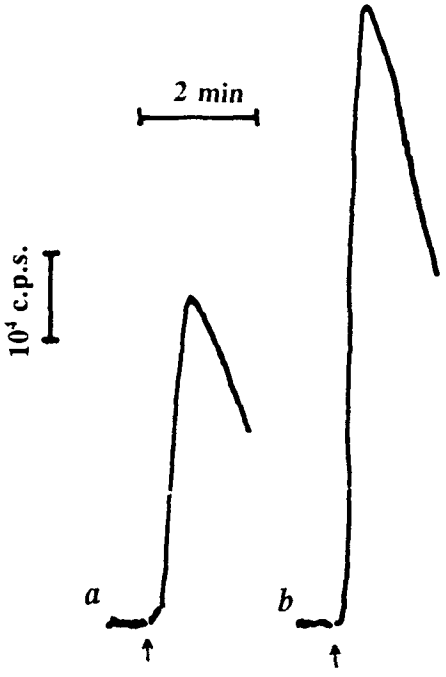

Figure 1. Records of the chemiluminescence response obtained in neutrophils stimulated with PMA. 5×10^4 neutrophils were incubated at 37 °C in 1 ml PBS supplemented with 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$ in the absence (trace *a*) and in presence (trace *b*) of 10^{-8} M VIP. Incubation was in the thermostatted chamber of the luminometer and after 2 min PMA was added, see arrows, to a final concentration of 50 nM. Traces are hand-drawn copies of the actual records, representative of at least five other cell batches.

The priming effect of VIP on the respiratory burst of neutrophils was further analyzed by measuring the production of superoxide anion by means of the reduction of cytochrome *c*. We compared the enhancement by VIP and complement of PMA-induced respiratory burst in human neutrophils. The results (see table) show that potentiation was higher in cells preincubated in the presence of complement. It should be pointed out that in the absence of PMA, VIP and complement by themselves failed to trigger the onset of the respiratory burst (see also table).

We describe here a very significant priming effect of VIP on the respiratory burst of neutrophils which shows two main characteristics:

1) It is produced in neutrophils, a phagocytic cell which does not express VIP receptors[8]. Moreover, the adenylate cyclase of neutrophils is not activated by VIP[10]. We performed VIP-binding experiments in plasma mem-

Enhancement by VIP and complement of PMA induced respiratory burst in human neutrophils

Preincubation	Incubation	Chemiluminescence	Superoxide
None	None	0	0.1 ± 0.01
VIP	None	0	0.1 ± 0.03
Complement	None	0	0.09 ± 0.02
None	PMA	100	2.5 ± 0.2
VIP	PMA	200 ± 15	5.1 ± 0.4
Complement	PMA	365 ± 26	8.8 ± 0.7

5×10^4 neutrophils were preincubated for 2 min at 37 °C in 1 ml PBS supplemented with 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$ in the presence of the indicated compounds. Incubation refers to the stimulation of neutrophils by 50 nM PMA. The chemiluminescence reaction was continuously recorded (see fig. 1) and the values were normalized giving, within each experiment, a value of 100% to the rate obtained following PMA stimulation of cells preincubated in the absence of any compound. The rate of superoxide anion production, calculated from the changes in the absorbance due to cytochrome *c* reduction, is given as nmol/10^6 neutrophils $\times$ min^{-1}. Results are the mean $\pm$ SEM of five separate cell preparations.

branes obtained as previously described[15] from human neutrophils as well as in intact neutrophils[6], and we failed to find specific binding of VIP (not shown). From the experiments with plasma membranes we excluded the possibility that VIP receptors were hindered by endogenous VIP[16], although we could not rule out the presence of VIP receptors at too low a level to be detected.

2) The concentration of VIP required to prime the neutrophils is in the physiological range and far below concentrations which may be expected in VIP-secreting nerve endings.

VIP has been described as an effective singlet oxygen quencher in vitro in a cell free system, and the possible modulation by VIP of the oxidative tissue injury caused by this reactive species of oxygen has been raised[17]. VIP does not appear to behave as a scavenger of reactive oxygen species in the experiments with human neutrophils described in the present work, as may be deduced from the similar degree of stimulation obtained by both methods: the oxidation of luminol and the reduction of cytochrome *c*. The high concentrations of VIP used in the in vitro experiments[17] could explain the difference between these and our results in freshly isolated cells.

The triggering of signals leading to activation of cells following receptor occupancy is regarded as the main path which couples effectors and a physiological cell response. The bypassing of a receptor-operated mechanism is regarded in some instances as a pharmacological response which is achieved at high 'non-physiological' effector concentrations. We propose here that the priming effect of VIP, at physiological concentrations, in a cell which does not express its receptor and therefore bypasses receptor-operated mechanisms cannot be considered a pharmacological response. Rather, it should be regarded from the point of view of the pathology. The high reactivity of oxygen metabolites produced following the respiratory burst of neutrophils could trigger a number of processes leading to cell injury and may be significant in neuroimmunopathology. The actual mechanism of the priming effect of VIP on neutrophils remains to be studied; nonetheless a possible mediation by cytosolic free calcium can be disregarded since VIP failed to increase $[Ca^{2+}]_i$ in Fluo3/AM loaded neutrophils (not shown).

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1 Said, S. I., and Mutt, V., Science *169* (1970) 1217.
2 Giachetti, A., Said, S. I., Reynolds, R. C., and Koniges, F. C., Proc. natl Acad. Sci. USA *74* (1977) 3424.
3 O'Dorisio, M. S., Wood, C. L., and O'Dorisio, T. M., J. Immun. *135* (1985) 792.
4 Ottaway, C. A., Ann. N.Y. Acad. Sci. *527* (1988) 486.
5 Tseng, J., and O'Dorisio, M. S., in: Neuroimmune Networks: Physiology and Disease, p. 105. Eds E. F. Goetzl and N. H. Specter. Alan R. Liss, New York 1989.
6 Guerrero, J. M., Prieto, J. C., Elorza, F. L., Ramirez, R., and Goberna, R., Molec. cell. Endocr. *21* (1981) 151.
7 Danek, A., O'Dorisio, M. S., O'Dorisio, T. M., and George, J. M., J. Immun. *131* (1983) 1173.
8 Wiik, P., Opstad, P. K., and Boyum, A., Regul. Pept. *12* (1985) 145.
9 Wiik, P., Regul. Pept. *35* (1991) 19.
10 O'Dorisio, M. S., Hermina, N. S., O'Dorisio, T. M., and Balcezak, P., J. Immun. *127* (1981) 2551.
11 Wiik, P., Regul. Pept. *25* (1989) 187.
12 López-González, M. A., Guerrero, J. M., and Lucas, M., Life Sci. *51* (1992) 1803.
13 Lucas, M., and Solano, F., Biochem. Pharmac. *42* (1991) 425.
14 Boyum, A., Scand. J. clin. Lab. Invest. *21* (1968) 77.
15 Segura, J. J., Guerrero, J. M., Goberna, R., and Calvo, J. R., Peptides *13* (1992) 953.
16 O'Dorisio, M. S., O'Dorisio, T. M., Cataland S. M., and Balcerzak, S. P., J. Lab. clin. Med. *96* (1980) 662.
17 Misra, B. R., and Misra, H. P., J. biol. Chem. *265* (1990) 15371.