Translocation Inhibitors Define Specificity of Protein Kinase C Isoenzymes in Pancreatic β-Cells*

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The protein kinase C (PKC) family consists of 11 isoenzymes. Following activation, each isoenzyme translocates and binds to a specific receptor for activated C kinase (RACK) (Mochly-Rosen, D. (1995) Science 268, 247–251) that provides an anchoring site in close proximity to the isoenzyme’s specific substrate. Pancreatic islet cells contain at least six PKC isoenzymes (Knutson, K. L., and Hoenig, M. (1994) Endocrinology 135, 881–886). Although PKC activation enhances insulin release, the specific function of each isoenzyme is unknown. Here we show that following stimulation with glucose, αPKC and εPKC translocate to the cell’s periphery, while δPKC and PKC translocate to perinuclear sites. βC2–4, a peptide derived from the RACK1-binding site in the C2 domain of βPKC, inhibits translocation of αPKC and reduces insulin response to glucose. Likewise, εV1–2, an εPKC-derived peptide containing the site for its specific RACK, inhibits translocation of εPKC and reduces insulin response to glucose. Inhibition of islet-glucose metabolism with mannoheptulose blocks translocation of both αPKC and εPKC and diminishes insulin response to glucose while calcium-free buffer inhibits translocation of αPKC but not εPKC and lowers insulin response by 50%. These findings illustrate the unique ability of specific translocation inhibitors to elucidate the isoenzyme-specific functions of PKC in complex signal transduction pathways.

Protein kinase C is a family of 11 lipid-dependent serine/threonine kinases involved in a wide spectrum of signal transduction (7, 8). Upon activation, PKC isoenzymes translocate to new cellular sites, including the plasma membrane (9, 10), cytoskeletal elements (11, 12), and the nucleus (13, 14), as well as other subcellular compartments (15). Many cells are known to contain several isoenzymes (16, 17), each localizing to a different cellular site upon stimulation (18). The multiplicity of isoforms of a single enzyme renders the analysis of enzyme-function relationship difficult. Recent work revealed that activated PKC isoenzymes bind anchoring proteins termed RACKs (1–3), believed to be positioned in close proximity to the isoenzyme’s substrate. It was further shown that the functional specificity of the PKC isoenzyme is determined, in part, by the differential localization of the isoenzyme-specific RACKs (19). The RACK for βPKC, RACK1, has been cloned, and at least part of its binding site on βPKC has been mapped to a short sequence within the C2 domain (1). βC2–4, a nonpeptide derived from this region, inhibits phorbol ester-induced translocation of the C2-containing isoenzymes but not the translocation of C2-less isoenzymes such as δ- and εPKC when tested in intact cells (1). A short peptide derived from the V1 region of εPKC, εV1–2, was similarly shown to inhibit the translocation of εPKC, but not α-, β-, and δPKC (20). Furthermore, these isozyme-specific inhibitors blocked the specific function of individual isoenzymes; for example, εV1–2, but not βC2–4, inhibited phorbol 12-myristate 13-acetate-induced regulation of the contraction rate in intact cardiomyocytes. Here we use these novel PKC isozyme-specific inhibitors to determine that PKC activation is part of the signals involved in the regulation of glucose-induced insulin secretion and to identify the specific isoenzymes that mediate this glucose effect.

MATERIALS AND METHODS

Islets obtained from 200-g male Sprague-Dawley rats were cultured for 3–5 days in glass chamber slides coated with extracellular matrix of bovine corneal endothelial origin (21). When more than 75% of the islet cells spread out to form a monolayer, the media were replaced with modified Krebs Ringer solution (KRB) (22) containing either 2.5 or 20 mM glucose. Following a brief wash, the islets were fixed in cold acetic, blocked with 1% normal goat serum for 1 h, and treated overnight with the specific anti-PKC isoenzyme antibody. Freshly isolated islets were used for insulin release studies. Islets were preincubated with excess amounts of their corresponding antigen derived from the V5 region of the isoenzyme (PKC-εisoform-(728–737) or PKC-α-(Tyr663–(664–672)), Research and Diagnostic Antibodies, Berkeley, CA). Fluorescein isothiocyanate-linked goat-anti-rabbit IgG (Sigma Israel Chemicals, Rehovot, Israel) was applied for 2 h, and the slides were mounted in 90% glycerol, 10% phosphate-buffered saline, 0.1% sodium azide, 3% dextran blue (2.25 g/l), pH 9.0, for microscopic imaging. Histochemical imaging was conducted on a PhoBio 1000 confocal microscope (Sarastro Inc., Ypsilanti, MI), equipped with Zeiss Universal Optics and argon laser illumination. The anti-PKC antisera each exhibited a single band of the appropriate size on Western blot (10 μg of rat brain or rat islet homogenate). No bands were observed when the antibodies were preincubated with excessive amounts of their corresponding antigens.

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for confocal imaging. α- and εPKC translocated to the cell periphery while δPKC concentrated asymmetrically in a perinuclear region and ζPKC formed a ring structure around the cell nuclei. Color gradients depicted as black, blue, yellow, and red indicate, in increasing order, the relative concentration of the isoenzyme.

islet cells was achieved by transient permeabilization (“skinning”) (4). Ten-minute skinning, followed by a 1-h incubation of isolated rat islets with Krebs buffer containing 2.5 mM glucose, had no effect on islet viability as examined by trypan blue exclusion or by the exhibition of intact islet secretory response to glucose in either batch or perifusion studies.

Insulin release was measured by radioimmunoassay using specific guinea pig anti-rat insulin antiserum (Linco Research, St. Charles, MO) and rat insulin standard (Novo Research Institute, Bagsvaerd, Denmark) (22). Data presented are mean net insulin values after subtraction of non-stimulated level (2.5 mM glucose). Statistical significance was determined by paired, non-parametric comparison to control or to control skinning test, using the Wilcoxon test.

RESULTS AND DISCUSSION

The role of PKC as an amplifier of the glucose-generated signal to release insulin has been well established (5, 23–25). Six PKC isoforms, α, β, δ, ε, ζ, and η were found thus far in rat pancreatic islets of Langherans (5, 6, 25). The specific function of the individual isoforms remains unknown. We used adult rat islet cultures (21) to assess the direct effect of glucose on PKC isoforms in intact islet cells. Site-specific translocation could be demonstrated for α-, δ-, ε-, and ζPKC (Fig. 1). Confocal microscopy imaging revealed that αPKC and εPKC redistributed following glucose stimulation to the cell’s periphery, δPKC concentrated in an asymmetric structure in perinuclear region (possibly the Golgi apparatus), and ζPKC concentrated as a ring around the cell nucleus (Fig. 1).

Isoenzyme α is a member of the calcium-sensitive cPKC subfamily (7, 8). Alterations in cytosolic calcium levels play a prominent role in β-cell stimulus-secretion coupling for insulin release (for review, see, for example, Ref. 26). We therefore examined whether modulation of the calcium-sensitive PKC subtypes affects glucose-induced insulin release in rat islets. βC2–4 (Ser-Leu-Asn-Pro-Glu-Trp-Asn-Glu-Thr) is a nonopeptide derived from the binding site (amino acids 218–226) of β2IPKC to RACK1 (19), a well conserved sequence within the C2 domain of members of the cPKC subfamily. Introduction of βC2–4 into islet monolayers by means of transient permeabilization at low temperature (skinning) (4) abolished the glucose-induced translocation of αPKC to the cell’s periphery (Fig. 2c), whereas βC2–4-s, a scrambled control peptide (Trp-Asn-Pro-Glu-Ser-Leu-Asn-Thr), did not prevent translocation (Fig. 2d).

Moreover, administration of βC2–4 into freshly isolated islets resulted in 35% reduction in the insulin response to glucose stimulation (Fig. 2e); introduction of the scrambled analog, βC2–4-s, had no inhibitory effect on insulin secretion. To rule out the possibility that the partial inhibition of the insulin response was the result of unequal penetration of the peptide, freshly isolated islets in suspension were skinning in the absence or presence of 10 μM βC2–4 and subsequently incubated with 20 mM glucose. At the end of a 60-min stimulation, the islets were fixed in paraformaldehyde, dehydrated in ethanol, and further treated in propylene oxide Surr mixture. Following resin polymerization, 5- and 10-μm slices were prepared and stained with anti-αPKC antibodies. Confocal imaging revealed uniform inhibition of the glucose-induced isozyme translocation throughout the sections (not shown), indicating homogeneous penetration of the octapeptide throughout the islet. βC2–4 has been shown to be equally effective against all members of the classical PKC subfamily (19). While αPKC often has been reported to be the predominant PKC isozyme in islet β-cells, βPKC exhibits only scant expression in these cells (5); we, however, assume that all cPKC isoforms were
Moreover, introduction of the isoenzyme (20). Introduction of Ser-Glu-Thr-Lys-Pro-Ala-Val had no effect (Fig. 3). Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr) derived from the RACK-1 islet incubated in KRB containing 20 mM glucose (d). 

e. Inhibition of glucose-dependent translocation of aPKC and ePKC (amino acids 14–21 within the V1 region) studied in freshly isolated islets in calcium-free (and in the presence of EGTA) abolished glucose-dependent translocation of aPKC and ePKC (Fig. 4a,b). Mannoheptulose (30 mM), a glycolytic inhibitor, impeded glucose-stimulated translocation of both aPKC and ePKC (not shown). 

5 μM was found to be a maximally effective concentration for both βC2–4 and ePKC in 1-h incubations, never exceeding 43% inhibition of glucose-induced insulin release. The effect of the two peptides was additive at that concentration in fresh islets incubated for 60 min at 20 mM glucose (Fig. 4b). The fact that both peptides together inhibited only 67% of the glucose-mediated insulin release suggests that aPKC and ePKC are each independently involved in one of several distal coupling systems (see below). Stimulation of islet monolayer in the absence of Ca2+ and in the presence of EGTA abolished glucose-dependent translocation of aPKC but not of ePKC (Fig. 4a). It also diminished the glucose-induced insulin response in fresh islets by 85% (Fig. 4b), providing further evidence that aPKC but not ePKC is involved in the calcium-mediated regulatory signal to release insulin. However, addition of mannoheptulose, an inhibitor of glucose metabolism, completely abolished the glucose-induced translocation of both aPKC and ePKC. The data are shown of islet cultures skinned and stained with anti-aPKC. Immunohistochemical images are shown of islet cultures skinned and stained with anti-ePKC following a 60-min incubation in KRB containing 2.5 mM glucose (a), skinned islets incubated in KRB containing 20 mM glucose (b), skinned islets treated with eV1–2 then incubated in KRB containing 20 mM glucose (c), or skinned islets treated with the control scrambled peptide eV1–2-s then incubated in KRB containing 20 mM glucose (d). e. Inhibition of glucose-induced insulin secretion by eV1–2 in isolated rat islets. Insulin response was determined following a 60-min stimulation with 20 mM glucose in untreated islets (Control), control skinned islets without peptide added (Skinned), skinned islets in the presence of 10 μM eV1–2 (eV1–2), or skinned islets in the presence of 10 μM scrambled peptide (eV1–2-s). * denotes statistical significance of p < 0.004 compared with glucose-stimulated control.

equally inhibited by βC2–4. The fact that maximally effective concentrations of βC2–4 had only a partial inhibitory effect on glucose-induced insulin release may therefore indicate that in addition to the ePKC subfamily, other PKC isoenzymes or non-PKC stimulus amplifiers are involved in the glucose-generated stimulus-secretion coupling.

Despite the pivotal role of cytosolic calcium mobilization in the control of insulin release, data are accumulating supporting the role of additional, calcium-independent signals in the control of insulin secretion (27–29). We therefore examined the role of ePKC, a calcium-insensitive PKC isoenzyme in glucose-induced insulin secretion utilizing eV1–2, the octapeptide (Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr) derived from the RACK-binding site of ePKC (amino acids 14–21 within the V1 region of the isoenzyme) (20). Introduction of eV1–2 into islet monolayers by means of skinning abolished glucose-induced translocation of ePKC (Fig. 3c); the scrambled analog eV1–2-s (Leu-Ser-Glu-Thr-Lys-Pro-Ala-Val) had no effect (Fig. 3d). Moreover, introduction of eV1–2 into freshly isolated islets in suspension inhibited the insulin response to glucose by 40% (Fig. 3c), whereas eV1–2-s, the scrambled analog, had no inhibitory effect (Fig. 3d). In histochemical studies, eV1–2 had no effect on glucose-dependent translocation of aPKC and, vice versa, βC2–4 had no effect on the translocation of ePKC (not shown).
the duration of glucose stimulation (30), it is possible that different PKC isoenzymes have distinct roles in the different phases of release. This subject is presently under investigation. Islet coupling signals originating from activation of adenylate cyclase, phospholipase C, and phospholipase A_2 are among the more thoroughly investigated potentiating signals involved in the modulation of the insulin response to glucose (31–33). Messengers generated from these key metabolic pathways are known to activate multiple PKA, PKC, and CaM kinases, each controlling a specific amplifying branch of the insulin stimulus-secretion coupling pathway, resulting in the multiphasic dynamics of hormonal secretion in response to glucose stimulus (30, 31). The identification of PKC isoenzyme-specific binding proteins offers novel tools to resolve the specific contribution of each isoform to this complex of interrelated signals. Furthermore, RACK-binding translocation inhibitors should prove to be valuable tools in resolving the specific function of the individual PKC isoenzyme in cells expressing multiple forms of the enzyme as well as in identifying their specific substrates.

REFERENCES
1. Ron, D., Chen, C. H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 839–843
2. Mochly-Rosen, D., Khaner, H., and Lopez, J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3997–4000
3. Mochly-Rosen, D. (1995) Science 268, 247–251
4. Johnson, J. A., Gray, M. O., Karliner, J. S., and Mochly-Rosen, D. (1996) Circ. Res., in press
5. Knutson, K. L., and Hoenig, M. (1994) Endocrinology 135, 881–886
6. Selfie, L. A., Schmitz, P. C., Sheng, Y., and Binn, T. J. (1993) J. Biol. Chem. 268, 24296–24302
7. Dekker, L. V., and Parker, P. J. (1994) Trends Biochem. Sci. 19, 73–77
8. Nishizuka, Y. (1992) Science 255, 607–614
9. Shoji, M., Girard, P. R., Mazzei, G. J., Vogler, W. R., and Kuo, J. F. (1986) Biochem. Biophys. Res. Commun. 135, 1144–1149
10. Parker, P. J., Kour, G., Marais, R. M., Mitchell, F., Pears, C., Schaap, D., and Stabel, S. (1989) Mol. Endocrinol. 65, 1–11
11. Mochly-Rosen, D., Heinrich, C. J., Cheever, L., Khaner, H., and Simpson, P. C. (1996) Cell Regul., 1, 693–706
12. Kiley, S. C., and Jaken, S. (1990) Mol. Endocrinol. 4, 59–68
13. Disatnik, M. H., Winnier, A. R., Mochly-Rosen, D., and Arteaga, C. L. (1994) Cell Growth Differ. 5, 873–880
14. Miura, U. K., and Sabyoun, N. (1987) Biochem. Biophys. Res. Commun. 145, 760–768
15. Boneh, A., and Tennenhouse, H. S. (1988) Biochem. Cell Biol. 66, 262–272
16. Baldassare, J. J., Henderson, P. A., Burns, D., Loomis, C., and Fisher, G. J. (1992) J. Biol. Chem. 267, 15385–15390
17. Genot, E. M., Parker, P. J., and Cantrell, D. A. (1995) J. Biol. Chem. 270, 9853–9859
18. Disatnik, M. H., Burnagi, G., and Mochly-Rosen, D. (1994) Exp. Cell Res. 210, 287–297
19. Ron, D., Luo, J., and Mochly-Rosen, D. (1995) J. Biol. Chem. 270, 24180–24187
20. Johnson, J. A., Gray, M. O., Chen, C.-H., and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24962–24966
21. Kaiser, N., Corcos, A. P., Tur-Sinai, A., Ariav, Y., and Cerasi, E. (1988) Endocrinology 123, 834–840
22. Nesher, R., Abramovitch, E., and Cerasi, E. (1985) Diabetologia 28, 233–236
23. Zawalich, W. S., Zawalich, K. C., Ganesan, S., Calle, R., and Rasmussen, H. (1991) Biochem. J. 279, 807–813
24. Arkhammar, P., Juntti, B. L., Larsson, O., Welsh, M., Nanberg, E., Sjoholm, A., Kohler, M., and Berggren, P. O. (1994) J. Biol. Chem. 269, 2743–2749
25. Ganesan, S., Calle, R., Zawalich, K., Greenawalt, K., Zawalich, W., Shulman, G. I., and Rasmussen, H. (1992) J. Cell Biol. 119, 313–324
26. Ashcroft, F. M., and Ashcroft, S. J. H. (1992) in Insulin: Molecular Biology to Pathology: a Practical Approach (Ashcroft, F. M., and Ashcroft, S. J. H., eds) pp. 97–139, Oxford University Press, London
27. Ammala, C., Ashcroft, F. M., and Rorsman, P. (1994) Nature 363, 356–358
28. Komatsu, M., Schermerhorn, T., Aizawa, T., and Sharp, G. W. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10728–10732
29. Ammala, C., Eliason, L., Bokvist, K., Berggren, P.-O., Honkanen, R. E., Sjoholm, A., and Rorsman, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4343–4347
30. Parker, R., and Cer, E. (1994) in Insulin Secretion and Pancreatic B-Cell Research (Flatt, P. P., and Lenzen, S., eds) pp. 411–419, Smith-Gordon, London
31. Zawalich, W. S., and Rasmussen, H. (1990) Mol. Cell. Endocrinol. 70, 119–137
32. Turk, J., Wolf, B. A., and McDaniel, M. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 9157–9161
33. Jones, P. M., and Persaud, S. J. (1993) J. Endocrinol. 137, 7–14