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Hydrolysis of cyclic GMP in rat peritoneal macrophages★✪

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Intact rat peritoneal macrophages (rPM) treated with 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterases (PDEs), accumulated more cGMP than untreated cells. A PDE activity toward [^{3}H]cGMP was detected in the soluble and particulate fractions of rPM. The hydrolysis of cGMP was Ca^{2+}/calmodulin-independent but increased in the presence of cGMP excess. Similar results were obtained when [^{3}H]cAMP was used as a substrate. The hydrolytic activity towards both nucleotides was inhibited in the presence of IBMX. Therefore, the PDEs of families 2, 5, 10 and 11 are potential candidates for cGMP hydrolysis in the rPM. They may not only regulate the cGMP level in a feedback-controlled way but also link cGMP-dependent pathways with those regulated by cAMP.

Cyclic GMP (cGMP) is a key messenger molecule in several signal transduction pathways involved in vision, regulation of blood pressure, platelets aggregation, the function of kidney and heart. The nucleotide is an activator of protein kinases, can activate or inhibit phosphodiesterases of cyclic nucleotides and directly regulates the opening of cationic channels (for recent reviews see: Kobiałka & Gorczyca, 2000a; Lucas *et al.*, 2000). The intracellular level of cGMP is controlled by the opposite action of guanylyl cyclases (GCs) and phosphodiesterases (PDEs). It is generally accepted that the PDE activity is the main factor

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Abbreviations: ANP, atrial natriuretic peptide; GC, guanylyl cyclase; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; PMSF, phenylmethylsulfonyl fluoride; PM, peritoneal macrophage; rPM, rat peritoneal macrophage.

responsible for the duration of the cGMP signal (Conti, 2000; Essayan, 2001).

Cyclic nucleotide phosphodiesterases catalyse hydrolysis of the 3′-phosphodiester bond in cyclic 3′,5′-nucleoside monophosphates (cAMP and/or cGMP) converting them to respective 5′-nucleoside monophosphates. To date more than 40 different isoforms of PDEs have been identified and classified into 11 families (PDE1-11). They exhibit distinct tissue, cellular, and subcellular expression and differ in structure, substrate specificity, mechanisms of activation, and sensitivity to inhibitors (Beavo, 1995; Francis *et al.*, 2000; Houslay & Milligan, 1997; Soderling & Beavo, 2000). Among the multiple isoforms of phosphodiesterases, the enzymes belonging to four families (PDE3, 4, 7 and 8) are believed to be only cAMP-specific, three families (PDE5, 6 and 9) are cGMP-specific and four (PDE1, 2, 10, 11) display dual substrate specificity. Recently published data suggest that the cAMP-specific PDE4 also hydrolyses cGMP (Bellamy & Garthwaite, 2001). Therefore, at least seven families of PDEs are able to hydrolyse cGMP.

Macrophages participate in all stages of the immune response. Following stimulation they migrate to the site of the initiated immune reaction, phagocyte microorganisms and secrete several cytokines and mediators. It is well documented that cAMP is involved in all of these processes (Barnes, 1995; Gantner *et al.*, 1997; Okonogi *et al.*, 1991; Rossi *et al.*, 1998). Although also cGMP has been shown to mediate some of them, the mechanisms regulating the metabolism of this nucleotide are still not well recognized and there are only few reports concerning activity of cGMP-hydrolysing PDEs in macrophages (Dent *et al.*, 1994; Tenor *et al.*, 1995; Turner *et al.*, 1993). We have previously reported (Kobiałka & Gorczyca, 2000b) that in rat peritoneal macrophages cGMP is synthesised mainly in response to atrial natriuretic peptide (ANP), which is a known activator of particulate GC type A (GC-A). In this study we address the question how cGMP is hydrolysed in these cells.

MATERIALS AND METHODS

Chemicals. ANP was obtained from Calbiochem-Novabiochem. [^{3}H]cAMP and [^{3}H]cGMP were from New England Nuclear Life Science Products. *Crotulus atrox* snake venom was from Serva Feinbiochemica GmbH. AG1-X2 resin was from Bio-Rad Laboratories. Aprotinin, bicinchoninic acid solution, Hepes, IBMX, leupeptin, pepstatin A, PMSF, soybean trypsin inhibitor and other chemicals were from Sigma-Aldrich Co.

Animals. Wistar male rats (150–200 g) were obtained from the colony at the Institute of Immunology and Experimental Therapy in Wrocław. The animals were treated in accordance with the procedures approved by the local Ethics Committee For Animal Experimentation in Wrocław.

Cells. The peritoneal macrophages were prepared as previously described (Gorczyca *et al.*, 1989). The rats were intraperitoneally injected with 10 ml of thioglycollate. Four days later the animals were sacrificed and peritoneal exudate cells were harvested. The cells were centrifuged and remaining erythrocytes lysed by osmotic shock. After next two washes with Hank's balanced salt solution (HBSS), the cells were resuspended in RPMI 1640 Medium containing 2% heat inactivated autologous serum. More than 95% of the isolated cells were macrophages as determined by morphological evaluation and their viability was always >95% as determined by trypan blue exclusion.

Induction and measurement of intracellular cGMP. Induction of cGMP synthesis and its measurement were performed according to a procedure described elsewhere (Kurowska *et al.*, 2002). Briefly, the macrophages were transferred into a 48-well microplate and allowed to rest for 30 min at 37°C before the experiments. Each well con-

tained 2.5×10^5 cells in a final volume of 0.5 ml. The inhibitor of PDE was added and after 10 subsequent minutes of incubation at 37°C the cells were supplemented with an activator of particulate guanylyl cyclase – ANP. All samples were prepared in quadruplicate. After 30 min incubation, the reaction was terminated, the cells were disintegrated, and intracellular cGMP was determined using a competitive ELISA method based on rabbit anti-cGMP antibodies obtained in our laboratory.

Preparation of subcellular fractions. The procedures were performed at 4°C according to Germain and co-workers (1998). Freshly isolated macrophages were resuspended at 2.5×10^8 cells/ml in homogenisation buffer (10 mM Tris/HCl, pH 7.5, containing 1 mM $MgCl_2$, 1 mM DTT, 5 μM pepstatin A, 10 μM leupeptin, 50 μM PMSF, 10 μM soybean trypsin inhibitor and 1 mM benzamidine) and sonicated on ice. The homogenate was centrifuged at 100 000 × ***g*** for 1 h. The supernatant was collected and the pellet resuspended in equal volume of homogenisation buffer. The supernatant and resuspended pellet were used in determination of PDE activity as the soluble and particulate fractions, respectively. Concentration of protein in both fractions was determined by the bicinchoninic acid method (BCA), using BSA as a standards.

Determination of phosphodiesterase activity (PDE assay). The PDE activity was determined in the obtained fractions (soluble or particulate) using the modified two-step radioisotope method of Thompson and co-workers (1974). Assays were performed at 37°C. Reaction samples (100 μl final volume) were prepared in duplicate and contained 40 mM Hepes, pH 7.6, 5 mM $MgCl_2$, 0.1% bovine serum albumin, 0.5 μM [^{3}H]cGMP or [^{3}H]cAMP (40 000 c.p.m.), activators and inhibitors. The reaction was initiated by addition of 10 μl of the tested fraction containing approximately 20 μg of protein, and was terminated after 15 min by boiling. To separate the product from the substrate, in the first step phosphate was liberated from the monophosphate nucleosides by adding *Crotulus atrox* snake venom (50 μg) followed by further incubation for 30 min at 37°C. The reaction was stopped by addition of 250 μl of a 1:3 slurry of AG1-X2 Bio-Rad resin in H_2O. After 15 min equilibration, all mixture was added to 1.5 ml of Bray's scintillation cocktail and the radioactivity of samples was measured in a Wallac-LKB liquid scintillation counter.

RESULTS

Accumulation of cGMP in rPM stimulated with ANP is higher in the presence of PDE inhibitor

Since the intracellular level of cGMP is controlled by the opposite actions of GCs and PDEs, we first tested whether accumulation of the nucleotide in intact rat peritoneal macrophages depends on the presence of a PDE inhibitor. The accumulation of the nucleotide in rPM treated with the activator of cGMP synthesis was markedly (about five-fold) higher in the presence of IBMX, the non-selective inhibitor of most PDEs (Fig. 1). This result clearly indicated that in rPM there was or were present active enzyme(s) hydrolysing cGMP. To determine which isoforms of phosphodiesterases might be responsible for cGMP degradation in rat macrophages, the next experiments were performed using different cellular fractions, substrates, and activating factors.

PDE activity depends on cGMP concentration and is present in both the soluble and particulate fractions of rPM

Using [^{3}H]cGMP as the substrate, the PDE activity was determined in the soluble and particulate fractions of rPM. In both fractions hydrolysis of cGMP was independent of Ca^{2+}/calmodulin but depended on cGMP concentration (Fig. 2A and C). At 5 μM concentra-

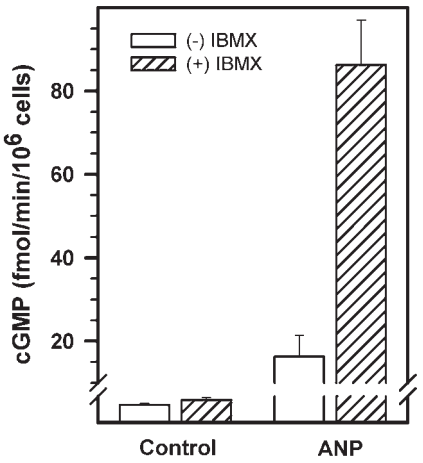

Figure 1. The level of synthesized cGMP in rPM is higher in the presence of PDE inhibitor.

The cells unstimulated (control) or stimulated with 10 nM ANP were incubated either in the absence or in the presence of 0.5 mM IBMX and then intracellular content of cGMP was determined as described in Materials and Methods. The data shown are the means ±S.D. of at least three independent measurements. The results are normalized and expressed as femtomoles of cGMP formed per min in samples containing 1×10^6 cells. The reaction was linear up to 30 min of incubation.

tion of cGMP, the PDE activity in the soluble and in the particulate fraction was about 14-fold and about 9-fold, respectively, higher than in the control (0.5 μM cGMP). The same type of experiment was performed using [^{3}H]cAMP as the substrate (Fig. 2B and D). Again, there was no difference between the PDE activities measured either in the presence or in the absence of Ca^{2+}/calmodulin. The hydrolysis of cAMP also appeared to be concentration-dependent, however, a higher (5 μM) concentration of the nucleotide caused only a seven- and less than three-fold elevation of the PDE activity in soluble and particulate fractions, respectively. The PDE activity toward both substrates was strongly inhibited by 0.2 mM IBMX. These results indicated that in rat PMs active Ca^{2+}/calmodulin-dependent PDE1 was absent, but possibly PDEs of families 2, 5, 10 or 11 hydrolysing cGMP in a cGMP-dependent way were present. Three of them are known to hydrolyse also cAMP and we compared the hydrolysis of [^{3}H]cAMP measured in the absence or in the presence of 1 μM concentration of cGMP to see whether the PDE activity toward cAMP also depends on cGMP. Conversely, to see if cAMP influences hydrolysis of cGMP, the hydrolysis of cGMP was measured in the presence of 1 μM cAMP. The results obtained (Fig. 3) showed that cAMP had no effect on cGMP hydrolysis in both fractions of rPM while cGMP caused a further elevation (by a factor of 1.2 and 1.7 in the soluble and particulate fractions, respectively) of the PDE activity toward cAMP.

Thus in both fractions PDEs were present which were able to hydrolyse cGMP and cAMP in a cGMP-dependent way.

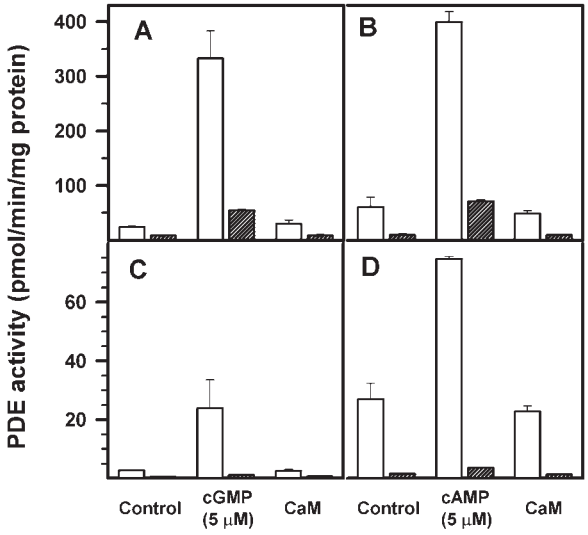

Figure 2. PDE activity is present in the soluble and particulate fractions of rPM.

Cells were homogenized, the homogenate was resolved to soluble (A and B) and particulate (C and D) fractions and in both fractions PDE activity was determined using either [^{3}H]cGMP (A and C) or [^{3}H]cAMP (B and D) as the substrates, according to procedures described in Materials and Methods. In both fractions the PDE activity was additionally measured in the presence of 250 μM Ca^{2+} and 2.4 μM bovine calmodulin (CaM). Control experiments were performed at 0.5 μM concentrations of the substrates. Filled bars represent the PDE activity measured in the presence of 0.2 mM IBMX, while open bars represent the enzyme activity in the absence of the inhibitor. The data shown are the means ±S.D. of three independent measurements. The normalised results are expressed as picomoles of cyclic nucleotide hydrolysed per min in a sample containing 1 mg of total protein.

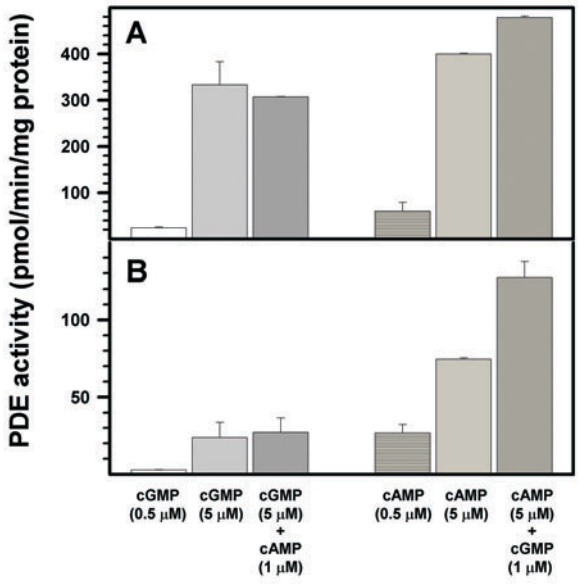

Figure 3. PDE activity is stimulated by cGMP but not by cAMP.

The PDE activity in the soluble (**A**) and particulate (**B**) fractions of rat peritoneal macrophages was determined at the indicated concentrations of the substrates and additionally in the presence of cAMP when [^{3}H]cGMP was the substrate and in the presence of cGMP when [^{3}H]cAMP was the substrate. Other details are as in Fig. 2.

DISCUSSION

Using a sensitive assay of PDE activity we show that in rat peritoneal macrophages several different phosphodiesterases are possibly involved in cGMP hydrolysis. The insensitivity of the nucleotide degradation to Ca^{2+}/calmodulin and to EGTA (not shown) indicates that in rPM there is no active isoform of PDE1, which was reported to be present in human alveolar and guinea pig peritoneal macrophages (Tenor *et al.*, 1995; Turner *et al.*, 1993). The fact that hydrolysis of cGMP is strongly elevated in the presence of its excess, suggests the presence of PDEs which are not only cGMP-specific but may be also cGMP-stimulated by binding the nucleotide to allosteric sites. PDEs of four families (PDE2, 5, 10 and 11) are regulated in this manner. One of them (PDE5) is specific only toward cGMP and three (PDE2, 10, 11) are able to hydrolyse cGMP as well as cAMP (Corbin & Francis, 1999; Fawcett *et al.,* 2000; Rosman *et al.*, 1997; Soderling *et al.*, 1998; 1999). It is known also that PDE9 is a cGMP-specific enzyme with a very low K_m for cGMP (Soderling *et al.*, 1998). However, it is insensitive to IBMX (Fisher *et al.*, 1998; Soderling *et al.*, 1998) and since our experiments show that PDEs in both cellular fractions are very sensitive to this inhibitor, we rather exclude the possibility that the PDE9 is present in rPM. At the same time we can not exclude the presence of PDE5 in the analysed cells. This cGMP-stimulated enzyme is also highly specific to cGMP, is inhibited by IBMX, and was shown to be present in human alveolar macrophages (Dent *et al.*, 1994; Tenor *et al.*, 1995). The observation that the PDE activity toward cGMP and cAMP in the particulate fraction of rPM may be additionally stimulated by cGMP strongly indicates that PDE2, known to be the only membrane-bound cGMP-stimulated PDE (Sonnenburg *et al.*, 1991), is present there. The fact that the soluble fraction also contains PDEs hydrolysing both nucleotides in a cGMP-dependent manner allows us to postulate that two additional phosphodiesterases (PDE10 and PDE11) might be present in rPM. To prove this further experiments are, however, required.

In summary, our results indicate that the level of cGMP in rat peritoneal macrophages is strictly controlled by the activity of PDEs, apparently in a feedback-regulated way. Since antibodies specific to cGMP-dependent protein kinases are not able to detect these enzymes in rPM (not shown), we speculate that in rPMs, the cGMP-regulated PDEs are the main effector enzymes for cGMP. They possibly play a dual role: control cGMP concentration in the cells and also influence the level of cAMP, thus constituting a link between these two signal transduction systems.

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