6. K. A. Roth, I. M. Mefford, J. D. Barchas, *Brain Res.* **239**, 417 (1982).
7. H. Nishino, *Folia Pharm.* **72**, 941 (1976).
8. This concentration of α-methyl-*p*-L-tyrosine blocks CA synthesis for at least 3 hours [K. G. Höhn and W. O. Wuttke, *Brain Res.* **156**, 241 (1978)].
9. The interval of 2 hours was chosen because the rate of CA disappearance is linear during this period, therefore, the slope of CA decay permits a reliable calculation of turnover rates and constants [P. M. Wise, N. Rance, C. A. Barraclough, *Endocrinology* **108**, 2186 (1981)].
10. M. Da Prada and G. Zürcher, *Life Sci.* **19**, 1161 (1976).
11. H. O. Besedovsky, A. del Rey, E. Sorkin, *J. Immunol.* **126**, 385 (1981).
12. We thank L. Brunner for technical assistance. This work was supported by the Swiss National Science Foundation (grant 3.603.80).

26 November 1982; revised 6 April 1983

Thrombin Stimulation of Guanosine 3′,5′-Monophosphate Formation in Murine Neuroblastoma Cells (Clone N1E-115)

Abstract. *Thrombin, the central regulatory enzyme in coagulation, when incubated in nanomolar concentrations with murine neuroblastoma cells produced a rapid and marked increase in tritiated guanosine 3′,5′-monophosphate (cyclic GMP) formation that was blocked by hirudin and competitively antagonized by dansylarginine N-(3-ethyl-1,5-pentanediyl)amide. Diisopropylphosphofluoridate-inactivated thrombin as well as the serine protease trypsin were markedly less potent and less effective than α-thrombin in producing this effect. Thrombin-stimulated cyclic GMP formation was inhibited by mepacrine and nordihydroguaiaretic acid but unaffected by indomethacin, suggesting that lipoxygenase metabolites of arachidonic acid are involved in the response. These results suggest that a thrombin-like protease in the brain may be involved with the function of neurons or that thrombin interactions with nerve cells, such as those following cerebral hemorrhage or other trauma of the central nervous system, may be important in the subsequent neuropathology.*

Clones of murine neuroblastoma cells in culture have been widely studied as model systems for neurobiology. Such clones possess many of the properties of normal differentiated neurons (*1*). Cells of one of these clones (N1E-115) respond to stimulation of the muscarinic or histamine H_1 receptor by increasing their concentration of guanosine 3′,5′-monophosphate (cyclic GMP). This response represents a convenient means by which receptor-mediated cellular activation can be investigated. During a study of the mechanism of receptor-mediated cyclic GMP formation, we found that the serine protease thrombin (E.C. 3.4.21.5), a pivotal enzyme in the regulation of hemostasis, also stimulated cyclic GMP formation in neuroblastoma cells. Here we characterize the nature of this effect and propose that thrombin receptors on neuroblastoma cells mediate this phenomenon. We also provide evidence that phospholipase A_2 activation and lipoxygenase metabolites are involved in this neuronal cyclic GMP response. The results suggest that thrombin plays a role in the neuropathology of cerebral hemorrhage or other traumas of the central nervous system in which the blood-brain barrier has been compromised.

Thrombin caused a rapid and marked increase in tritiated cyclic GMP formation by neuroblastoma cells with a peak around 30 to 60 seconds (Fig. 1). This time course is similar to that reported for the formation of cyclic GMP in this cell type in response to stimulation of the muscarinic and histamine H_1 receptors (*2*). Moreover, the response induced by thrombin was markedly dependent on extracellular Ca^{2+} as has also been observed for other agents (for example, carbachol, histamine, KCl, and Ca^{2+} ionophores) that enhance cyclic GMP synthesis (*2, 3*). The EC_{50} (median effective concentration) for thrombin ranged from 0.08 to 0.44 NIH units per mililiter (mean = 0.20 ± 0.04 U/ml in ten concentration-response studies with each point assayed in duplicate). Maximal thrombin concentrations (1 to 10 U/ml) increased cyclic GMP levels from 4.1 to 19 times more than the basal levels measured in these cells (mean = 11.5 ± 1.2 times; N = 17 experiments, each performed in duplicate or triplicate). Thrombin-stimulated increases in cyclic GMP were not affected by atropine ($10^{-7}M$) or pyrilamine ($10^{-6}M$), thus ruling out the possibility that muscarinic or histamine H_1 receptors were involved. In addition, a highly purified preparation of bovine thrombin (*4*) gave results indistinguishable from those obtained with thrombin from Sigma Chemical Company. Thus, the effect on cyclic GMP synthesis was not due to a contaminant of the commercially available preparation.

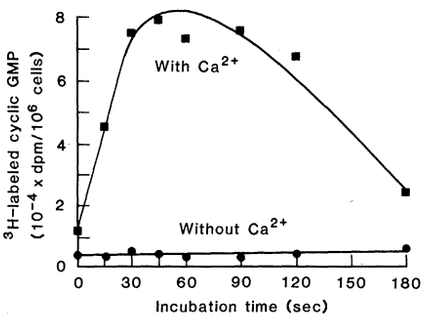

Fig. 1. Time course of the effect of thrombin (3 U/ml) on the formation of tritiated cyclic GMP in cultured murine neuroblastoma cells. Clone N1E-115 cells (subculture 9–11) were grown and assayed for relative changes in cyclic GMP formation with the use of a radioactively labeled precursor in intact cells as previously described (*18*). Symbols: ■, assay conducted in normal phosphate-buffered saline (PBS) solution containing 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2 mM Na_2HPO, 25 mM glucose, and 70 mM sucrose (pH 7.3 to 7.4, osmolality 335 to 340 mOsm); ●, assayed in PBS in the absence of Ca^{2+}, but containing 2 mM $MgCl_2$ and 1.0 mM EGTA with the other ions and conditions remaining unchanged. This figure depicts a representative result from one of four experiments.

Thrombin consistently produced the most potent (EC_{50} = 1.0 to 1.5 nM) response whereas trypsin and diisopropylphosphofluoridate-inactivated thrombin (DIP-thrombin) were 50 to 100 times and 300 to 500 times less potent in stimulating this neuronal cyclic GMP response, respectively (Fig. 2A). Thus, this effect showed a marked degree of specificity for catalytically active thrombin; trypsin, another serine protease with a broad specificity, was much less potent and less effective in stimulating this response. That thrombin was the mediator of these effects was further established by the finding that hirudin, the specific thrombin inhibitor isolated from the leech salivary gland (*5*), inhibited thrombin-stimulated cyclic GMP formation in this preparation in a concentration-dependent fashion (Fig. 2B). In addition, the competitive inhibitor of thrombin dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) shifted the response curve for thrombin concentration and cyclic GMP formation to the right in a parallel manner (Fig. 2C). Thus, DAPA was a competitive inhibitor of thrombin in this neuronal system and from a direct plot (*6*) of the dose ratio (DR) data for two concentrations of DAPA, we found a K_d (equilibrium dissociation constant) of $1.67 \times 10^{-8}M$ (standard error = $\pm 0.14 \times 10^{-8}M$). This value is in agreement with the competition of DAPA with thrombin in other systems (*7*). Finally, C-6 glioma cells that have β-adrenergic receptors that mediate the synthesis of both adenosine 3′,5′-monophosphate (cyclic AMP) and cyclic GMP (*8*) failed to produce cyclic GMP in response to thrombin (data not shown), suggesting

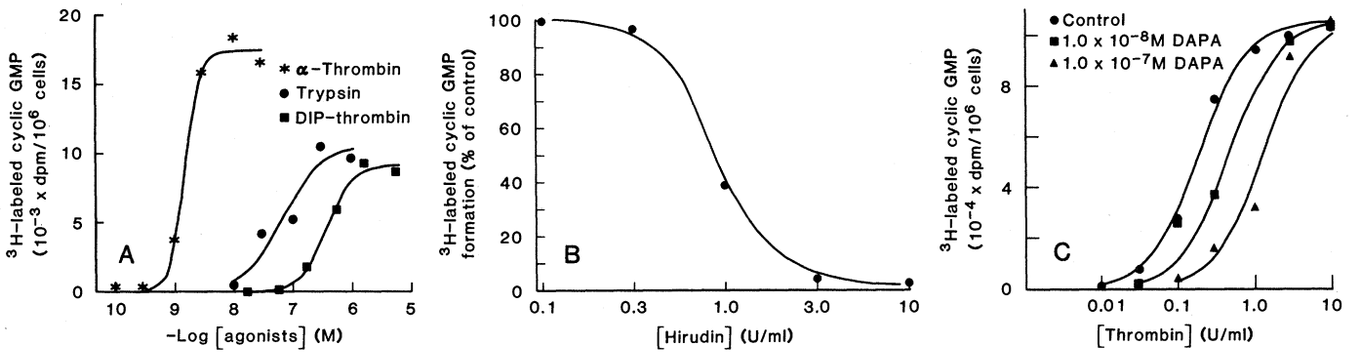

Fig. 2. (A) Relation between concentration of tritiated cyclic GMP, thrombin, trypsin, and DIP-thrombin in murine neuroblastoma NIE-115 cells. The assay was conducted in normal PBS with the agonists, and incubation was for 45 seconds as previously described (*18*). This experiment was replicated with essentially identical results three times. (B) Hirudin-inhibition of thrombin (1 U/ml) stimulated cyclic GMP formation. Cells in normal PBS were incubated with various hirudin concentrations for 3 to 5 minutes prior to the addition of thrombin (1 U/ml for 45 seconds). This experiment was repeated twice. (C) The effect of dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) on thrombin-stimulated cyclic GMP formation in murine neuroblastoma clone N1E-115 cells. Cells in normal PBS were incubated with DAPA for 30 minutes prior to the addition of thrombin for 45 seconds. The K_d for DAPA was calculated to be $1.67 \times 10^{-8}M \pm 0.14 \times 10^{-8}M$ by using the dose ratio data for two concentrations of DAPA and the direct plot method in which DR-1 = [DAPA]/K_d. Plotting DR-1 versus [DAPA] with the line constrained to pass through the origin gives a straight line with a slope of $1/K_d$. The K_d above is the mean ± standard error from three separate experiments of the type represented with each point assayed in duplicate.

that the effect of this peptide in neuroblastoma cells is specific to neurons and is not a general phenomenon of transformed cells.

Thrombin stimulation of platelets results in the liberation of arachidonic acid by the action of phospholipase A_2 (*9*). Experiments were conducted to evaluate the involvement of arachidonic acid metabolites in the thrombin-induced increase in cyclic GMP in neuroblastoma cells. The phospholipase A_2 inhibitor mepacrine antagonized the thrombin-stimulated cyclic GMP response with an IC_{50} (median inhibitory concentration) of about 0.1 m*M*. Similarly, lipoxygenase inhibition with nordihydroguaiaretic acid (NDGA) noncompetitively antagonized the thrombin response with an IC_{50} of approximately 30 μ*M*. By contrast, cyclooxygenase inhibition with indomethacin (concentrations up to $10^{-6}M$) failed to produce any consistent effect on thrombin-stimulated cyclic GMP formation. Mepacrine, NDGA, and indomethacin were tested for any direct effects on guanylate cyclase and were found to produce only slight inhibition (30 percent) at concentrations that totally blocked cyclic GMP synthesis in intact cells (data not shown).

These results suggest that arachidonic acid metabolites, specifically those of the lipoxygenase pathway, mediate the facilitatory effects of thrombin on cyclic GMP formation in neuroblastoma cells. Goldberg and co-workers (*10*) reported that thrombin augments cyclic GMP synthesis in platelets. This is interesting since thrombin has also been shown to release arachidonic acid in platelets (*9*). It is possible that lipoxygenase products are the final intracellular messengers that link many of the wide array of cyclic GMP stimulants (for example, muscarinic, histaminic, and α-adrenergic receptors or cellular depolarization and Ca^{2+}-ionophores) with guanylate cyclase in various cellular and tissue preparations (*11*). This hypothesis is also consistent with observations on the role of lipoxygenase metabolites in guanylate cyclase activation in rat ductus deferens (*12*) and guinea pig myometrial (*13*) preparations. By analyzing the phospholipid metabolites formed by neuroblastoma cells incubated with thrombin it should be possible to determine which arachidonic acid metabolites mediate these effects.

These results support the hypothesis that murine neuroblastoma cells have functional thrombin receptors that also occur on other cell types such as platelets (*14*) and endothelial cells (*15*). The binding of ^{125}I-thrombin to intact murine neuroblastoma cells occurs with either a high affinity (K_d = 1.4 n*M*) and low capacity (30,000 receptors per cell) or with a low affinity and high capacity, as reported for the binding of ^{125}I-thrombin to platelets (*14, 16*).

The implications of this finding are intriguing. It is unlikely that under normal conditions blood-borne clotting factors ever come into direct contact with nerve cells in the brain. However, it is possible that one or more proteases with thrombin-like specificity are synthesized within the brain and act locally. It is also possible that thrombin interactions with nerve cells play a role in the neuropathology that follows cerebrovascular accidents or similar events in which the blood comes into direct contact with the brain. Since cellular cyclic GMP is believed to modify neuronal activity and excitability (*17*), this finding has important implications in the fields of stroke and spinal cord injury.

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References and Notes

1. E. Richelson, in *International Review of Biochemistry*, vol. 26, *Neurochemistry and Biochemical Pharmacology*, K. F. Tipton, Ed. (University Park Press, Baltimore, 1979), p. 81.
2. H. Matsuzawa and M. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3472 (1975); E. Richelson, F. G. Prendergast, S. Divinetz-Romero, *Biochem. Pharmacol.* **27**, 2039 (1978); E. Richelson, *Science* **201**, 69 (1978).
3. T. Bartfai, X. O. Breakfield, P. Greengard, *Biochem. J.* **176**, 119 (1978); R. E. Study, X. O. Breakfield, T. Bartfai, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 6295 (1978).
4. Purified thrombin was kindly provided by K. G. Mann and E. Nesheim, Department of Hematology Research, Mayo Clinic.
5. F. Marquardt, *Methods Enzymol.* **19**, 924 (1960); D. Bagdy, E. Barabas, L. Graf, T. E. Petersen, S. Magnusson, *ibid.* **45**, 669 (1976); S. W. Tam, J. W. Fenton II, T. C. Detwiler, *J. Biol. Chem.* **254**, 8723 (1979).
6. R. J. Tallarida, A. Cowan, M. W. Adler, *Life Sci.* **25**, 637 (1979); the dose ratio (DR) data of Fig. 2C for two concentrations of DAPA were plotted as follows: DR-1 versus [DAPA]; the resultant line constrained to pass through the origin gives a straight line with a slope of $1/K_d$.
7. M. E. Nesheim, F. G. Prendergast, K. G. Mann, *Biochemistry* **18**, 996 (1979).
8. J. P. Schwartz, *J. Cyclic Nucleotide Res.* **2**, 287 (1976).
9. M. M. Billah and E. G. Lapetina, *J. Biol. Chem.* **257**, 5196 (1982); E. G. Lapetina, M. M. Billah, P. Cuatrecasas, *ibid.* **256**, 5037 (1981).
10. J. G. White, N. D. Goldberg, R. D. Estensen, M. K. Haddox, G. H. R. Rao, *J. Clin. Invest.* **52**, 89a (1973).
11. N. D. Goldberg and M. K. Haddox, *Annu. Rev. Biochem.* **46**, 823 (1977).
12. C. Spies *et al.*, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **311**, 71 (1980).
13. D. Lieber and S. Harbon, *Mol. Pharmacol.* **21**, 654 (1982).
14. D. M. Tollefsen, J. R. Feagler, P. W. Majerus, *J. Biol. Chem.* **249**, 2646 (1974); P. Ganguly, *Nature (London)* **247**, 306 (1974); B. M. Martin, W. W. Wasiewski, J. W. Fenton II, T. C. Detwiler, *Biochemistry* **15**, 4886 (1976).
15. B. J. Awbrey, J. C. Hoak, W. G. Owen, *J. Biol. Chem.* **254**, 4092 (1979).

16. R. M. Snider *et al.*, in preparation.
17. M. J. Berridge, in *The Neurosciences Fourth Study Program*, F. O. Schmitt and F. G. Worden, Eds. (MIT Press, Cambridge, Mass., 1979), p. 873.
18. E. El-Fakahany and E. Richelson, *J. Neurochem.* **35**, 941 (1980). In brief, intact cells were washed free of growth medium, harvested for assay and suspended in 2 ml of phosphate-buffered saline (PBS) solution. They were then incubated with [^{3}H]guanine (10 μCi/ml) and rotated at 37°C for 45 minutes at 60 rev/min. The cells were briefly centrifuged, the radioactive supernatant was discarded, and then the cells were resuspended in either normal PBS or Ca^{2+}-free PBS and distributed into wells of a multiwell tray in 270-μl portions containing approximately 1×10^5 cells. After incubation of cells at 37°C for 15 to 20 minutes in the shaker bath, at 80 rev/min, thrombin (3 U/ml) was added in a 30-μl volume for a specified time. Basal concentrations of tritiated cyclic GMP were determined by adding 30 μl of PBS. The reaction was terminated by the addition of 30 μl of 50 percent (weight to volume) trichloroacetic acid. After adding to each well 0.6 nCi (1400 dis/min) of ^{14}C-labeled cyclic GMP as an internal standard, the contents of each well were applied to an AG50 W-X2 ion exchange column (0.8 by 8.0 cm) which had been equilibrated with 0.1*N* HCl. The columns were then washed with 4.4 ml of 0.1*N* HCl (eluate discarded) and 1.0 ml of H_2O (eluate discarded), and finally with 1.5 ml of H_2O which was collected in plastic Microfuge tubes. To this eluate, equal (30 μl) volumes of 2.67*M* $ZnSO_4$ and 2.67*M* Na_2CO_3 were added to precipitate any residual [^{3}H]GTP or [^{3}H]GDP. The tubes were then vortexed and centrifuged in a Beckman Microfuge for 2 minutes. The supernatant was transferred to a scintillation vial containing 7 ml of scintillation cocktail and the radioactivity determined in a Searle Isocap/300 liquid scintillation counter. All samples were corrected for the recovery of ^{14}C-labeled cyclic GMP (70 to 80 percent) and for quenching by using the external standard ratio technique.
19. Supported by the Mayo Foundation and PHS grants HL07111 and MH27692.

18 January 1983

Coping and Immunosuppression: Inescapable but Not Escapable Shock Suppresses Lymphocyte Proliferation

Abstract. *Rats were given series of escapable shocks, identical inescapable shocks, or no shock. The subjects were reexposed to a small amount of shock 24 hours later, after which an in vitro measure of the cellular immune response was examined. Lymphocyte proliferation in response to the mitogens phytohemagglutinin and concanavalin A was suppressed in the inescapable shock group but not in the escapable shock group. This suggests that the controllability of stressors is critical in modulating immune functioning.*

Exposure to a variety of psychosocial and environmental stressors can alter the functioning of the immune system (*1*). For example, in humans, lymphocyte proliferation in response to mitogens is suppressed 6 weeks after the death of a spouse (*2*). Such suppression is also seen after exposure to loud noise in mice (*3*), electric shock in rats (*4*), infant-mother separation in bonnet monkeys (*5*), and peer separation in pig-tailed monkeys (*6*). Similarly, acceleration, restraints, and overcrowding in mice reduce the plaque-forming response to sheep red blood cells (*7*).

These stressors are diverse, and it is not known which aspect is critical for the impairment of immune functioning. Research on the relation between stress and cancer suggests that an organism's inability to exert control over the events in question may be important. Psychological states that involve a loss of control, such as bereavement and severe depression, are associated with an increased incidence of cancer (*8*). In fact, an inability to cope with aversive events is frequently part of the definition of stress. It is often argued that an event will induce stress only if the organism cannot or anticipates that it cannot cope with the event (*9*). Stressors that have been shown to impair immune functioning in vitro, such as noise, electric shock, and separation, have been uncontrollable (inescapable and unavoidable).

In a number of experiments the impact of the psychological dimension of controllability on tumor growth and tumor rejection has been evaluated. Sklar and Anisman (*10*) injected mice with syngeneic P815 tumor cells and then gave them 60 shocks. Some of the mice were allowed to terminate each shock by performing an escape response, and thus had a degree of control over the shock. Other mice were given inescapable shocks, and thus had no control. Even though both groups received identical shocks in physical terms, tumor growth was enhanced in the inescapable shock group while the escapable shocks had no effect. Visintainer *et al.* (*11*) reported similar results for the rejection of Walker 256 sarcomas in rats.

However, tumor growth and regression do not necessarily reflect immune system functioning. They can be directly affected by such factors as vascular flow, steroids, and prolactin, all of which are increased by stress (*8*). The purpose of this study was to directly determine whether the controllability of stressors is important in modulating the activity of the immune system. We compared the effects of equal amounts and distributions of escapable and inescapable shock on mitogen-induced proliferation of lymphocytes in vitro. In order to enhance comparability with the tumor studies described above, we used only one session of shocks with qualities similar to those used in the tumor studies. Previous studies of the effects of stressors on in vitro measures of immune functioning have tended to involve multiple sessions of stressor exposure, much more prolonged sessions, or, where shock was used, much more intense shock.

It is not obvious how soon immune functioning should be measured after exposure to shocks of differing controllability. The immediate effects of shock could easily mask any differences between escapable and inescapable shock soon after the session. Suppression could simply be maximum. Indeed, Keller *et al.* (*4*) found no differences in lymphocyte proliferation after shocks of very different intensities when blood was drawn immediately after the session. Suppression was maximum in both high and low shock conditions. In addition, many of the behavioral effects of inescapable shock are typically measured 24 hours after shock exposure rather than soon after exposure. For example, Jackson *et al.* (*12*) reported that inescapably shocked subjects became analgesic 24 hours later on reexposure to a small amount of shock (itself insufficient to produce analgesia). Subjects for whom the initial shocks were escapable did not become analgesic when given shock 24 hours later. However, both escapably and inescapably shocked subjects are analgesic immediately after shock (*13*). Shavit *et al.* (*14*) found footshock that produces an opioid form of analgesia (reversed by opiate antagonists and cross-tolerant with morphine) to be immunosuppressive, whereas footshock that produces a nonopioid form of analgesia was not. The analgesia that emerges upon a brief reexposure to shock 24 hours after inescapable but not escapable shock is completely reversed by opiate antagonists and completely cross-tolerant with morphine (*15*). Thus we measured immune functioning immediately after a brief reexposure to shock 24 hours after experience with equal amounts of escapable or inescapable shock. The procedures used were identical to those found to produce opioid analgesia.

Twelve rats were given an average of one escapable shock per minute, for a total of 80 shocks. Shock intensity began at 0.8 mA and was increased 0.2 mA every 20 trials. Final intensity was thus 1.6 mA. The rats were placed in a small "wheel-turn" box (*16*) and shock was