Protein Kinase C Isoform-specific Differences in the Spatial-Temporal Regulation and Decoding of Metabotropic Glutamate Receptor1a-stimulated Second Messenger Responses*

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Metabotropic glutamate receptors (mGluRs) coupled via Gq to the hydrolysis of phosphoinositides stimulate Ca\(^{2+}\) and PKC\(\beta\) oscillations in both excitable and non-excitable cells. In the present study, we show that mGluR1a activation stimulates the repetitive plasma membrane translocation of each of the conventional and novel, but not atypical, PKC isozymes. However, despite similarities in sequence and cofactor regulation by diacylglycerol and Ca\(^{2+}\), conventional PKCs exhibit isoform-specific oscillation patterns. PKCa and PKC\(\beta\) display three distinct patterns of activity: 1) agonist-independent oscillations, 2) agonist-stimulated oscillations, and 3) persistent plasma membrane localization in response to mGluR1a activation. In contrast, only agonist-stimulated PKC\(\beta\) translocation responses are observed in mGluR1a-expressing cells. PKC\(\beta\) expression also promotes persistent increases in intracellular diacylglycerol concentrations in response to mGluR1a stimulation without affecting PKC\(\beta\)II oscillation patterns in the same cell. PKC\(\beta\)II isoform-specific translocation patterns are regulated by specific amino acid residues localized within the C-terminal PKC V5 domain. Specifically, Asn-625 and Lys-668 localized within the V5 domain of PKC\(\beta\)II cooperatively suppress PKC\(\beta\)II-like response patterns for PKC\(\beta\)II. Thus, redundancy in PKC isoform expression and differential decoding of second messenger response provides a novel mechanism for generating cell type-specific responses to the same signal.

The spatial-temporal patterning of Ca\(^{2+}\) release from intracellular stores contributes to the regulation of a diverse array of cellular responses including insulin secretion, sustained activation of mitochondrial function, and the selective activation of transcription factors required for fine-tuning gene expression and differential decoding of second messenger signals. The synchronization of Ca\(^{2+}\) oscillations at least two modes of Ca\(^{2+}\) signaling: repetitive baseline Ca\(^{2+}\) spikes and sinusoidal-type Ca\(^{2+}\) oscillations (6). These Ca\(^{2+}\) oscillations are faithfully recapitulated by the repetitive redistribution of protein kinase C (PKC) between the cytosol and plasma membrane (7–10). The molecular mechanism(s) underlying the repetitive baseline plasma membrane translocation of PKC are best characterized for Group 1 metabotropic glutamate receptors (mGluRs). PKC\(\beta\)II and PKC\(\gamma\) oscillations are regulated by mGluR-stimulated oscillations in diacylglycerol (DAG), inositol 1,4,5-triphosphate (InsP\(_3\)), and Ca\(^{2+}\) release from intracellular stores (8, 9). Glutamate-activated Ca\(^{2+}\) and/or PKC oscillations are observed in most cell systems including immature neuronal cultures, developing neocortex, astrocytes, and mGluR-transfected heterologous cell cultures (8, 9, 11–14).

PKC isoforms are classified into three groups based on structural properties and cofactor requirements and exhibit specific in vivo activity as well as spatial organization within the cell (15–17). The activation and plasma membrane localization of the conventional PKC isoforms (\(\alpha, \beta, \beta', \gamma\), and \(\eta\)) is regulated by Ca\(^{2+}\) and DAG, whereas the activity and subcellular localization of the novel PKC isoforms (\(\epsilon\), \(\eta\), and \(\theta\)) is regulated by DAG, but not Ca\(^{2+}\) (17, 18). The atypical PKCs do not respond to either Ca\(^{2+}\) or DAG (17, 18). Although PKC structure/function has been studied extensively, it is unknown whether PKC subtype-specific differences in Ca\(^{2+}\)- and/or DAG-binding affinities contribute to PKC isoform-specific activity and/or spatial-temporal distribution within cells.

In the present study, we explore whether mGluR1a-stimulated oscillations in intracellular DAG and/or Ca\(^{2+}\) concentrations regulate conventional and novel PKC isoform activity in an identical manner. We find that, although all conventional and novel PKC isoforms oscillate in response to mGluR1a activation, conventional PKC isoforms exhibit isozyme-specific translocation response patterns that we classify as either PKC\(\beta\)- or PKC\(\beta\)II-like responses. Specifically, we have identified two discrete amino acid residues localized within the V5 domain of PKC\(\beta\)II that function to suppress PKC\(\beta\)II-like responses for PKC\(\beta\)II. Thus, structural differences in the PKC V5 domain allow conventional PKC isoforms to differentially decode and influence receptor-stimulated DAG and Ca\(^{2+}\) signals. Consequently, the expression of multiple conventional PKC isoforms in either the same cell or within different cells provides a novel mechanism by which cell type-specific responses to an identical signal may be established.

*This work was supported by Canadian Institutes of Health Research Grant MA-15506 (to S. S. G. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ Recipient of a Canadian Hypertension Society/CIHR fellowship.

** Recipient of Heart and Stroke Foundation of Canada MacDonald Scholarship, Premier’s Research Excellence Award, and Canada Research Chair in Molecular Neuroscience.

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The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; InsP\(_3\), inositol 1,4,5-triphosphate; mGluR, metabotropic glutamate receptor; HEK, human embryonic kidney; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; PHI, pleckstrin homology; PLC, phospholipase C.
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FIG. 1. Conventional GFP-tagged PKC isoform membrane translocation responses to mGluR1α activation. HEK 293 cells were transfected with cDNA encoding mGluR1α and either GFP-PKCα (A), GFP-PKCβI (B), GFP-PKCβII (C), or GFP-PKCγ (D). Shown are representative images selected from a time series of 200–400 laser scanning confocal microscopic images collected at 6.8–12.5 s intervals. The images demonstrate the repetitive translocation of GFP-PKCα (13/33 cells), GFP-PKCβI (10/27 cells), GFP-PKCβII (62/82 cells), and GFP-PKCγ (16/49 cells) proteins between the cytosol and plasma membrane in cells responding to persistent mGluR1α activation with 30 μM quisqualate.

MATERIALS AND METHODS

Materials—Restriction enzymes were obtained from Promega and New England Biolabs Inc. The pcDNA3.1/Amp expression vector was acquired from Invitrogen. DsRed2-C1, pEGFP-C1, pEGFP-C2, and pEGFP-C3 expression vectors were purchased from Clontech. The QuikChange™ Site-directed Mutagenesis kit was from Stratagene. The human universal Quick-Clone™ cDNA library was obtained from Clontech. Human embryonic kidney cells (HEK 293) were from American Type Culture Collection (ATCC). Fetal bovine serum was from HyClone Laboratories Inc. Gentamicin, minimal essential medium, and 0.05% Trypsin containing 0.5 mM EDTA were acquired from Invitrogen. The calcium indicator, Oregon Green 488 BAPTA-1 AM, was obtained from Molecular Probes. Quisqualate was from Tocris Cookson Inc. All other biochemical reagents were purchased from Sigma, Fisher Scientific, and VWR.

Plasmid Constructs—To construct EGFP-tagged PKCα, PKCβI, PKCγ, PKCδ, PKCa, PKCb, PKCc, PKCd, PKCe, and PKCf/α the cDNA for each of the PKC isoforms were first amplified by PCR from the human universal Quick-Clone™ library (Clontech). The PCR products generated were digested with the appropriate restriction enzymes and subcloned into the appropriate pEGFP-C1, pEGFP-C2, and pEGFP-C3 vectors (Clontech). The PKCβI cDNA was also cloned into the BglII/XhoI sites of the vector DsRed2-C1 (Clontech). The construction of EGFP-PKCβI (28), and EGFP-PLCγ1 PH domain were previously described (8). The EGFP-PKCβI C1 domain was a generous gift from Dr. Sergio Grinstein. PKCα/βIII, α/βII, and βII/βII chimeras were constructed by “two-step PCR” as described previously (19) and the resulting PCR products subcloned into either the pEGFP-C1 or pEGFP-C3 vector. PKCa and βI point and deletion mutants were constructed using the QuikChange™ Site-directed Mutagenesis kit (Stratagene). Sequence integrity of all PCR-generated products was confirmed by automated DNA sequencing.

Cell Culture and Transfection—HEK 293 cells were maintained in minimal essential medium supplemented with 10% (v/v) fetal bovine serum and 100 μg/ml gentamicin at 37 °C in a humidified atmosphere containing 5% CO2. Cells used in each of the experiments were transfected using a modified calcium phosphate method as described previously (20). Following transfection (~18 h), cells were incubated with fresh medium and allowed to recover 8 h and allowed to grow an additional 18 h before any experimentation. In all experiments, cells were transfected with 10 μg of pcDNA3.1 plasmid cDNA containing FLAG-mGluR1α with and without 1–5 μg of each PKC construct expressed in either pEGFP or DsRed2 expression vectors.

Confocal Microscopy—Following transfection with plasmid cDNAs encoding EGFP-PKC constructs and mGluR1α, cells were re-seeded on collagen-coated 15-mm glass-cover slips designed for use in a perfusion system (Warner Instrument Corporation). All experiments were conducted at 37 °C, and prior to visualization or additional treatments the cells were perfused with at least 5 ml of HEPES-buffered salt solution (1.2 mM KH2PO4, 5 mM NaHCO3, 20 mM HEPES, 11 mM glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, pH 7.4). Cellular InoP, and DAG levels were measured indirectly through the use of the EGFP-PLCγ1 PH domain (8) and EGFP-PKCδ C1 domain reporter constructs (9). Confocal-based Ca2+ imaging was performed by preloading cells for 30 min with 10 μM Oregon Green 488 BAPTA-1 AM prior to receptor activation according to the manufacturer's specifications (Molecular Probes). Confocal microscopy was performed on a Zeiss LSM-510 laser-scanning microscope using Zeiss 63× 1.4 numerical aperture oil immersion lens. Enhanced GFP and Oregon Green 488 BAPTA fluorescence was visualized with excitation at 488 nm and emission 515–540 nm emission filter set. DsRed2 fluorescence was visualized with excitation at 543 nm and emission 590–610 nm filter set. Fluorescent signals were collected sequentially every 6.8–12.5 s using the Zeiss LSM software time scan function.

Data Analysis—PKC translocation time courses and Ca2+, DAG, and InoP responses were recorded as time series of 150–300 confocal images for each experiment. Image analysis was performed using the Zeiss LSM-510 physiology analysis software and was defined as the relative change in cytoplasmic fluorescence intensity over time in a 5-μm-diameter region of interest. All time course data were plotted using GraphPad Prism. The statistical significance of the data presented in Fig. 6 was analyzed using a non-parametric SIGN test.

RESULTS

PKC Isozyme-specific Plasma Membrane Translocation Responses—Because individual conventional (α, β, βIII, and γ), novel (δ, ε, η, and θ), and atypical (α/ and γ) PKC isoforms may exhibit differences in their receptor-activated translocation profiles, we have examined the translocation response patterns for each PKC isoform following mGluR1α agonist activation. We find that each of the conventional PKC isoforms displays the capacity to oscillate between the cytosol to the plasma membrane in response to mGluR1α activation. Shown in Fig. 1 is an agonist-stimulated oscillatory response pattern that is exhibited by all four conventional PKC isoforms. This agonist-stimulated transient, but repetitive, translocation of enzyme to the plasma membrane is the only response pattern that is observed for PKCβII (PKCβII-like). However, PKCa and PKCβ display additional translocation responses (PKCβII-like) that are never observed for PKCβII. Although some cell to cell variability is observed, PKCβII-like translocation patterns can
be categorized into three distinct patterns that are illustrated in Fig. 2. First, GFP-PKCa and GFP-PKCB exhibit the capacity to oscillate between the cytosol and plasma membrane in the absence of mGluR1a activation (Fig. 2A). In these cells, mGluR1a agonist activation serves to increase the oscillation frequency of the entire cellular complement of GFP-tagged PKCa and PKCB (Fig. 2A). The removal of the agonist results in the return of the oscillation frequency to the pre-agonist-stimulated oscillatory rate (Fig. 2A, PKCa). These agonist-independent oscillations are only observed following mGluR1a expression, indicating that they occur in response to spontaneous mGluR1a activity. Second, in response to mGluR1a activation, the entire pool of either GFP-PKCa or GFP-PKCB translocates from the cytosol to the plasma membrane where it remains persistently localized (at steady state) until the agonist is removed by perfusion (Fig. 2B). Third, mGluR1a activation stimulates the translocation of the entire GFP-PKCa and GFP-PKCB pools to the plasma membrane, and a fraction of the enzyme returns to the cytosol and subsequently oscillates in the presence of the agonist (Fig. 2C). PKCγ also exhibits very weak PKCβI-like responses (13/49 cells), but unlike what is observed for the other conventional PKC isoforms the most common PKCγ response pattern is a single transient plasma membrane translocation (20/49 cells) (data not shown). For PKCa, PKCβI, and PKCβII transient translocation responses were rarely observed (< 5% of cells imaged). Taken together, these observations suggest that PKCa, PKCβI, and PKCγ exhibit the capacity to decode subtly different changes in second messenger responses that are not recognized by PKCβII. Alternatively, the expression of PKCa, PKCβI, and PKCγ may lead to multiple distinct second messenger responses to mGluR1a activation.

Each of the novel PKC isoforms also oscillate in response to mGluR1a activation (Fig. 3). The response for each of the novel PKC isoforms is similar to the third PKCβI-like response pattern outlined above (Fig. 2C). Following the agonist-dependent translocation of the entire pool of GFP-PKC, a fraction of each of the novel GFP-PKC isoforms remains localized to the plasma membrane, and the remaining enzyme oscillates in the continuous presence of agonist (Fig. 3). The atypical PKC (α and ζ) do not respond to mGluR1a activation (data not shown). Because the conventional PKCs exhibit the greatest behavioral complexity and diversity to mGluR1a activation, we focused subsequent experimentation on the conventional PKC isoforms.

PKCβI-dependent Alterations in mGluR1a-stimulated Second Messenger Responses—It is possible that the expression of either PKCa or PKCβI leads to alterations in the patterning of mGluR1a-stimulated second messenger responses and that this may underlie the multiplicity of PKCβI-like response patterns. To address this possibility, we examined the patterning of red fluorescent protein tagged-PKCβI (DsRed2-PKCβI) responses at the same time as we measured changes in intracellular DAG, InsP3, and Ca2+ concentrations.

DAG responses were measured using a GFP-PKCa C1 domain construct, which translocates from the cytosol to the plasma membrane in response to increases in intracellular DAG concentrations (9). We found that mGluR1a-stimulated GFP-PKCa C1 domain translocation patterns were synchronized exactly with DsRed2-PKCβI membrane translocation responses (Fig. 4A). Following mGluR1a activation, the GFP-PKCa C1 domain either oscillated between the cytosol and plasma membrane in synchrony with DsRed2-PKCβI (Fig. 4A, upper panel) or accumulated with DsRed2-PKCβI at the plasma membrane (Fig. 4A, lower panel). InsP3 responses were measured using a GFP-PLCβ1 PH domain construct, which under basal conditions was localized at the plasma membrane due to its interaction with membrane phosphatidylinositol 4,5-bisphosphate (PIP2) (8). Following mGluR1a-stimulated PIP2 hydrolysis and InsP3 formation the GFP-PLCβ1 PH domain was released from the plasma membrane and redistributed to the cytosol (Fig. 4B, upper panel). Identical to what was observed for DAG responses, the GFP-PLCβ1 PH domain either oscillated between the plasma membrane and cytosol at the same frequency at which DsRed2-PKCβI translocated from the cytosol to plasma membrane (Fig. 4B, upper panel), or the GFP-PLCβ1 PH domain accumulated in the cytosol with the same time course as DsRed2-PKCβI accumulated at the plasma membrane (Fig. 4B, lower panel). Thus, the
spatial-temporal localization of PKCβI at the plasma membrane was coordinated with alterations in both intracellular DAG and InsP₃ concentrations. Changes in intracellular Ca²⁺ concentrations were measured using the Ca²⁺ indicator dye Oregon Green 488 BAPTA-1 AM. We found that oscillatory DsRed2-PKCβI responses were synchronized with Ca²⁺ oscillations (Fig. 4C, upper panel). However, unlike what was observed for DAG and InsP₃ responses, Ca²⁺ oscillations persisted when DsRed2-PKCβI was persistently localized to the plasma membrane (Fig. 4C, lower panel).
Taken together, our observations indicate that expression of PKCβI alters the patterning of DAG and InsP₃ formation, but not the patterning of Ca²⁺ release from intracellular stores in response to mGluR1α activation. This is different from cells expressing PKCβII where only synchronized oscillatory DAG, InsP₃, and Ca²⁺ responses are observed following mGluR1α activation (data not shown and Ref. 8).

**Effect of PKCβI Expression on PKCβII Plasma Membrane Translocation Responses**—PKCβI and PKCβII isoforms are thought to be regulated in the same manner by DAG (17). Therefore, if differences in PKCβI versus PKCβII response patterns are solely the consequence of PKCβI expression-dependent alterations in DAG formation or differences in mGluR1α expression levels between cells, GFP-PKCβII should exhibit PKCβI-like translocation patterns in cells co-expressing DsRed2-PKCβI. When co-expressed together in HEK 293 cells, we observe two distinct DsRed2-PKCβI and GFP-PKCβII responses to mGluR1α activation: 1) DsRed2-PKCβI and GFP-PKCβII exhibit synchronized oscillatory plasma membrane translocation responses (Fig. 5A); and 2) DsRed2-PKCβI accumulates at the plasma membrane, whereas GFP-PKCβII continues to oscillate between the plasma membrane and cytosol (Fig. 5B). These observations suggest that, although PKCβI expression alters the patterning of mGluR1α-stimulated DAG and InsP₃ response patterns, PKCβII is apparently insensitive to PKCβI-induced changes in DAG formation. Moreover, the differences in PKCβI versus PKCβII membrane translocation patterns observed in the same cell indicate that differences in mGluR1α expression levels between cells cannot account for the different PKCβI-like translocation patterns.

**Molecular Determinants for Isozyme-specific Translocation Response Patterns**—The patterning of conventional PKC isoform responses to mGluR1α activation are sub-classified as either PKCβI-like (agonist-independent oscillations, agonist-stimulated oscillations, and persistent plasma membrane localization) or PKCβII-like (only agonist-stimulated oscillations). We have used these definitions to characterize the structural determinants underlying differences in conventional PKC isoyme response patterns. PKCβI and PKCβII differ by only 53 amino acids comprising the alternatively spliced V5 domains of the kinases (Fig. 6A). Furthermore, the exchange of the last 54 amino acid residues of PKCα with the corresponding residues from PKCβII generates a PKCα/βII 620–673 chimera that displays a PKCβII-like response pattern (Fig. 6B). Thus, PKC isoyme-specific response patterns must be regulated by amino acid residues localized within V5 domains of conventional PKC isoforms.

Sequence alignment of PKCα, PKCβI, and PKCβII reveals considerable carboxyl-terminal sequence conservation (Fig. 6A). The last 13 amino acids of the V5 domain exhibit the greatest sequence disparity (Fig. 6A). By swapping either the last 13 amino acids of PKCβII for the last 15 amino acid residues of PKCα (PKCβII/α 657–672) or deleting the last 13 amino acid residues from the carboxyl-terminal of PKCβII (PKCβII–S660D), we create PKCβII chimeras with PKCβI-like response patterns (Fig. 6B). Serial truncation analysis of PKCβII between amino acid residues 660 and 672 reveals that PKCβII-like responses are lost if the final six (PKCβII–L667Δ) but not the final three (PKCβII–E670Δ) PKCβII amino acids are deleted (Fig. 6C). The deletion of Lys-668–Glu-670 (PKCβII–KPEΔ) from PKCβII also establishes a PKCα-like response pattern for PKCβII (Fig. 6C). The mutation of Lys-668, Pro-669, and Glu-670 individually to glycine residues reveals that only Lys-668 is required to maintain PKCβII-like responses and to suppress PKCβI-like response patterns (Fig. 6D). When expressed together in HEK 293 cells, we find that DsRed2-PKCβI and GFP-PKCβII–K668G exhibit identical response patterns to mGluR1α activation (Fig. 7).

We found that the establishment of a PKCβII-like response pattern in PKCα required the exchange of the entire PKCβII V5 domain. Furthermore, neither the exchange of the last 13 amino acid residues from PKCβII into PKCα (PKCα/βII 660–673) nor the introduction of the KPE motif into PKCβII established PKCβII-like responses for either PKCα or PKCβI (Fig. 6, B and C). Therefore, there must be additional amino acid residues localized within the PKCβII V5 domain that cooper-
FIG. 6. Structure-Function Analysis of V5 domain residues regulating PKC isoform response patterns. A, schematic representation of the domain structure for conventional PKC isoforms and the alignment of the amino acid sequences of the V5 domains from PKC\(\alpha\), PKC\(\beta\)I, and PKC\(\beta\)II. Non-conserved amino acid residues are boxed. B–D, characterization of the translocation patterns of GFP-PKC\(\alpha\), GFP-PKC\(\beta\)I and GFP-PKC\(\beta\)II chimeras, truncation and point mutations in mGluR1a-expressing cells in either the absence or presence of agonist. The response patterns assessed were plasma membrane oscillations in either the absence (−) or presence (+) of agonist and persistent plasma membrane translocation. The last 13 PKC\(\beta\)II amino acids are underlined. Asterisks indicate PKC\(\beta\)II mutants displaying differences in translocation pattern compared with the expected wild-type PKC\(\beta\)II isoform response patterns. A non-parametric SIGN test, \(p > 0.07\) supports the null hypothesis that the asterisked PKC\(\beta\)II mutants exhibit no differences in oscillation patterns with the expected patterns observed for PKC\(\alpha\).

FIG. 7. Synchronization of GFP-PKC\(\beta\)II K668G and DsRed2-PKC\(\beta\)I responses. HEK293 cells were transfected with cDNA encoding mGluR1a, DsRed2-PKC\(\beta\)I, and GFP-PKC\(\beta\)II K668G. Shown are: synchronized agonist-stimulated DsRed2-PKC\(\beta\)I and GFP-PKC\(\beta\)II translocation responses (8/14 cells) (A), synchronized agonist-independent DsRed2-PKC\(\beta\)I and GFP-PKC\(\beta\)II oscillations that increase in frequency following the treatment of cells with 30 mM quisqualate (3/14 cells) (B), and persistent localization of both DsRed2-PKC\(\beta\)I and GFP-PKC\(\beta\)II K668G at the plasma membrane in response to agonist stimulation (3/14) (C).

In PKC\(\alpha\) and found that only PKC\(\beta\)II-N625G exhibited PKC\(\alpha\)-like behavior patterns (Fig. 6D). In summary, extensive structure-function analysis identified Asn-625 and Lys-668 as essential amino acid residues within the PKC\(\beta\)II V5 domain required for the establishment of PKC\(\beta\)II translocation responses. The mutation of either residue releases the suppression of PKC\(\alpha\)-like response behaviors for PKC\(\beta\)II.

DISCUSSION

In the present study, we show that each of the conventional (\(\alpha\), \(\beta\), \(\beta\)I, and \(\gamma\)) and novel (\(\delta\), \(\varepsilon\), \(\eta\), and \(\theta\)) PKC isoforms respond to mGluR1a activation by repetitively translocating between the cytosol and plasma membrane. The detailed analysis of conventional PKC isoform translocation responses reveals that PKC\(\alpha\) and PKC\(\beta\)I exhibit a variety of unique response patterns to mGluR1a activation that are not observed for PKC\(\beta\)II. Because PKC\(\beta\)I and PKC\(\beta\)II exhibit distinct translocation patterns to the same stimulus, even when expressed in the same cell, it is unlikely these differences can be attributed to differences in mGluR1a expression levels or G protein coupling efficiency. Rather PKC\(\alpha\) and/or PKC\(\beta\)I expression alters the patterning of mGluR1a-stimulated second messenger responses. Furthermore, the sensitivity of conventional PKC isoforms to intracellular DAG and \(\text{Ca}^{2+}\) concentrations appears to be regulated by residues localized to the V5 domains of the kinases. Thus, we conclude that the spatial-temporal dynamics of mGluR signaling will not only be determined by the identity of the mGluR isoform that is activated but will also be controlled by the PKC isoform that is decoding and modulating the mGluR1a-generated second messenger signals. As a consequence, the expression of multiple conventional PKC isoforms in either the same cell or within different cells provides a novel mechanism by which cell type-specific responses to an identical signal may be established.

Both PKC\(\alpha\) and PKC\(\beta\)I exhibit agonist-independent oscillations in cells expressing mGluR1A, and this may be related to the observation that mGluR1A exhibits significant basal activity in the absence of agonist (21, 22). Intrinsic mGluR1A activity leading to the spontaneous activation of PLC may result in sub-threshold changes in DAG and InsP\(_3\) formation. If PKC\(\alpha\) and PKC\(\beta\)I exhibit heightened sensitivity to changes in intracellular DAG concentrations as compared with PKC\(\beta\)II, this...
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may be translated into isoform-specific agonist-independent membrane translocation. The crystal structure of the mGluR ligand-binding domain predicts that in the absence of agonist the ligand-binding domain exists in equilibrium between active and inactive conformations (23). Agonist binding likely stabilizes the active (closed) conformation of the ligand-binding domain at equilibrium, which is then translated as an increase in both the efficacy and frequency of G protein coupling. Similarly, the mutation of an aspartic acid residue at position 854 in the G protein-coupling domain of mGluR1a to an alanine residue creates a receptor that gains the capacity to drive agonist-independent PKCβII oscillations (8). Conversely, in cells expressing mGluR1b, a mGluR1 splice variant that exhibits reduced spontaneous G protein coupling activity (21, 24), PKCα and PKCβII do not exhibit agonist-independent oscillations, but retain the capacity to both oscillate and accumulate at the cell surface in response to mGluR1a activation (data not shown). Taken together, these observations suggest that agonist-independent PKCα and PKCβI oscillations are driven by basal mGluR1a activity.

Unlike PKCβII, PKCα and PKCβI exhibit the capacity to be persistently localized at the plasma membrane in response to mGluR1a activation, and this response persists until the cells are perfused with agonist-free medium. Persistent PKCα translocation responses have also been reported previously (25) in response to thyrotropin-releasing hormone receptor activation. Constitutive plasma membrane localization of PKCβII can also be achieved following the mutation of two autophosphorylated amino acid residues, Thr-641 and Ser-660, to alanine residues or by treating cells with PKC inhibitors (8, 26-28). In contrast, the autophosphorylation of equivalent residues within the carboxyl-terminal variable domain of PKCα prolongs its activation (29, 30), which may account in part for the persistent localization of the enzyme at the plasma membrane in response to mGluR activation. However, the persistent localization of PKCα and PKCβII at the plasma membrane cannot be completely explained by PKC subtype-regulated differences in autophosphorylation, because the spatial-temporal dynamics of DAG and InsP3 formation are also altered in these cells. One potential explanation for the observed changes in DAG and InsP3 formation in PKCα and PKCβII-expressing cells is that the enzymes participating in the regulation of intracellular DAG levels may serve as PKC isoform-specific substrates and that the V5 domain may control substrate-specificity. Consistent with this idea, the activation of endogenous conventional PKC with thymeletoxin results in PLCβ2 phosphorylation and attenuation of oxytocin receptor-stimulated phosphatidylinositol turnover (31). However, it is unknown which conventional PKC isoforms contribute to the phosphorylation-dependent inactivation of PLC. An alternative explanation may involve the differential ability of conventional PKC isoforms to associate with membrane anchoring proteins due to structural differences in their V5 domains. For example, the association of PKCβII with receptor for activated C kinase 1 (RACK1) is regulated by three regions within the V5 domain of PKCβII (32), and two of these regions encompass the amino acid residues (Asn-625 and Lys-668) that regulate PKCβII translocation patterns. The relative contributions of PKC autophosphorylation, PLC phosphorylation, and membrane anchoring proteins to the regulation of PKC subtype-specific translocation patterns will require extensive future study.

An important observation made in the present study is that PKCβII and PKCβIII exhibit distinct activation patterns even when expressed in the same cell. These differences in activity are abolished by either the mutation of Asn-625 or Lys-668 in PKCβII. These two residues appear to cooperate with one another to repress PKCa-like response patterns for PKCβIII. This indicates the amino acid composition of the V5 domains of otherwise identical PKC isoforms regulates the relative sensitivity of the enzymes to both changes in intracellular DAG and Ca2+ concentrations. Previously, Keranen and Newton (33) demonstrated that the PKCβII and PKCβIII V5 domains regulate differences in the enzymes Ca2+-dependent affinity for acidic membranes. Thus, the PKCβII-like versus PKCβIII-like response patterns may reflect V5 domain-regulated differences in both DAG and Ca2+ affinity. Thus, the periodicity of activation for different conventional PKC isoforms is not only regulated by the duration and strength of DAG and Ca2+ signals, but is also determined by the relative sensitivity of the kinases to alterations in intracellular DAG and Ca2+ concentrations.

In summary, we have discovered that homologous PKC isoforms, even when expressed in the same cell, display distinct patterns of activation in response to the same receptor stimulus. This observation is of fundamental importance to our understanding of cell signaling and clearly illustrates that the expression of multiple kinase isoforms in the same cell does not result in redundancy of cellular function. Rather, our results provide concrete evidence that the stimulation of a single receptor subtype, in a single cell, has the potential to activate distinct patterns of PKC isozyme activation, which may be translated into distinct cellular responses. We speculate that this may be particularly important in the developing and adult nervous system where Ca2+ spikes and PKC translocation responses are linked to both synapse formation and synaptic plasticity required for memory and learning (34, 35).

Acknowledgments—We thank Drs. S. J. D’Souza, L. Dagnino, R. F. Roldan, J. R. Rylett, and M. W. Salter for critical reading of the manuscript. We also thank Dr. M. Taljaard for help with statistical analysis.

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