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C-Terminal Fragments of Parathyroid Hormone-Related Protein, PTHrP-(107-111) and (107-139), and the N-Terminal PTHrP-(1-40) Fragment Stimulate Membrane-Associated Protein Kinase C Activity in Rat Spleen Lymphocytes

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Membrane-associated protein kinase C (PKC) activity in lymphocytes freshly isolated from rat spleen was stimulated by the C-terminal parathyroid hormone-related protein fragments, PTHrP-(107–111) and PTHrP-(107–139), at concentrations from 10^{-3} to 10^{4} pM. By contrast, the same concentrations of PTHrP-(120–139), without the 107–111 TRSAW (-Thr-Arg-Ser-Ala-Trp-) sequence of the other C terminal fragments, did not stimulate spleen lymphocyte PKC. Low concentrations of the N-terminal PTHrP-(1–40) fragment also stimulated membrane-associated PKC activity in the spleen lymphocytes. These results suggest that PTHrP might be an important physiological regulator of the immune response.

Fenton et al. (1991a,b, 1993) reported that C-terminal fragments of the parathyroid hormone-related protein, PTHrP-(107–111) and PTHrP-(107–139), having a 107–111 TRSAW (-Thr-Arg-Ser-Ala-Trp-) amino acid sequence, potently inhibited the bone-resorbing activity of rat osteoclasts and they proposed, but did not demonstrate, that these TRSAW fragments inhibited osteoclast activity by activating protein kinase C (PKC), because their inhibitory activity was mimicked by a specific PKC activator, 12-0-tetradecanoyl phorbol-13-acetate (TPA), and blocked by two relatively nonspecific PKC inhibitors, 1-(5-isoquinoline-sulfonyl)2-methylpiperazine (H7) and sphingosine. Dudley et al. (1992) then cast doubt on these findings by reporting that in their hands PTHrP-(107–139) inhibited osteoclast formation, but not osteoclast activity. Further doubt was cast by Sone et al. (1992) who reported that neither hPTH-(107–111) nor hPTHrP-(107–139) inhibited the resorption of neonatal mouse calvariae by osteoclasts under conditions where calcitonin was strongly inhibitory. The reason for these conflicting results is unknown, but they might have been due to a difference between mouse and rat osteoclasts or to complex interactions between osteoclasts and other cells that occur in cultured bone but not in the isolated osteoclast assay used by Fenton et al. (1991a,b, 1993). However, Gagnon et al. (1993) have recently shown that other rat cells can respond to these C-terminal PTHrP fragments. They showed that picomolar concentrations of PTHrP-(107–111) and PTHrP-(107–139), but not a TRSAW-less PTHrP fragment, PTHrP-(120–139), consistently and strongly stimulated membrane-associated PKC activity in ROS 17/2 rat osteosarcoma cells.

In this article, we report the results of experiments which, for the first time, show that C-terminal PTHrP-(107–111) and PTHrP-(107–139) fragments as well as the N-terminal PTHrP-(1–40) fragment stimulate membrane-associated PKC in freshly isolated rat spleen lymphocytes and thus raise the possibility of PTHrP and certain of its fragments being physiological regulators of the proliferative and other activities of lymphocytes.

MATERIALS AND METHODS

PTH and PTHrP fragments

High performance liquid chromatography (HPLC)-purified human hPTH-(1–34) and [$Nle^{8,18}$, Tyr^{34}]bovine bPTH-(3–34)amide were purchased from Sigma Chemical Co. (St. Louis, MO). hPTH-(13–34) was purchased from Star Biochemicals, Inc. (Torrance, CA). HPLC-purified hPTHrP-(1–40) was also purchased from Star Biochemicals. PTHrP-(107–111)-amide, AcPTHrP-(107–139)-amide, and AcPTHrP-(120–139)-

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amide were synthesized in a MilliGen model 9050 synthesizer using fluorenylmethoxy-carbonyl (Fmoc)-protected amino acids and a Rink amide continuous flow solid support resin (Fields and Noble, 1990; Rink, 1987; Sieber, 1987). Fmoc-protected amino acids and the resin were purchased from Amino Technology (Ottawa, Ontario, Canada). The following side-chain-protecting groups were used: asp and glu, t-butyl; thr and ser, trityl; arg, 2,2,5,7,8-pentamethyl-chroman 6-sulfonyl. Coupling was carried out with active pentafluoroesters or 2,3-benzotriazine esters (ser, thr). Arginine was coupled after being activated with 2-(1H-benzotriazol1yl)1,1,3,3 tetramethyluronium tetrafluoroborate, N-hydroxybenztriazole, and N',N-diisopropylethylamine according to Knorr et al. (1989) and Bernatowicz et al. (1989). The products were cleared from the resin with 95% trifluoroacetic acid (TFA) for 4 h at room temperature using phenol and thioanisole as scavengers (Bernatowicz et al., 1989). Following evaporation of the solvents the peptides were precipitated with diethyl ether and dried. The amide peptides were purified to greater than 95% by HPLC on a reversed-phase PLRP column (Polymer Laboratories, Amherst, MA) with a 1%/minute gradient of 0.1% TFA/acetonitrile in 0.1% TFA/H_2O.

Preparation of spleen lymphocyte suspensions

Male, Sprague-Dawley rats, weighing 180g, were first anesthetized with fluothane and then killed by cervical dislocation. Their spleens were promptly removed and immersed in ice-cold, serum-free Eagle's minimal essential medium (E-MEM) containing 0.05 mM Ca^{2+} from Biofluids (Rockville, MD). A total of five spleens were needed to get enough lymphocytes for one experiment.

The chilled, freshly removed spleens were then minced and their cells released into 2 ml of ice-cold E-MEM. The 2-ml mixed cell suspension was carefully layered on 2 ml of Ficoll-Paque (supplied in a lymphocyte isolation kit from Pharmacia LKB Biotechnology Inc., Piscataway, NJ) in a 15-ml plastic centrifuge tube. Ficoll-Paque is a ready-to-use Ficoll-sodium diatrizoate solution of the proper density, viscosity, and osmotic pressure for the rapid isolation of lymphocytes. The mixed cell suspension, layered on top of the Ficoll-Paque solution, was centrifuged for 5 minutes at 600g. The erythrocytes in the cell suspension sedimented through the Ficoll-Paque solution into a pellet while the B and T lymphocytes collected into a layer at the E-MEM/Ficoll-Paque interface. The lymphocytes were carefully aspirated from the surface and diluted to 10 ml with cold (4°C) phosphate-buffered saline. The purified lymphocytes were then sedimented by centrifugation at 600g for 5 minutes and resuspended at a concentration of about 3×10^7 cells/ml in 3 ml of fresh, prewarmed (37°C) E-MEM containing a PTH or PTHrP fragment. The suspensions were incubated for 10 minutes, by which time the PKC activity was at its peak as illustrated in Figure 1 by the typical changes of membrane-associated PKC activity with time after exposure to 10^4 pM PTHrP-(107–111) in one of five separate experiments.

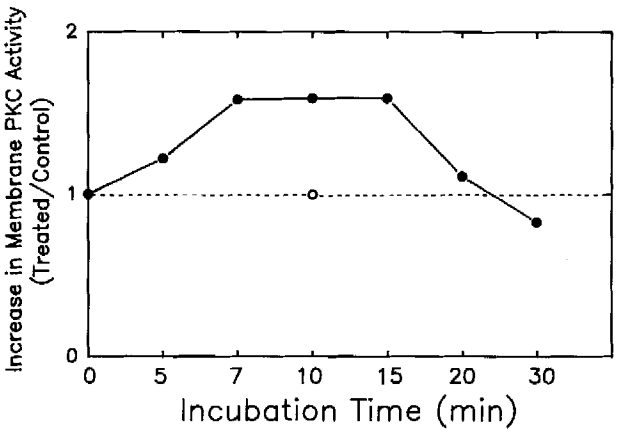

Fig. 1. Changes with time in the membrane-associated PKC activity in rat spleen lymphocytes after the addition of 10 nM PTHrP-(107–111). (●), 10 nM PTHrP-(107–111) was added to time 0; (○), control, no addition. The points are the means of the values in triplicate suspensions.

Measurement of membrane-associated PKC activity

After incubation for 10 minutes at 37°C in E-MEM containing a PTH or PTHrP fragment the 10^8 lymphocytes in each 3-ml culture were sedimented by centrifugation at 4°C for 5 minutes at 600g. The supernatant E-MEM was discarded and the cells were resuspended in ice-cold hypotonic lysis buffer (Chakravarthy et al., 1990, 1991). The cells were lyzed, the lysate centrifuged for 5 minutes at 600g, and the membranes in the post-nuclear supernatant fraction were sedimented by centrifugation at 100,000g (Chakravarthy et al., 1991). The PKC activity associated with these membranes was measured directly without prior extraction, reconstitution, or artificial activation according to Chakravarthy et al. (1991). The active enzyme on such membranes did not need added Ca^{2+}, phospholipid, and/or a PKC activator such as TPA for its optimal activity (Chakravarthy et al., 1991). The level of membrane-associated PKC activity was indicated by the extent of phosphorylation of a peptide, Ac-FKKSFKL-NH_2 (acetyl-phe-Lys-Lys-Ser-phe-Lys-Leu-NH_2) which was made in this Institute by Dr. R.E. Williams (Chakravarthy et al., 1991). This peptide corresponds to residues 160–166 of the phosphorylation site domain (151–175) of the MARCKS protein, the standard marker for PKC activation in intact cells (Blackshear, 1993; Heemserk et al., 1993; Orr et al., 1992). It and the MARCKS protein's phosphorylation site domain to which it belongs, are highly selective substrates for PKC: Their phosphorylation is not significantly catalyzed by other protein kinases such as cyclic AMP- and cyclic GMP-dependent protein kinases or calmodulin-dependent protein kinases I, II, and III (Chakravarthy et al., 1991; Blackshear, 1993; Heemserk et al., 1993; Orr et al., 1992; Williams et al., 1992). Moreover, the substrate peptide is phosphorylated equally by the several PKC isoforms (Heemserk et al., 1993).

The 100 μl assay mixture contained 1 μg of spleen cell membrane protein (measured according to Bradford [1976]) in assay buffer and 10 μl of 900 μM MARCKS-derived Ac-FKKSFKL-NH_2 PKC-selective substrate peptide (in 50 mM Tris-HCl buffer, pH 7.5). The reaction was started by adding 10 μl of 500 μM [γ-^{32}P]ATP (220 cpm/pmole in Tris buffer; 0.5 μCi/assay tube; from New England Nuclear-DuPont Canada, Mississauga, Ontario, Canada). The reaction was stopped after 10 minutes at 27°C by adding 10 μl of 5% acetic acid. The resulting 110 μl mixture was microfuged for 1 minute at 4°C and then put on ice. Samples (90 μl) were spotted on 2 cm squares of Whatman P81 filter paper which were then washed twice by gentle stirring for 10 minutes in 5% acetic acid (10 ml/square). The ^{32}P radioactivity remaining on the filter paper was measured with an LKB 1217 RACKBETA scintillation spectrophotometer. Membranes from untreated or hormone-stimulated lymphocytes were also incubated in the absence of the Ac-FKKSFKL-NH_2 peptide substrate, and the amount of ^{32}P radioactivity remaining on the washed P81 Whatman filter paper was measured. This background or blank value was between 10% and 20% of the basal value and was always subtracted from the value obtained in the presence of the substrate peptide. It should be noted that the background value was never higher in membranes isolated from fragment-treated cells.

Statistical analysis

All data were expressed as the means ± S.E.M. (standard error of the mean). The significance of the differences between groups was determined by analysis of variance (ANOVA). P values of 0.05 or less were considered to be significant.

RESULTS

Exposure of freshly isolated spleen lymphocytes to PTHrP-(107–111) or PTHrP-(107–139) increased the ability of membranes isolated from these cells to phosphorylate the PKC-specific, MARCKS-derived Ac-FKKSFKL-NH_2 peptide substrate in vitro (Figs. 1, 2). These PTHrP fragments, with their TRSAW sequence, significantly ($P < 0.05$) stimulated membrane-associated PKC activity 1.4–3.0 times at concentrations between 10^{-3} and 10^4 pM (Fig. 2). This was a specific response, most probably of the lymphocytes' PKC-activating mechanism, to the C-terminal TRSAW fragments because another C-terminal peptide, PTHrP-(120–139), without the TRSAW sequence, did not stimulate membrane-associated PKC over the same range of concentrations (Fig. 2).

The likelihood of the TRSAW-stimulated, MARCKS-peptide-phosphorylating protein kinase(s) being PKC(s) was supported by its sensitivity to the highly PKC-specific RFARKGALRQKVNHEVKN inhibitor peptide. This peptide corresponds to the PKCs' autoinhibitory pseudosubstrate region (House and Kemp, 1987; Smith et al., 1990). It has been found to inhibit the Ac-FKKSFKL-NH_2-phosphorylating activity in membranes from various types of cell stimulated by PTH, PTHrP, TPA, and other agents (Chakravarthy et al., 1991; Gagnon et al., 1993; Jouishomme et al., 1992; Smith et al., 1990; Whitfield et al., 1992). It also

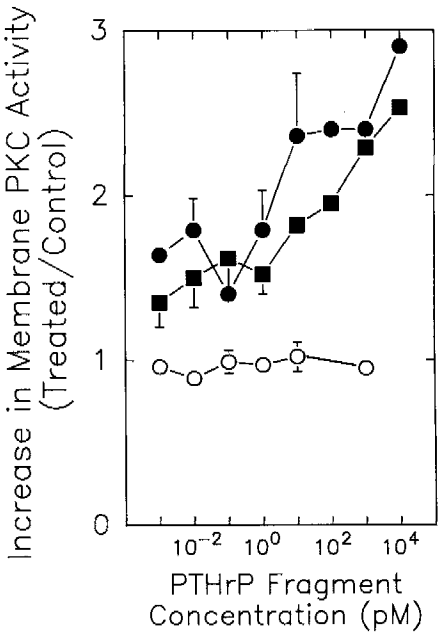

Fig. 2. The stimulation of membrane-associated PKC activity by PTHrP-(107–111) (■) and PTHrP-(107–139) (●), but not by PTHrP-(120–139) (○), in freshly isolated rat spleen lymphocytes. The cells were isolated, purified, and their membrane-associated PKC activity measured as described in Materials and Methods. The points for 10^{-3} to 10^3 pM are the means ± S.E.M. of the values from at least two separate experiments, each of which was obtained with the pooled cells from five spleens and is the mean of values from triplicate cultures. Each of the points for the stimulation of PKC by 10^2 to 10^4 pM PTHrP-(107–111) and PTHrP-(107–139) is the mean of values from triplicate cultures in one experiment.

inhibited the enhanced phosphorylation of the Ac-FKKSFKL-NH_2 peptide substrate by membranes from spleen lymphocytes treated with PTHrP-(107–111). Thus, for example, including 10 μM inhibitor peptide in the assay mixture (see Materials and Methods) reduced the 10-min phosphorylation of the Ac-FKKSFKL-NH_2 substrate by membranes from untreated spleen lymphocytes from 692 to 0 cpm/1 μg of membrane protein, and it reduced the phosphorylation of the PKC-specific substrate by membranes isolated from lymphocytes stimulated by 10^4 pM PTHrP-(107–111) from 1,267 to 0 cpm/1 μg (n = 2) of membrane protein.

Exposure of the spleen lymphocytes to a wide range of concentrations of the N-terminal PTHrP fragment, PTHrP-(1–40), also increased the ability of membranes isolated from these treated cells to phosphorylate the PKC-specific Ac-FKKSFKL-NH_2 peptide substrate but there appeared to be two peaks in the dose-response curve (Fig. 3). The fragment was significantly effective ($P < 0.02$) at 0.1 pM, invariably ineffective at 1 pM, and again significantly ($P < 0.02$) effective at 10 pM (Fig. 3). Spleen lymphocyte PKC activity was also stimulated by three N-terminal PTH fragments just as it was in other cells such as ROS 17/2 rat osteosarcoma cells (Jouishomme et al., 1992). For example, 10 pM PTH-(1–34) increased membrane-associated PKC activity 1.89 ± 0.13 (n = 5) times. The same concentration of PTH-(3–34) and PTH-(13–34) stimulated membrane

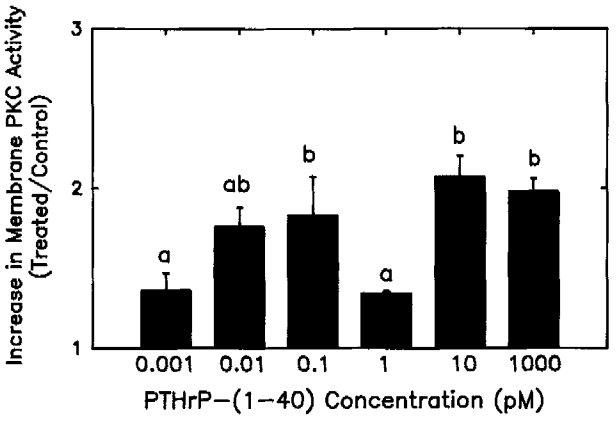

Fig. 3. Stimulation of membrane-associated PKC activity by PTHrP-(1–40) in freshly isolated rat spleen lymphocytes. The cells were isolated, purified, and their membrane-associated PKC activity measured as described in Materials and Methods. The bar heights are the means ± S.E.M. of the values from at least three separate experiments, each of which was obtained with the pooled cells from five spleens and is the mean of values from triplicate cultures. Bars with different superscripts are significantly different ($P < 0.05$).

PKC activity 2.0 ± 0.7 (n = 3) and 2.1 ± 0.2 (n = 3) times, respectively. Thus, the freshly isolated spleen lymphocytes may express the conventional receptor that binds both PTH and PTHrP (Jüppner et al., 1988; Nissenson et al., 1988).

Finally, it should be noted that maximally effective concentrations of all of the N- and C-terminal PTH and PTHrP fragments (except, of course, PTHrP-[120–139]) increased the ability of the membranes from the fragment-treated lymphocytes to phosphorylate the Ac-FKKSFKL-NH_2 substrate as much as, or almost as much as, the specific PKC activator TPA. Thus, treating the spleen lymphocytes with the maximally effective 1 μM TPA increased the ability of their membranes to phosphorylate the peptide substrate 2.8 ± 0.2 (n = 24) times. As expected the ability of the membranes from the TPA-treated cells to phosphorylate the substrate was completely inhibited by adding 10 μM PKC-specific inhibitor peptide to the assay mixture.

DISCUSSION

It seems from these results that normal rat spleen lymphocytes might express the conventional PTH/PTHrP receptors because their membrane-associated PKC activity was stimulated by similar, low concentrations of N-terminal fragments of PTH and PTHrP. The responsiveness of spleen lymphocyte PKC to PTHrP-(1–40) was biphasic like the two-peak response of ROS 17/2 rat osteosarcoma cells and mouse keratinocytes to much higher concentrations of PTHrP-(1–40) as well as to PTH-(1–84) holoprotein and various N-terminal PTH fragments reported by Gagnon et al. (1993), Jouishomme et al. (1992), and Whitfield et al. (1992). The reason for this two-peak, dose-response is unknown, but Gagnon et al. (1993) and Jouishomme et al. (1992) have suggested that in the case of ROS 17/2 osteosarcoma cells it might be due to the existence of two different PTH/PTHrP receptors or one receptor with widely different affinity states.

The responsiveness of the spleen lymphocytes to extremely low concentrations of PTHrP-(107–111) and PTHrP-(107–139) suggests that these cells might also express another receptor which is specific for PTHrP's 107–111 TRSAW domain. PTH has only one PKC-activation domain located in the 28–34 part of its 20–34 receptor-binding region, but the much larger PTHrP molecule has PKC-activation domains in its 28–34 region and its 107–111 TRSAW region (Gagnon et al., 1993). Although the amino acid sequences of the 20–34 receptor-binding regions of PTH and PTHrP are different, their higher order configurations are similar and therefore they can bind to, and activate, the same PTH/PTHrP receptor (Cohen et al., 1991; Halloran and Nissenson, 1992; Jüppner et al., 1988; Nissenson et al., 1988; Shigeno et al., 1988). However, PTHrP's 107–111 TRSAW region is different from its own and PTH's 20–34 receptor-binding region which suggests that it may bind to a different PTHrP receptor. Another reason for suspecting that there may be a different receptor for PTHrP's TRSAW region is the extreme sensitivity to PTHrP-(107–111) and PTHrP-(107–139) of rat osteoclasts (Fenton et al., 1991a,b, 1993) which are widely believed not to express conventional PTH/PTHrP receptors (Chambers, 1991; Nijweide et al., 1986).

Regardless of how many PTHrP receptors rat spleen lymphocytes might have, their responsiveness to extremely low concentrations of C-terminal and N-terminal PTHrP fragments suggests that this multidomain hormone is involved in the regulation of the immune system. Some support for this suggestion is provided by evidence indicating that PTH is a wound hormone which is released from the parathyroid glands in response to surgical and other injuries, to stimulate an immune response and promote hemopoiesis presumably by activating PTH/PTHrP receptors on precursor cells (Whitfield, 1990; Whitfield et al., 1992). Indeed, PTH stimulates the proliferation of rat thymic lymphoblasts and bone marrow CFU-S progenitor cells, enhances the phytohemagglutinin-induced expression of interleukin-2 (IL-2) receptors and the proliferation of human peripheral blood T lymphocytes, and promotes the primary immune response in rats to the injection of sheep red blood cells (Atkinson et al., 1987; Klinger et al., 1990; Swierenga et al., 1976; Whitfield, 1990, 1992; Whitfield et al., 1971, 1992). PTHrP holoprotein and N- and/or C-terminal PTHrP fragments generated by local proteases might be potent autocrine or paracrine modulators of the proliferation of both normal lymphocytes and lymphocytes in PTHrP-expressing tumors. Indeed, activated normal T cells and IL-2-stimulated T-leukemia cells make and secrete enough PTHrP to affect their proliferative activity (Adachi et al., 1990; Ikeda et al., 1993; McCauley et al., 1992).

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