DANGER, a Novel Regulatory Protein of Inositol 1,4,5-Trisphosphate-Receptor Activity*

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We report the cloning and characterization of DANGER, a novel protein which physiologically binds to inositol 1,4,5-trisphosphate receptors (IP3R). DANGER is a membrane-associated protein predicted to contain a partial MAB-21 domain. It is expressed in a wide variety of neuronal cell lineages where it localizes to membranes in the cell periphery together with IP3R. DANGER interacts with IP3R in vitro and co-immunoprecipitates with IP3R from cellular preparations. DANGER robustly enhances Ca2+-mediated inhibition of IP3R Ca2+ release without affecting IP3 binding in microsomal assays and inhibits gating in single-channel recordings of IP3R. DANGER appears to allosterically modulate the sensitivity of IP3R to Ca2+ inhibition, which likely alters IP3R-mediated Ca2+ dynamics in cells where DANGER and IP3R are co-expressed.

The inositol 1,4,5-trisphosphate receptor (IP3R)3 is a large, endoplasmic reticulum (ER) resident protein, which is a key regulator of intracellular Ca2+ signaling (1, 2). Inositol 1,4,5-trisphosphate (IP3) is formed in response to the activation of G protein-coupled receptors or receptor tyrosine kinase receptors located in the plasma membrane (1), which elicit IP3R-mediated Ca2+ release from ER stores.

The IP3 recognition site of IP3R includes amino acids (aa) 225–578 in the N-terminal portion of the protein, while the Ca2+ channel domain comprises ~300 aa in the extreme C terminus (2). The IP3 binding site and the Ca2+ channel are separated by ~2,000 aa, providing a large area for interactions with multiple regulatory proteins including calmodulin, chromogranins, glyceraldehyde-3-phosphate dehydrogenase, RACK1, and caldendrin (3). While these proteins regulate IP3R function in diverse ways, only two regulators have been shown to influence Ca2+ sensitivity. Cytochrome c, which binds to the extreme C terminus of the IP3R, relieves the inhibitory actions of Ca2+ upon the channel (4), and Bcl-XL binding to the C terminus also influences the Ca2+ dependence (5).

We have identified a novel vertebrate protein, designated DANGER, which was isolated by yeast two-hybrid analysis with the regulatory region of the IP3R as bait. DANGER physiologically binds to IP3R and allosterically enhances the potency of Ca2+-inhibition of IP3R-mediated Ca2+ release without affecting ligand binding.

**EXPERIMENTAL PROCEDURES**

_Yeast Two-hybrid Analysis_—The Matchmaker3 yeast-2-hybrid system from Clontech (Palo Alto, CA) was employed. AH109 β-galactosidase yeast was used. IP3R fragments were cloned into pGADT7 (β-galactosidase acceptor domain) vector and were screened against a rat brain and human fetal kidney library (Clontech) as per the manufacturer’s specifications. Expression of these fragments was determined by Western blotting using antibodies from Clontech, corresponding to the expression vector. Positive clones grew on minimal SD agar (Clontech) lacking adenine, histidine, leucine, and tryptophan and had β-galactosidase activity.

_Calcium Release Measurements and Electrophysiology_—Calcium release through recombinant type I (SII+) IP3R was measured exactly as described previously (13, 14). Calcium concentrations in all solutions were calibrated with a calcium-selective minielectrode, and confirmed with fluorescent dyes. When the effects of DANGER were examined, it was added simultaneously with IP3. In the absence of IP3, SERCA activity was not affected by the addition of DANGER. At the concentrations used in this study, DANGER did not change the calcium concentration of the assay solution as determined by a calcium minielectrode.

_Spodoptera frugiperda_ (Sf9) cells (Invitrogen) were grown and maintained in SF-900I serum-free media (Invitrogen) as described previously (9). An ice-cold nuclear isolation solution, containing (in mm): 140 KCl, 250 sucrose, 1.5 β-mercaptoethanol, 10 Tris-HCl (pH to 7.4), with complete protease inhibitor mixture (Roche Applied Science) and 0.05 mm phenylmethylsulfonyl fluoride (PMSF).
DANGER Modulates IP₃R Ca²⁺ Dependence

A

IP₃R: Trypsin and Y-2-H bait fragments

0 500 1000 1500 2000 2749 a.a

S1 S2

N PKA G ATP Ca²⁺

I II III IV V

Trypsin Fragments

1-346 346-923 923-1662 1662-1932 1932-2276 2609-2749

B

coomassie

188 → 188

? -IP₃R Type 3

188

98

Beads alone

GST-pulldown

Load

Beads alone

Beads alone

Blot: IP₃R

Blot: GST

DANGERS: Conserved Domains

Coiled-Coiled Domains

partial

1 547

Signal Peptide

MAB-21

C

+myc DANGER +myc DANGER

PC12 lysate

PC12 lysate

α-DANGER α-myc

+ control + control

siRNA DANGER + siRNA DANGER

α-DANGER α-actin

Antibody peptide: a.a. 496-514 RAEPNLFRPFVLQR SLYR

D

IP IP₃R

IP DANGER -control

α-IP₃R α-DANGER

Subcellular Fractionation
PC12 cells

E

P1 P2 P3 S3

188 188

98 98

65 65

49 49

P1 P2

50/10 kDa

28 28
sulfonyl fluoride, was added to the flask, and the cells were detached by gentle scraping. Homogenization of 1–2 ml of the mixture was performed using 2–4 strokes of the pestle in an ice-cold Dounce homogenizer. 20–30 µl of the homogenized mixture was added to 1 ml standard bath solution (in mM: 140 KCl, 10 HEPES, 0.5 BAPTA, pH 7.3, and free [Ca\(^{2+}\)] adjusted to \(\sim 300 \text{nM}\) in an experimental chamber on the stage of an inverted microscope. Isolated nuclei are 5–10 µM in diameter and were distinguished from intact cells based on their unique morphology (9). The standard pipette solution contained (in mM): 140 KCl, 0.5 Na\(_2\)ATP, 10 HEPES, pH 7.3, 0.07 mM Ca\(^{2+}\), and 100 nM InsP\(_3\). All solutions were carefully buffered to desired free [Ca\(^{2+}\)] (8) confirmed by fluorometry. Data were acquired as described (8). Segments of current traces exhibiting [Ca\(^{2+}\)] and 1 mM free InsP\(_3\) were cultured in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum, 5% horse serum, and 2 mM l-glutamine, and 1% penicillin-streptomycin. PC12 cells were cultured in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 1% penicillin-streptomycin.

Expression Constructs—The type 1 IP\(_3\)R SII+ splice variant and SERCA-2b in pcDNA.3 have been described elsewhere (17) and were kindly provided by Dr. Suresh K. Joseph (Thomas Jefferson University, Philadelphia, PA). DANGER was cloned from human, rat, and mouse cDNA libraries generated from brain using the Roche reverse transcriptase. PCR was performed using 5’ and 3’ 21-mer primers corresponding to the following sequences human (GI: 29789287), Mouse (GI: 39645725), and Rat (GI: 304666774), which have 9-mer overlaps on each end to engineer the appropriate restriction sites. Two siRNA sequences were used for DANGER deletion, each with similar efficacy: 5’-aagaatgccccagcgctcatt-3’ (human) and 5’-aaatatcaggtgagcgtctg-3’ (rat).

Antibody Generation—Rabbit polyclonal antiserum against DANGER was generated by injecting New Zealand White male rabbits with peptide RAPELNI4FPPFLVQR SLYR coupled to keyhole limpet hemocyanin (Pierce). Initial injection was with Complete Freund’s adjuvant, and boost injections at days 14, 21, and 49 were with Incomplete Freund’s Adjuvant. Rabbit injections, bleeds, and housing were performed by Cocalico Biologicals (Reamstown, PA).

Monoclonal type 1 IP\(_3\)R antibody m18A10 was a kind gift from M. Mikoshiba. Anti-Myc antibody was from Sigma.

Subcellular Fractionation—After inducing differentiation by NGF, cells were harvested by gently scraping plates with a cell scraper, and were washed once with cold PBS. The washed pellet was subjected to one freeze-thaw cycle in liquid nitrogen. Pellets were resuspended in 1 ml of Buffer A (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, one protease inhibitor pill). Cells were then homogenized on ice using a 1-ml glass Dounce homogenizer with a tight fitting pestle until \(\sim 95\%\) of cells were disrupted as indicated by trypan blue staining. Crude lysates were centrifuged at 1,000 \(\times g\) for 15 min at 4°C to remove nuclei and unbroken cells. The supernatant was collected and the pellet (P1) discarded. The low speed supernatant was then subjected to 10,000 \(\times g\) centrifugation for 15 min, which yielded the 10,000 \(\times g\) pellet (P2). The supernatant from the P2 pellet was centrifuged at 15,000 \(\times g\) to completely rid the supernatant of any remaining mitochondria. Finally the 15,000 \(\times g\) supernatant was separated into cytosol (S3) and light membrane (P3) fractions by centrifugation at 100,000 \(\times g\) for 1 h. The 100,000 \(\times g\) supernatant was collected as the S3 fraction, and the pellet was resuspended in 40–70 µl of Buffer A. P2 was washed twice by resuspending cells in 100 µl of Buffer A and pelleting (10,000 \(\times g\)) for 15 min. After the final wash, P2 was resuspended in 50–100 µl of Buffer A. All fractionations were repeated a minimum of four times with essentially identical results. Subcellular fractions were characterized using nucleoporin (P1, nuclear), cytochrome c oxidase (P2, mitochondria, heavy ER), heme oxygenase 2 (P3, light ER), and lactate dehydrogenase (S3, cytosol).

Co-immunoprecipitation—Cell lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.8, 1% Triton, 1 mM EDTA) was added to 100 µg of cell lysate to bring samples to a total volume of 500 µl. Samples were cleared of insoluble debris by centrifugation at 10,000 \(\times g\). Anti-IP\(_3\)R-1, pre-immune sera, mouse IgG, or anti-DANGER antibodies, and protein A-Sepharose beads were added and incubated on a rotator for 30 min at 4°C. The protein A-Sepharose beads were washed three times with lysis buffer and quenched with 20 µl of SDS sample buffer. Co-immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot analysis.
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Immunofluorescence and Immunohistochemistry—PC12 cells were grown on coverslips for 56 h then fixed with 0.4% paraformaldehyde in 2× PBS for 30 min and washed three times in 2× PBS for 15 min. Coverslips were quenched in 50 mM NH₄Cl for 15 min and washed three times in 2× PBS for 15 min. Cells were permeabilized in saponin solution (2× PBS, 1% bovine serum albumin, 1% goat serum, and 0.075% w/v saponin) for 1 h. Coverslips were washed three times in 2× PBS for 15 min and subsequently inverted on 250 μl of anti-DANGER and IP₃R in a wet chamber overnight at 4°C. Coverslips were washed three times in 2× PBS for 15 min and inverted on 250 μl of secondary antibody solution 1:300 at room temperature. Finally, coverslips were washed three times in 2× PBS for 15 min and mounted onto slides with Slo-fade. Fluorescence signals were detected with a Zeiss LSM410 confocal laser scanning microscope, using a 63×/NA 1.4 objective.

Formalin-fixed, paraffin-embedded 5 μm sections were de-waxed, rehydrated, and subjected to antigen retrieval by incubation in 10 mM sodium citrate, pH 6.0, at 98°C for 20 min. Sections were then immunostained using the Vectastain™ ABC system according to the manufacturer instructions using affinity purified anti-DANGER antibody described above at a dilution of 1/50.

Antibodies and Reagents—Plasmids were from the following sources: EYFP, Matchmaker®, and Myc-CMV vector cDNA from CLONETECH and human M5 muscarinic receptor cDNA from L. Birnbaumer (National Institutes of Health). Human Northern blot from Clontech (Palo Alto, CA). Carbachol, protein A-agarose, GST-agarose, Sigma. Fura-2/AM was from Molecular Probes (Eugene, OR). UTP was from Invitrogen. siRNA duplex was from Qiagen (Valencia, CA). Ca⁴⁺ was from Amersham Biosciences.

RESULTS AND DISCUSSION

We conducted a yeast two-hybrid analysis using multiple fragments of IP₃R as described previously (6) (Fig. 1A). A novel interacting sequence, corresponding to a previously uncharacterized expressed sequence tag designated KIAA1754 (7), bound to aa 923–1581 fragment of IP₃R. This expressed sequence tag, which we now denote as DANGER, maps to a human genomic sequence on chromosome 10q25.1 containing expressed sequence tag designated KIAA1754 (7), which we now denote as DANGER, maps to a human genomic sequence on chromosome 10q25.1 comprising a single coding exon (4.3 kb). The open reading frame codes for a protein of 547 aa and is predicted to contain several coiled-coil regions, an N-terminal signal peptide for ER/plasma-membrane retention, and a partial MAB-21 domain (Fig. 1A, schematic).

Direct binding of DANGER to full-length IP₃R was detected in vitro by co-immunoprecipitation with both overexpressed and endogenous DANGER proteins. We observe robust interactions between a GST-DANGER fusion protein and IP₃R (type 1 and type 3) from cell lysates as well as purified type 1 IP₃R from rat cerebellum (Fig. 1B). Binding to type 2 IP₃R could not be confirmed as the type2 IP₃R antibodies we employed failed to demonstrate immunoreactivity to the samples assayed. To study endogenous DANGER, we developed a polyclonal antibody against C-terminal aa 496–514 of DANGER (Fig. 1C). Using a Myc-tagged DANGER expression construct in PC12 cells, the polyclonal antibody recognizes both endogenous and over-expressed DANGER at the same molecular weight (Fig. 1C) as shown by anti-Myc staining of overexpressed DANGER. Western analysis of PC12 cell lysates blotted with polyclonal anti-DANGER reveals a single major band (59 kDa) corresponding to the full-length protein as well as two other bands of lower intensity (49 and 41 kDa). To ensure the specificity of the antibody, we employed DANGER-specific siRNA to deplete endogenous DANGER from PC12 cells (Fig. 1C). The siRNA abolishes DANGER immunoreactivity indicating that the three bands observed by our antibody arise from the same mRNA. Next, we tested the interaction of endogenous DANGER and IP₃R in intact cells. We observed co-immunoprecipitation of DANGER with IP₃R using either our polyclonal antibody to DANGER or a monoclonal anti-IP₃R type 1 (right). Bottom: higher magnification image of Purkinje cells showing co-localization of DANGER and the type1 IP₃R.

FIGURE 2. Endogenous DANGER co-localizes with IP₃R. A–C, immunofluorescence of type I IP₃R (red) and DANGER (green) in PC12 cells treated with 10 ng/ml NGF for 2 days. Merged image demonstrates co-localization of DANGER with the IP₃R near the plasma membrane. C1 and C2, magnification of the cell body and neurites. Discrete regions of co-localization can be observed. D, top: sequential sagittal sections of mouse cerebellum probed with anti-DANGER (left) and polyclonal anti-IP₃R type1 (right). Bottom: higher magnification image of Purkinje cells showing co-localization of DANGER and the type1 IP₃R.
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DANGER, which has a high degree of co-localization with type 1 IP₃R (54% ± 8% concurrence in 50 cells measured) (Fig. 2, A–C). We also observe a small amount of DANGER staining in the nucleus, consistent with the results from the subcellular fractionation. Higher magnification reveals substantial co-localization of DANGER with type 1 IP₃R near the plasma membrane (Fig 2C1). DANGER and type 1 IP₃R also co-localize in NGF-induced neurites to a similar extent as in the cell body (46% ± 11%) (Fig. 2C2). DANGER is concentrated in cerebellar Purkinje cells, which contain the highest densities of IP₃R in the brain (Fig. 2D). Whereas cerebellar immunoreactivity for IP₃R is confined to Purkinje cells, DANGER is also concentrated in basket cells. DANGER is also expressed throughout the body in numerous terminally differentiated cell types including neurons, neuroendocrine cells, crypt cells, muscle cells, B-cells, and T-cells (data not shown).

To ascertain a possible modulatory role of DANGER on IP₃R function, we monitored IP₃-induced release of Ca²⁺ from microsomal preparations of COS7 cells transfected with type 1 IP₃R and SERCA2b (Fig. 3, A–C) as described previously (4). Compared with GST alone (black), incubation with purified GST-tagged DANGER (500 nM) has little effect on IP₃-induced Ca²⁺ release in the presence of 300 nM Ca²⁺ over a wide range of IP₃ concentrations (Fig. 3A). In addition, overexpression or depletion (siRNA) of DANGER does not alter IP₃ production in response to muscarinic and purinergic receptor stimulus (data not shown).

At low Ca²⁺ (~150–200 nM) concentrations, Ca²⁺ enhances IP₃R channel activity, while supraphysiologic concentrations of Ca²⁺ inhibit IP₃R activity, reflecting feedback regulation (8). DANGER (500 nM) has no effect on IP₃-induced Ca²⁺ release from microsomal preparations of COS7 cells transfected with type 1 IP₃R (Fig. 3A). In addition, overexpression or depletion (siRNA) of DANGER does not alter IP₃ production in response to muscarinic and purinergic receptor stimulus (data not shown).

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from changes in binding affinity of IP$_3$ for IP$_3$R as DANGER does not affect IP$_3$ binding affinity (Fig. 3B).

We further examined the effects of DANGER on activity of IP$_3$R by performing single IP$_3$R channel recordings with patch clamp electrophysiology. We utilized endogenous Sf9 IP$_3$R in the outer membrane of freshly isolated nuclei (5, 9). The cytoplasmic face of the channel is exposed to the pipette solution containing $7\mu M$ Ca$^{2+}$ and 100 nM InsP$_3$. In this preparation, the channel open probability ($P_o$) is $\sim$0.7 (Fig. 4), as observed previously (9). Inclusion of 1 $\mu M$ DANGER in the pipette solution reduces channel $P_o$ by $\sim$50%. Channel $P_o$ is diminished primarily as a consequence of an increase of the mean closed time ($t_c$), indicating that DANGER decreases the channel opening rate. In contrast, the rate of channel inactivation, monitored by the channel activity duration $T_a$ and the mean number of channels activated in each patch, $N_A$, is unaffected by DANGER (Fig. 4).

In summary, DANGER is a novel membrane-associated IP$_3$R binding protein that enhances the sensitivity of IP$_3$R to inhibition by Ca$^{2+}$. By contrast, cytochrome c and Bcl-XL, which also modulate Ca$^{2+}$ sensitivity of IP$_3$R, decreases the inhibitory actions of high Ca$^{2+}$ (4, 5). What might be the major physiologic role of DANGER? Its enhancement of Ca$^{2+}$ inhibition of IP$_3$R channel function is striking. In microsomal membranes treated with DANGER, IP$_3$R channel function decreases by 90% as [Ca$^{2+}$] increases from 300 to 800 nM, reflecting a highly cooperative process. This suggests that in DANGER-enriched cells, Ca$^{2+}$ is a major regulator of channel opening. DANGER occurs in numerous terminally differentiated tissues (data not shown), but its marked enrichment in cerebellar Purkinje cells is unique among neuronal lineages. IP$_3$R in Purkinje cells are highly insensitive to IP$_3$ (10–12). This decreased sensitivity fits well with the influences we have observed of DANGER on IP$_3$R channel function, which may reflect allosteric modulation of the Ca$^{2+}$ inhibition site. Last, many of the proteins which have been demonstrated to bind to the IP$_3$R have not been tested for their ability to alter IP$_3$R activity over a large range of external Ca$^{2+}$ concentrations. Thus it may be that regulation of IP$_3$R Ca$^{2+}$ dependence via IP$_3$R binding partners may be a more common function than has been described previously.

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