Selective Inhibition of Thrombin Receptor-mediated Ca$^{2+}$ Entry by Protein Kinase C β*

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Yanping Xu and J. Anthony Ware†

From the Cardiovascular Division and the Harvard-Thorndike Laboratories of the Department of Medicine, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02215

Thrombin initiates many physiological processes in platelets and other megakaryocyte-lineage cells by interacting with surface receptors and generating rises in cytoplasmic Ca$^{2+}$; these rises result from both Ca$^{2+}$ release from intracellular stores and receptor-mediated Ca$^{2+}$ entry. Regulators that limit Ca$^{2+}$ entry after its initiation by thrombin have not been identified. In this study, prevention of expression of a single protein kinase C isoenzyme (PKCβ) by antisense cDNA overexpressed in HEL cells, a human megakaryoblastic cell line that expresses thrombin receptors, promotes thrombin receptor-mediated Ca$^{2+}$ entry without altering thrombin-induced intracellular release of Ca$^{2+}$ and cytoplasmic Ca$^{2+}$ rise. The cytoplasmic Ca$^{2+}$ rise initiated by endoperoxide analogs was not affected by inhibiting PKCβ. Overexpression of a cDNA encoding wild-type PKCβ mutated to prevent recognition by the antisense cDNA abolished the enhancement of Ca$^{2+}$ influx following thrombin. Thus, PKCβ appears to be a specific negative regulator of thrombin receptor-mediated Ca$^{2+}$ entry.

The protease thrombin is generated at sites of vascular injury and is a central mediator of hemostasis, thrombosis, inflammation, and vascular proliferation. Thrombin stimulates several cell types, including platelets and other cells of megakaryocytic lineage, monocytes, endothelium, and vascular smooth muscle, by triggering surface receptors to generate intracellular messengers (2, 3). The addition of thrombin to smooth muscle, by triggering surface receptors to generate Ca$^{2+}$ entry, is a central mediator of hemostasis, thrombosis, inflammation, and vascular proliferation. Thrombin can also be activated by generation of lipid regulators (14) that are mobilized following thrombin stimulation (2); some isoenzymes of PKC (e.g., PKCβ) are regulated by a rise in Ca$^{2+}$ (15). Since limiting or negative mediators of Ca$^{2+}$ influx might be reasonably considered to be regulated by a process requiring Ca$^{2+}$, we asked whether selective inhibition of a Ca$^{2+}$-dependent PKC isoenzyme would modify the thrombin receptor-mediated Ca$^{2+}$ influx. For these experiments, we utilized human erythroleukemic (HEL) cells (17), a megakaryoblastic cell line that has functional thrombin receptors and shares with platelets many components of the thrombin signaling mechanism (12, 18). HEL cells offer an additional advantage for the present studies, in that they express only one (PKCβ) of the Ca$^{2+}$-dependent PKC isoenzymes (12, 19) and therefore required only a single intervention to elminate Ca$^{2+}$-dependent PKC activity from the cells. As there are few chemical PKC inhibitors that are specific for individual isoenzymes, a strategy based on antisense DNA was utilized to reduce selectively PKCβ in HEL cells.

MATERIALS AND METHODS

Construction, Expression, and Detection of anti-PKCβ—To achieve selective inhibition of PKCβ, a segment of cDNA (55 base pairs) specific to PKCβ (5'-TCCGGCTCCTGGCGGAAATGTCGACCCGCTGGGGGCACGCGCAAGATG3') was cloned into pcDNA1/Neo (Invitrogen) in an antisense orientation. The nucleotide sequence of the first 20 and the last 35 base pairs of the cDNA corresponded to that of the 5'-end of the untranslated and the beginning of the translated regions (respectively) of human PKCβ1 cDNA.

Low passage HEL cells (a generous gift of Thalia Papayannopoulou, University of Washington, Seattle) were either transfected with the antisense PKCβ construct or with the vector only (control cells) by electroporation. Cells that stably expressed anti-PKCβ cDNA (anti-β cells) were selected by limiting dilution and on the basis of cell survival in the presence of Geneticin (1.2 mg/ml). For Northern blot analysis, total RNA was prepared from the cells using the method described previously (12). The cDNA probes of PKCβ1, PKCε, and PKCγ are the same as described previously (19). The cDNA probes for α1α, β-thromboglobulin, and the thrombin receptor were generous gifts from Drs. Peter Newman (Blood Research Institute of Southeastern Wisconsin), Mortimer Pincz, and Lawrence Brass (University of Pennsylvania), respectively; the anti-thrombin receptor antibody was kindly provided by Dr. Brass.

Construction and Expression ofmut-PKCβ—to create a mutated PKCβ molecule that was not inhibited by the antisense PKCβ construct, the 5'-end of the untranslated and the beginning of the translated region of the cDNA encoding rat PKCβ (a generous gift of Dr. I. B. Weinstein, Columbia University) was mutated to reduce the complementarity to anti-β cDNA. The mutant cDNA was cloned into pREP4 vector (Invitrogen) carrying the hygromycin resistance gene. Anti-β cells were transfected with themut-PKCβ construct by electroporation. Stable transfectants were selected by limiting dilution and on the basis of cell survival in the presence of 100 μg/ml hygromycin.

[Ca$^{2+}$]i Measurements—Cytoplasmic ionized Ca$^{2+}$ ([Ca$^{2+}$]i), was measured in Fura-2 loaded HEL cells as described previously (12). Briefly, HEL cells were washed and resuspended in HEPES-Tyrode's buffer (12). Fura-2/AM was added in a final concentration of 2 μM to the cells, which were incubated for 30 min at 37 °C. Fluorescence measurements were obtained using a dual excitation wavelength spectrofluorometer (SPEX Fluorolog-2, Edison, NJ). Fura-2 signals were calibrated as described previously (20). All measurements were performed...
on cells suspended in HEPES-Tyrode's buffer containing 1 mM Ca\(^{2+}\); in some experiments, NiCl\(_2\), EGTA, or MnCl\(_2\) was added just before stimulation with thrombin.

RESULTS AND DISCUSSION

Expression of both RNA and protein of PKC\(\beta\) was significantly reduced in anti-\(\beta\) cells compared with that in either wild-type HEL cells or the control cells (Fig. 1, a and b). In addition to PKC\(\beta\), the predominant isoenzymes expressed by HEL cells are PKC\(d\) and PKC\(\varepsilon\), which are also expressed in platelets (12, 19); neither PKC\(d\) nor PKC\(\varepsilon\) was inhibited by anti-PKC\(\beta\) cDNA (Fig. 1a). Inhibition of PKC\(\beta\) provoked no obvious alteration of differentiation, as shown by the similar expression of integrin \(\alpha_{\text{IIb}}\), \(\beta\)-thromboglobulin, and thrombin receptor (Fig. 1c).

Because of the effect that overall PKC activation exerts on \([Ca^{2+}]_i\) homeostasis, we asked whether this sharp reduction in PKC\(\beta\) would alter \([Ca^{2+}]_i\) following thrombin. The thrombin-induced \([Ca^{2+}]_i\) in clonal populations of anti-\(\beta\) HEL cells loaded with the Ca\(^{2+}\)-sensitive fluorophore Fura-2 was significantly enhanced when compared with that of the control cells (Fig. 2, a and b). This result suggests that thrombin-induced elevation of \([Ca^{2+}]_i\) in HEL cells is normally inhibited by PKC\(\beta\); this effect appeared to be specific for thrombin, because \([Ca^{2+}]_i\) induced by the endoperoxide analog U46619, which activates the thromboxane A\(_2\) receptor, is not affected in anti-\(\beta\) cells (Fig. 2, c and d). Enhanced \([Ca^{2+}]_i\) does not result from altering the expression of thrombin receptors on anti-\(\beta\) cells, as assessed by flow cytometry utilizing a fluorescently labeled PKC\(\beta\) and Thrombin-induced \([Ca^{2+}]_i\)
anti-thrombin receptor antibody (data not shown) or by expression of mRNA encoding the thrombin receptor (Fig. 1).

To determine whether enhanced $[\text{Ca}^{2+}]_i$ resulted specifically from the reduction of PKCβ expression by the antisense construct, we restored the expression of PKCβ by stably transfecting cDNA encoding the full-length PKCβ into clonal populations of anti-β cells. To prevent inhibition of expression of the transfected PKCβ by the constitutively expressed anti-β cDNA, the degeneracy of the genetic code was exploited to generate a mutant PKCβ cDNA (mut-PKCβ) that had minimal complementarity with the anti-β cDNA but still encoded the same amino acid sequence as native PKCβ (Fig. 3a). Expression of the mut-PKCβ was not inhibited by the anti-β construct, as verified by cotransfection of the two constructs in COS7 cells, which do not normally express PKCβ, followed by Northern transfer analysis (data not shown). Transfection of mut-PKCβ into the anti-β cells restored the expression of PKCβ, as assessed by immunoblotting (Fig. 3b), and abolished the enhancement of thrombin-induced $[\text{Ca}^{2+}]_i$ in the anti-β cells (Fig. 3, c and d). Thus, the reversal of the changes induced by antisense restoration of PKCβ in the same clone of cells strengthens the argument that PKCβ specifically inhibits the thrombin-induced increase in $[\text{Ca}^{2+}]_i$.

This inhibition of the thrombin-induced increase in $[\text{Ca}^{2+}]_i$ by PKCβ might result from an effect on $\text{Ca}^{2+}$ entry, on $\text{Ca}^{2+}$ release from intracellular stores, or on both. To distinguish among these possibilities, the contribution of $\text{Ca}^{2+}$ entry from extracellular medium to $[\text{Ca}^{2+}]_i$ was eliminated by either briefly chelating extracellular $\text{Ca}^{2+}$ with EGTA or adding $\text{Ni}^{2+}$, which blocks the $\text{Ca}^{2+}$ entry via receptor-operated cation channels (5, 7). Following these interventions, $[\text{Ca}^{2+}]_i$ was not significantly different between anti-β and control cells (Fig. 4, a and b). Furthermore, the divalent cation entry, assessed by measuring the Mn$^{2+}$ quench of intracellular Fura-2 fluorescence (6) after addition of thrombin, was less in the control cells than in the anti-β cells (Fig. 4c). Thus, PKCβ reduces the magnitude of thrombin-induced $\text{Ca}^{2+}$ entry but has little effect on release of $\text{Ca}^{2+}$ from intracellular stores. These results also demonstrate that some aspects of thrombin receptor function were not affected by inhibition of PKCβ; additionally, PKC-mediated inhibition of thrombin-induced mobilization of $\text{Ca}^{2+}$ from intracellular storage sites (12, 13) does not require the presence of known $\text{Ca}^{2+}$-regulated PKC isoenzymes in HEL cells.

**Fig. 3.** Restoration of PKCβ expression with mutant PKCβ. a, wild-type human PKCβ sequence against which the antisense construct was targeted (top) and the sequence of the corresponding portion of the mutated full-length rat PKCβ that was overexpressed in the anti-β cells. b, immunoblot of PKCβ expression in control (left lane) and two clones of anti-β HEL cells transfected with a mutant PKCβ cDNA (mut-PKCβ). Immunoblotting was performed with a polyclonal anti-PKCβ antibody (Santa Cruz). c, changes in $[\text{Ca}^{2+}]_i$ upon adding thrombin (0.2 unit/ml) to control and mutant PKCβ cells. d, bar graph shows the mean ± S.E. of the differences between peak and basal levels of $[\text{Ca}^{2+}]_i$ in control (n = 3) and mut-PKCβ (n = 10) cells.

**Fig. 4.** Determination of the component of $[\text{Ca}^{2+}]_i$ regulated by PKCβ. a, bar graphs of the mean ± S.E. of the difference between peak thrombin-induced and basal levels of $[\text{Ca}^{2+}]_i$ in control and anti-β cells suspended in buffer to which 2 mM EGTA has been added (n = 3). b, bar graphs of the mean ± S.E. of the differences between peak thrombin-induced and basal levels of $[\text{Ca}^{2+}]_i$ in control and anti-β cells suspended in buffer to which 2 mM NiCl$_2$ has been added (n = 3). c, representative tracing of Fura-2 fluorescence changes (360 nm excitation wavelength) of control and anti-β cells suspended in medium containing Mn$^{2+}$ (0.1 mM) shortly after stimulation with thrombin (0.5 unit/ml).

After addition of thrombin (but not ADP) to platelets (5, 6, 7), a measurable delay precedes $\text{Ca}^{2+}$ entry, suggesting that the $\text{Ca}^{2+}$ channel is not directly linked to the receptor but instead is activated by intracellular mediators, generated perhaps by depletion of intracellular $\text{Ca}^{2+}$ stores (10, 11) or by phospholipid hydrolysis (21). Thus, PKCβ might interfere with a mediator that initiates or potentiates $\text{Ca}^{2+}$ influx; such a mediator would presumably be generated by thrombin but not endoperoxide analogs such as U46619. An alternative possibility is that
PKC\(\beta\), once activated, might phosphorylate a receptor-mediated Ca\(^{2+}\) channel specifically associated with the thrombin receptor. Either model has implications for the specificity of agonist effect. Thus, although other mechanisms for limiting Ca\(^{2+}\) entry may exist for other agonists, this reduction in thrombin receptor-mediated Ca\(^{2+}\) influx by PKC\(\beta\), a Ca\(^{2+}\)-regulated PKC, represents a novel and selective cross-regulatory mechanism that could prevent excessive accumulation of cytoplasmic Ca\(^{2+}\) following thrombin stimulation.

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REFERENCES

1. Coughlin, S. R., Vu, T. K., and Hung, D. T. (1992) J. Clin. Invest. 89, 351–355
2. Ware, J. A., and Colier, B. S. (1994) Williams’ Hematology, pp. 1161–1203, McGraw-Hill Book Co., New York
3. Coughlin, S. R. (1994) Semin. Hematol. 31, 270–277
4. Rink, T. J., and Sage, S. O. (1990) Annu. Rev. Physiol. 52, 431–449
5. Zschauer, A., van Bremmen, C., and Buhler, F. R. (1988) Nature 334, 703–705
6. Sage, S. O., Merritt, J. E., and Hallam, T. J. (1989) Biochem. J. 258, 923–926
7. Mahaut-Smith, M. P., Sage, S. O., and Rink, T. J. (1990) J. Biol. Chem. 265, 10479–10483
8. Randriamampita, C., and Tsien, R. Y. (1993) Nature 364, 809–814
9. Luckhoff, A., and Clapham, D. E. (1992) Nature 355, 356–358
10. Putney, J. W. (1993) Science 262, 676–678
11. Clapham, D. E. (1995) Cell 80, 259–268
12. Grabarek, J., Raychowdhury, M. K., Ravik, K., Kent, K. C., Newman, P. J., and Ware, J. A. (1992) J. Biol. Chem. 267, 10011–10017
13. Zavoico, G. B., Halenda, S. P., and Shaafi, R. I. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3859–3862
14. Bell, R. M., and Burns, D. J. (1991) J. Biol. Chem. 266, 4661–4664
15. Nishizuka, Y. (1992) Science 256, 607–614
16. Randriamampita, C., and Tsien, R. Y. (1995) J. Biol. Chem. 270, 29–32
17. Papayannopoulou, T., Nakamoto, B., and Yokochi, T. (1983) Blood 62, 832–845
18. Hoxie, J. A., Ahuja, M., and Brass, L. F. (1993) J. Biol. Chem. 268, 13756–13763
19. Chang, J. D., Xu, Y., Raychowdhury, M. K., and Ware, J. A. (1993) J. Biol. Chem. 268, 14208–14214
20. Johnson, P. C., Ware, J. A., and Salzman, E. W. (1989) Methods Enzymol. 169, 386–415
21. Berridge, M. J. (1993) Nature 361, 315–325