Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 345 (2006) 713–719
BBRC
www.elsevier.com/locate/ybbrc

Phosphodiesterase 8 (PDE8) regulates chemotaxis of activated lymphocytes

Hongli Dong, Venera Osmanova, Paul M. Epstein *,[1], Stefan Brocke [1]

Department of Pharmacology, University of Connecticut Health Center, Farmington, CT 06030-6125, USA

Received 19 April 2006
Available online 3 May 2006

Abstract

The immune system depends on chemokines to recruit lymphocytes to tissues in inflammatory diseases. This study identifies PDE8 as a new target for inhibition of chemotaxis of activated lymphocytes. Chemotactic responses of unstimulated and concanavalin A-stimulated mouse splenocytes and their modulation by agents that stimulate the cAMP signaling pathway were compared. Dibutyryl cAMP inhibited migration of both cell types. In contrast, forskolin and 3-isobutyl-1-methylxanthine each inhibited migration of unstimulated splenocytes, with little effect on migration of stimulated splenocytes. Only dipyridamole alone, a PDE inhibitor capable of inhibiting PDE8, strongly inhibited migration of stimulated and unstimulated splenocytes and this inhibition was enhanced by forskolin and reversed by a PKA antagonist. Following concanavalin A stimulation, mRNA for PDE8A1 was induced. These results suggest that in employing PDE inhibitor therapy for inflammatory illnesses, inhibition of PDE8 may be required to inhibit migration of activated lymphocytes to achieve a full therapeutic effect.

Keywords: Lymphocyte chemotaxis; Cyclic nucleotide phosphodiesterase; CXCL12; cAMP signaling; PDE8; Dipyridamole

cAMP has long been known to inhibit lymphocyte activation, proliferation, and function [1,2]. cAMP was also reported to inhibit motility of cytotoxic T lymphocytes [3]. Inasmuch as recruitment of lymphocytes to sites of inflammation, i.e., their migration through postcapillary endothelial layers and subsequent parenchymal accumulation [4] underlies the basis of a number of autoimmune diseases, such as multiple sclerosis [5–7], stimulating the cAMP signaling pathway in lymphocytes as a means to inhibit their migration, has been suggested as the basis of very effective treatments for these diseases [8]. cAMP levels in cells are controlled by their synthesis by adenylyl cyclases and degradation by cyclic nucleotide phosphodiesterases (PDEs). PDEs comprise a superfamily of related enzymes encoded by at least 21 different genes, grouped into 11 different gene families (PDEs 1–11), based on sequence similarity, mode of regulation, and preference for cAMP or cGMP as substrate [9–11]. PDE4 is one of the major PDE gene families expressed in both human [12–14] and mouse [15] lymphocytes, accounting for most of the hydrolysis of cAMP. With this observation in mind, the PDE4-selective inhibitor, rolipram, was tested for its effects on lymphocyte chemotaxis and was found to inhibit lymphocyte migration stimulated by platelet activating factor, interleukin-8, and CXCL12 (stromal cell-derived factor-1) [16,17].

These in vitro studies showing effects of rolipram on lymphocyte migration were done with unstimulated, quiescent lymphocytes, but it is widely accepted that the population of lymphocytes that migrate to the site of inflammation and across the endothelium in vivo mostly belong to previously activated lymphocyte subsets, and the expression profile and localizations of PDEs in activated lymphocytes may differ from that in unstimulated cells. Indeed, early studies had shown a long-term induction of 5–10-fold in lymphocyte PDE activity following

* Corresponding author. Fax: +1 860 679 3693.
E-mail address: epstein@nso1.uchc.edu (P.M. Epstein).
[1] Senior investigators P.M.E. and S.B. contributed equally to this work

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.143

stimulation by phytohemagglutinin [18] or concanavalin A (Con A) [18,19]. And subsequent studies reported the long-term induction in activated lymphocytes of the specific PDE forms, PDE1B1 [20], PDE1B2 [21], PDE4A4 [22], PDE4D1/D2 [22], PDE4D3 [22], PDE7A1 [23], PDE7A3 [24], and PDE8A1 [24]. Further, translocation of PDE4A4, PDE4B2, and PDE4D1/D2 to lipid rafts following lymphocyte activation was also reported recently [25]. Hence, given these changes in PDEs that occur following lymphocyte activation, the modulation of chemotactic responses of activated lymphocytes by agents that stimulate the cAMP signaling pathway could be quite different from that of unstimulated lymphocytes, and the focus of this study was to examine this. We found that while stimulated splenocytes, like unstimulated splenocytes, responded to the chemokine, CXCL12, a powerful chemoattractant for leukocytes [4], and were readily inhibited in their migration by the cAMP analogue, dibutyryl cAMP, unlike unstimulated cells, migration of stimulated splenocytes was hardly affected at all by activators of adenylyl cyclase or inhibitors of cAMP PDEs, except for dipyridamole, which differs from the other PDE inhibitors used, only in its ability to inhibit PDE8. Further, quantitative real-time RT-PCR reveals an induction of PDE8 mRNA in splenocytes following Con A stimulation. This suggests that for activated lymphocytes, the major therapeutic target in inflammatory autoimmune diseases, inhibition of PDE4 is not sufficient to block their recruitment into sites of inflammation, and it may be necessary to additionally inhibit PDE8 to achieve a full therapeutic response.

Materials and methods

Materials. Recombinant mouse CXCL12 was obtained from R&D Systems, forskolin and Rp-cAMPS from Biomol, and 3-isobutyl-1-methylxanthine (IBMX), dipyridamole, Con A, dibutyryl cAMP, and adenosine deaminase type X from Sigma. The PDE3 inhibitor motapizone, the PDE4 inhibitor piclamilast, and a proprietary PDE7-selective inhibitor were generously supplied by Drs. Christoff Zitt and Armin Hatzelmann, Altana Pharma, Konstanz, Germany.

Isolation of murine splenocytes. Splenocytes were isolated from 6 to 8-week-old C57BL/6 mice obtained from Jackson Laboratories, Bar Harbor, ME. Spleens were removed and a single-cell suspension prepared using 40 μm cell strainers (Fisher Scientific). Cells were washed with RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Gibco). Red blood cells were lysed using standard lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.4). Cells were then washed and used in chemotaxis assays either as unstimulated cells or after stimulation with Con A (3 μg/ml) as indicated.

Preparation of test reagents. Forskolin, IBMX, motapizone, piclamilast, and dipyridamole were dissolved as 1000× stock solutions in 100% DMSO and diluted into the chemotaxis assays to give a final DMSO concentration of 0.1%. This concentration of DMSO had no effect on migration of splenocytes in the presence or absence of CXCL12. CXCL12 was prepared as a 100 μg/ml stock solution in PBS + 0.1% BSA, Con A was prepared as a stock of 2.5 mg/ml in PBS, and dibutyryl cAMP and Rp-cAMPS were prepared as 50 mM stock solutions in water, and these reagents were diluted directly into the chemotaxis assays.

Chemotaxis assay. Chemotaxis assays were done in 24-well transwell plates with a pore size of 5 μm (Costar, Corning). Splenocytes were placed in medium at a concentration of 3×10^6 cells/ml. Where test agents were used, splenocytes were pretreated with the agent or vehicle for 45–60 min, following which 100 μl of the splenocyte suspension was placed into the upper chamber of transwell plates, and the lower chamber was filled with 600 μl of medium. When added to induce migration, CXCL12 (250 ng/ml) was added to the lower chamber only. Other test reagents were added as indicated to both the upper and lower chambers. After 4 h of incubation at 37 °C in 5% CO_2, transwell inserts were gently removed and the number of cells that migrated into the lower chamber was counted by withdrawing 500 μl of lower chamber medium, mixing it with 10 ml buffer and counting the cells on a Coulter Counter (Beckman Coulter Z series). Where results are expressed as % of CXCL12-stimulated migration, this was calculated as follows: (cells migrated in presence of CXCL12 and test reagent—cells migrated in medium alone)/(cells migrated in presence of CXCL12—cells migrated in medium alone) × 100. Experimental points for all chemotaxis assays were performed in triplicate.

Quantitative real-time RT-PCR. Total RNA was isolated from unstimulated splenocytes and from splenocytes stimulated by Con A for different lengths of time as indicated, using RNeasy mini kits (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using M-MLV reverse transcriptase (Promega). Primers were designed using ABI Primer Express Software v3.0. and synthesized by Invitrogen Life Technologies. Quantitative real-time RT-PCR was performed using an ABI 7500 fast system and data analyzed using 7500 fast system SDS software v3.0. Sets of primers with the following sequences were used: PDE4B2 primer sequences, forward: ACCTGAGCAACCCCACCAA, reverse: CCCCTCTCCCGTTCTTTGTC; PDE7A primer sequences, forward: TCAGCAGCAATCTTGATGCAA, reverse: AGAGGCTGG GCACTTCACAT; PDE8A primer sequences, forward: CCTGCAGCAT TCCCAAGTC, reverse: TGTATAAGGTTAGGCAGGTCAA; ribosomal protein L19 (RPL19) primer sequences, forward: CCAAGAAGAT TGACCGCCAT, reverse: CAGCTTGTGGATGTGCTCCAT. Amplicon sizes were 100 bp.

Statistics. Data are plotted as means ± SD of replicate determinations. Statistical significance of experimental conditions relative to control was analyzed by Student's t test and significance indicated by asterisks in the figure, with the p values given in the legend.

Results

CXCL12 induces migration of murine splenocytes

As expected, using the transwell assay system, CXCL12 (250 ng/ml) stimulated the migration of both unstimulated and Con A-stimulated mouse splenocytes (Fig. 1). In response to CXCL12, there was a 6.2-fold increase in the number of unstimulated splenocytes and a 2.5-fold increase in the number of Con A-stimulated splenocytes migrating to the side of the chamber containing CXCL12. The number of cells migrating to the chamber containing CXCL12 was about the same for unstimulated and Con A-stimulated splenocytes. The difference in fold stimulation between the two cell populations was primarily due to increased migration of Con A-stimulated splenocytes, relative to unstimulated splenocytes, in the absence of CXCL12.

Effect of cAMP analogue, adenylyl cyclase activator, and PDE inhibitors on CXCL12-induced splenocyte chemotaxis

The cell permeable cAMP analogue, dibutyryl cAMP (500 μM), significantly inhibited CXCL12-induced migration of both unstimulated and Con A-stimulated splenocytes by 54% and 29%, respectively (Fig. 2). In contrast,