RESEARCH COMMUNICATION

Double-stranded-RNA-activated protein kinase (PKR) regulates Ca^{2+} stores in *Xenopus* oocytes

David THOMAS[1], Hak Yong KIM, Roseline MORGAN and Michael R. HANLEY

Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616, U.S.A.

Expression of the double-stranded-RNA-dependent protein kinase (PKR) in *Xenopus* oocytes attenuated Ca^{2+} entry-dependent membrane currents activated by depletion of Ca^{2+} stores, whereas expression of a dominant-negative PKR mutant had the opposite effect. These results appeared to be due to perturbation of releasable Ca^{2+} stores, and not actions of PKR on protein synthesis. PKR may thus have novel protein substrates and cellular functions in Ca^{2+} storage and signalling.

INTRODUCTION

Receptors which couple to phosphoinositide hydrolysis generate at least two recognized messengers: Ins(1,4,5)P_3, which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C [1]. The events in intracellular Ca^{2+} release are now comparatively well defined [2]. However, in many, if not all, instances of receptor-activated Ca^{2+} discharge, there is a second, sustained phase of cytosolic Ca^{2+} elevation via Ca^{2+} entry arising from store-operated Ca^{2+} channels [2–4]. This 'capacitative' Ca^{2+} signalling pathway remains poorly understood.

Current debate focuses on the mechanism of capacitative Ca^{2+} entry. Crucial to this debate is the fundamental question of whether depleted stores couple directly or indirectly to the activation of Ca^{2+} entry channels [4–6]. A low molecular mass (< 600 Da) diffusible factor, denoted as calcium influx factor (CIF), has been proposed to couple depletion of Ca^{2+} stores to Ca^{2+} entry [7–11]. Moreover, pharmacological studies employing phosphatase or kinase inhibitors have implicated protein kinases in either the activation or inactivation of the Ca^{2+}-entry current triggered by depleted stores [7–9,11–13]. Thus one appealing possibility is that CIF acts through a novel CIF-activated protein kinase to achieve its biological effects.

Recently, it has been shown that depletion of Ca^{2+} stores potently activates interferon-inducible, double-stranded-RNA-regulated protein kinase (PKR) activity, leading to increased phosphorylation of the eukaryotic initiation factor 2α and thereby suppression of initiation of protein synthesis [14–16]. Significantly, this activation of PKR by stores depletion occurs in the absence of double-stranded RNA, suggesting that PKR may have several routes of activation [14,15]. It is also unclear whether PKR acts exclusively on the only identified protein substrate, eukaryotic initiation factor 2α [14]. Thus PKR was a candidate for the putative CIF-activated protein kinase which would couple Ca^{2+} stores depletion to activation of Ca^{2+} entry. If PKR were to play such a role, its overexpression should enhance capacitative Ca^{2+} entry, particularly as capacitative Ca^{2+} entry in oocytes has been shown recently to have 'spare' capacity [17].

Using ectopic overexpression in *Xenopus* oocytes, we report here that PKR does not respond to CIF, nor does PKR directly activate capacitative Ca^{2+} entry. However, PKR expression does, unexpectedly, dampen depletion-induced Ca^{2+} entry elicited through Ca^{2+}-mobilizing receptors or sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) inhibition. Furthermore, a physiological role for endogenous PKR is implied by the enhancement of depletion-induced Ca^{2+} entry by a dominant-negative PKR mutant. These results suggest that PKR may have novel roles in capacitative Ca^{2+} signalling distinct from its established function in control of the initiation of protein synthesis possibly at the level of stores.

EXPERIMENTAL

Electrophysiology and microinjection

Two-electrode whole-cell voltage clamp experiments using *Xenopus* oocytes were performed as described previously [9]. Oocytes were micro-injected with CIF fractions [9–11] and Ins(1,4,5)P_3 using the Picospritzer pressure injection apparatus (General Valve Corp., Fairfield, NJ, U.S.A.). The volume delivered by pressure injection was calibrated by measuring the diameter of the droplet expelled in air with an eyepiece micrometer. The injection pipette was broken off to give a tip diameter of approx. 20 μm.

Oocyte nuclear injection

The cDNA encoding the rat substance P receptor (SPR) was inserted into the eukaryotic expression vector pcDNA3 (Invitrogen). The cDNA encoding the wild-type and mutant PKR were gifts of Dr. Randal J. Kaufman (University of Michigan, Ann Arbor, MI, U.S.A.). The construction of the mutant PKR and wild-type expression vectors has been described previously [15,18,19]. Constructs were injected into oocyte nuclei as described previously [20]. Briefly, oocytes were layered on to a 35 % Ficoll bed in sterile OR2 medium (82 mM NaCl/2 mM $CaCl_2$/2.5 mM KCl/1 mM $MgCl_2$/1 mM Na_2HPO_4/5 mM Hepes, pH 7.4) and centrifuged for 10 min at 750 ***g***. The DNA was back-loaded into injection pipettes and approx. 50 pg of DNA was injected into the nucleus for each construct. The oocytes were kept in modified L15 medium for 3–5 days post-injection to allow for expression of the heterologous proteins.

Abbreviations used: CIF, calcium influx factor; PKR, RNA-regulated protein kinase; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SPR, rat substance P receptor; SP, substance P; LPA, lysophosphatidic acid.

[1] To whom correspondence should be addressed.

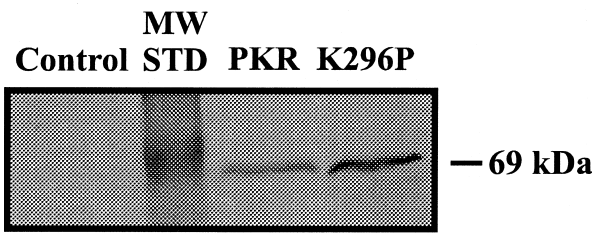

Figure 1 Overexpression of wild-type PKR and a dominant-negative PKR mutant (K296P) in *Xenopus* oocytes

Oocytes were nuclear injected with expression vectors encoding the wild-type and mutant PKR forms as described in the Experimental section. The lanes are, Control; uninjected oocytes; MW STD, molecular mass standards; PKR, wild-type PKR injected oocytes; K296P, dominant-negative mutant PKR injected oocytes. The cells were maintained for 3 days and then harvested for SDS/PAGE and immunoblotting as described in the Experimental section. A mouse polyclonal antibody to the dsRNA binding domain was used to detect the presence of the wild-type and mutant PKR.

Immunoblotting

Oocytes were homogenized in 20 mM Tris/HCl (pH 7.6)/50 mM NaCl/10 mM MgCl/1 mM EDTA/0.2 mM PMSF/75 nM aprotinin/1 μM pepstatin A/10 μM leupeptin three days after nuclear injection. Homogenates were extracted with an equal volume of Freon (1,1,2-trichlorotrifluoroethane; Sigma) to reduce yolk-platelet proteins [21]. Oocyte proteins were precipitated with acetone and resuspended in 1 × sample buffer [50 mM Tris/HCl/100 mM dithiothreitol/0.1 % (w/v) Bromophenol Blue/10 % (w/v) glycerol/2 % (w/v) SDS, pH 6.8] and subjected to SDS/PAGE. Proteins were blotted to PVDF membranes and expression of wild-type and mutant PKR was detected using a mouse polyclonal antibody targetted at the double-stranded RNA- binding domain of human PKR (a gift from Dr. Randall J. Kaufman).

Statistical analysis

The statistical significance of the mean Ca^{2+}-activated Cl^- currents was determined using the unpaired student's *t*-test on the peak Cl^- current responses and is denoted by the indicated *P* value; $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Nuclear injection of cDNAs encoding either wild-type PKR or a dominant negative mutant (K296P) resulted in the expression of an immunoreactive 69 kDa protein, not detectable in resting or stimulated oocytes, corresponding to the expected molecular mass of wild-type and a point-mutant PKR (Figure 1) [15,18,19].

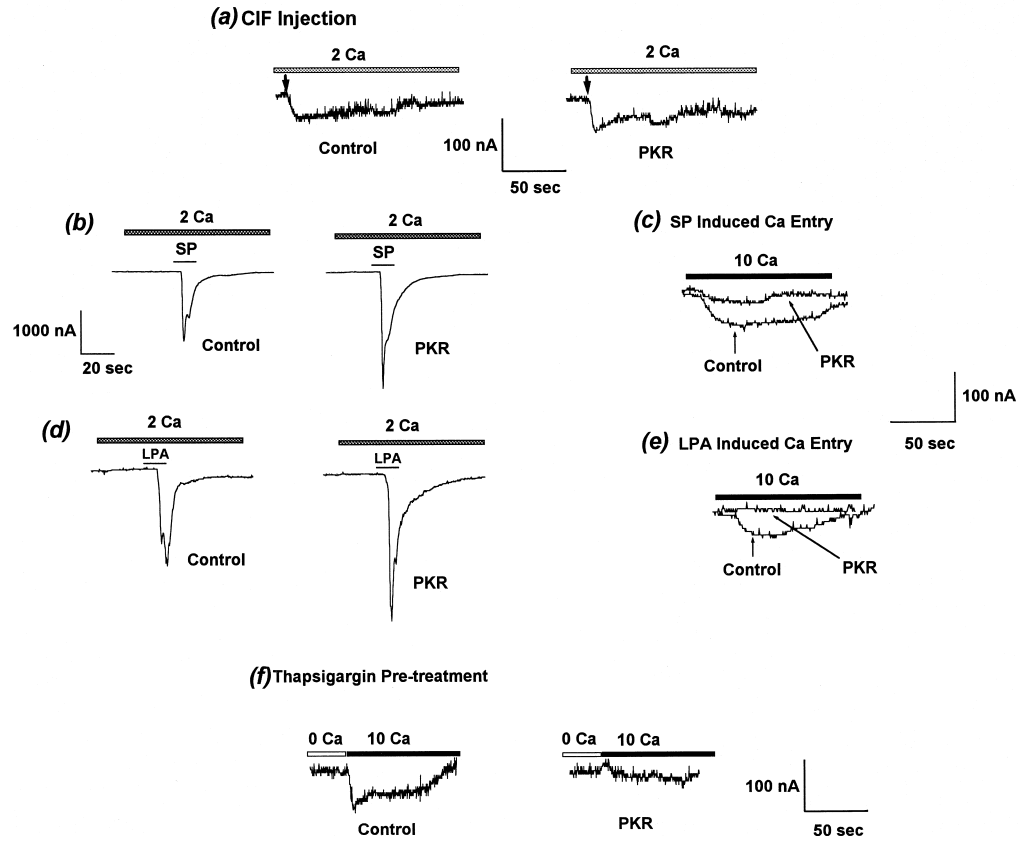

Figure 2 Overexpression of wild-type PKR fails to alter CIF-evoked current responses but uncouples Ca^{2+} entry from store depletion

(**a**) Oocytes were micro-injected with a 10 nl of CIF extract prepared as described previously [9–11] in OR2 containing 2 mM calcium. The arrows denote the time of CIF injections. (**b**) Oocyte responses to perfusion of 50 nM SP in OR2 containing 2 mM calcium. SP was perfused for the time interval indicated by the bar. (**c**) Ca^{2+} entry responses evoked by SP-induced depletion of Ca^{2+} stores. Current responses were elicited by raising extracellular Ca^{2+} to 10 mM after the decay of the discharge currents shown in (**b**). The bar indicates the duration of exposure of the cell to high calcium. (**d**) Oocyte responses to perfusion of 100 nM LPA in OR2 containing 2 mM calcium. (**e**) Ca^{2+} entry evoked by LPA-induced depletion of stores. (**f**) Oocyte Ca^{2+} stores were depleted by incubating cells with 1 μM thapsigargin for > 3 h in Ca^{2+}-free OR2. Oocytes were then voltage-clamped at -60 mV in Ca^{2+}-free frog Ringer's solution and the depletion-activated current responses were detected by perfusion of the oocyte in 10 mM Ca^{2+} containing OR2.

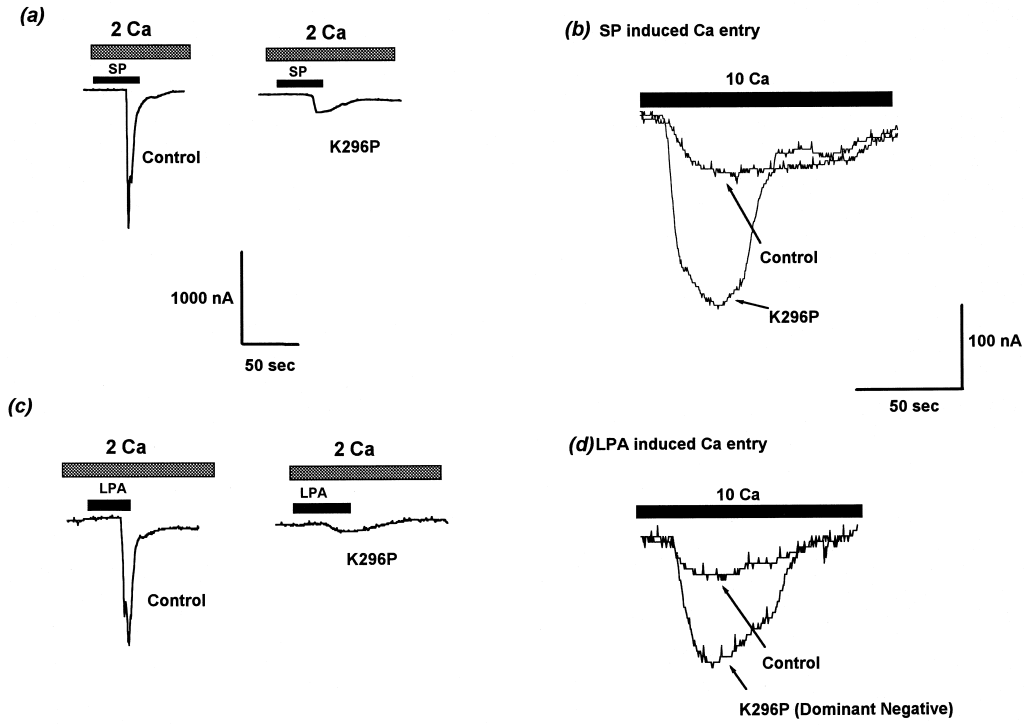

Figure 3 Overexpression of a dominant-negative PKR mutant attenuates agonist-activated Ca^{2+} discharge while enhancing agonist-activated Ca^{2+} entry responses

(**a**) Oocyte responses to perfusion of 50 nM SP in OR2 containing 2 mM calcium. SP was perfused for the time interval indicated by the bar. (**b**) Ca^{2+}-entry responses evoked by SP-induced depletion of Ca^{2+} stores. Current responses were elicited by raising extracellular Ca^{2+} to 10 mM after the decay of the discharge currents shown in (**a**). (**c**) Oocyte responses to perfusion of 100 nM LPA in OR2 containing 2 mM calcium. (**d**) Ca^{2+} entry evoked by LPA-induced depletion of stores.

PKR expression does not alter CIF-induced currents but attenuates Ca^{2+}-depletion induced currents

Direct microinjection of a purified CIF fraction, prepared and characterized as described previously [9–11], evoked Ca^{2+}-dependent Cl^- currents in *Xenopus* oocytes (50 ± 10 nA, $n = 5$; Figure 2a). To test whether CIF may regulate PKR function, CIF was injected into oocytes overexpressing wild-type or dominant-negative mutant PKR. No significant difference was observed in either case in CIF-induced Ca^{2+}-activated Cl^- currents (responses in PKR overexpressing oocytes; 49 ± 8 nA; $n = 5$, $P > 0.05$ compared with controls; Figure 2a).

To determine whether agonist-activated capacitative Ca^{2+}-entry responses were altered in oocytes overexpressing PKR, oocytes were co-injected with PKR and SPR cDNAs. Control oocytes expressing only SPR responded to substance P (SP) (50 nM) with large Ca^{2+}-activated Cl^- currents (1510 ± 405 nA; $n = 18$; Figure 2b) [20,22]. Oocytes co-expressing both SPR and PKR responded to SP application with discharge-dependent early currents (1885 ± 303 nA, $n = 15$; Figure 2b) which were not significantly different from controls ($P > 0.05$).

Following the decay of Ca^{2+} discharge-dependent currents, store-depletion evoked Ca^{2+} entry was assessed by measuring current activity induced by elevating extracellular [Ca^{2+}] from 2 to 10 mM (Figure 2c). Elevating extracellular [Ca^{2+}] has proved to be a reliable indirect approach for studying depletion-activated Ca^{2+} entry via Ca^{2+}-activated currents [9,12,23]. Control oocytes exhibited Ca^{2+} entry-dependent late current responses when stimulated by SP application (50 nM) and superfused with 10 mM Ca^{2+} (99 ± 16 nA, $n = 18$; Figure 2c). However, oocytes co-expressing SPR and PKR exhibited dramatically attenuated (75–80 %) current responses (26 ± 11 nA, $n = 15$, $P < 0.001$; Figure 2c). The effect of altered PKR activity was also tested on an endogenous Ca^{2+}-mobilizing lysophosphatidic acid (LPA) receptor [23–25]. LPA-induced discharge-dependent current responses were not significantly altered by wild-type PKR overexpression (control oocyte: 769 ± 222 nA, $n = 12$; PKR-expressing oocyte: 896 ± 223 nA, $n = 7$, $P > 0.05$; Figure 2d). However, Ca^{2+} entry-dependent current responses were reduced to the same degree (75–80 %) as those elicited by SPR activation (14 ± 13 nA versus 73 ± 26 nA, $n = 7$, $P < 0.001$; Figure 2e).

In interpreting these results, two points should be emphasized. First, the finding that oocytes co-expressing SPR and PKR mounted typical Ca^{2+} discharge-dependent early current responses to SP stimulation suggests that PKR overexpression had no effect on the translation of either an exogenous gene (SPR) or on endogenous Ca^{2+} signalling genes. Thus PKR-induced reduction of new protein synthesis was not the basis of the observed functional alterations. This point is further underscored by the observation that incubation in either cycloheximide (100 μM) or puromycin (500 μM), conditions known to inhibit oocyte protein synthesis [26,27], had no effect on the discharge or Ca^{2+} entry current responses (results not shown). Secondly, neither receptor-induced Ca^{2+} discharge-dependent current responses nor CIF-induced Ca^{2+} entry-dependent current responses were altered by expressing PKR. These results indicate that PKR overexpression was not acting directly upon channels used to detect Ca^{2+} changes in the oocyte, but rather appeared to be modifying releasable Ca^{2+} levels.

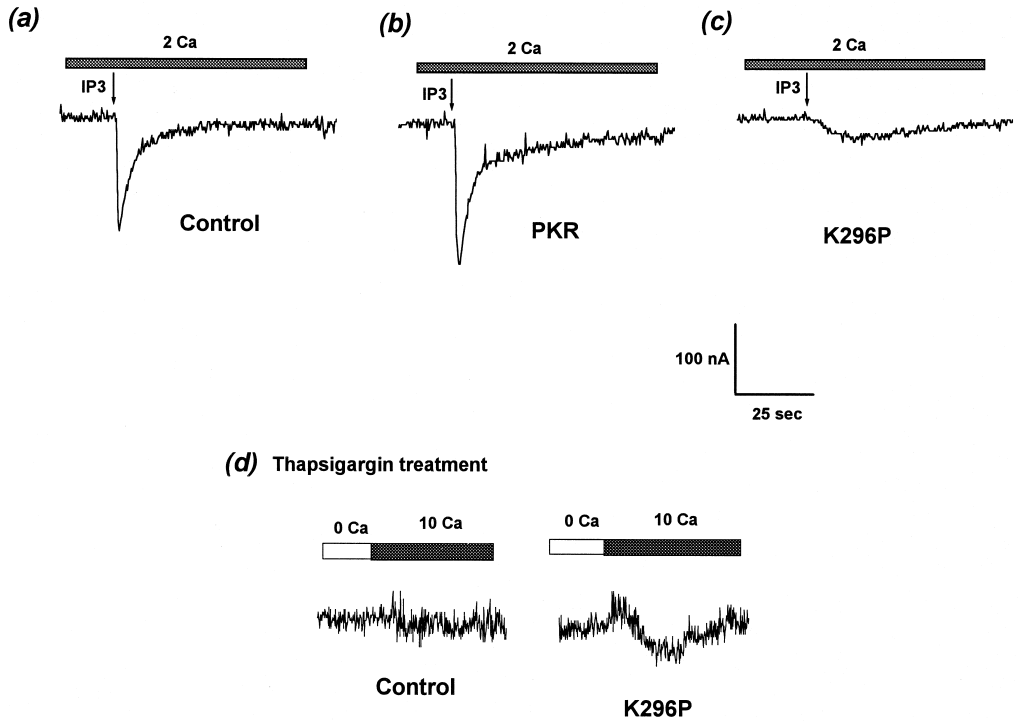

Figure 4 Overexpression of a dominant-negative PKR mutant attenuates current responses evoked by direct injection of Ins(1,4,5)P_3 and gives rise to oocytes which produce current responses after short-term thapsigargin treatment

(**a**) Response elicited by injection of Ins(1,4,5)P_3 (1 μM) in control oocyte not injected with PKR cDNA. Ins(1,4,5)P_3-stimulated currents were obtained in OR2 containing 2 mM calcium. (**b**) Response elicited by Ins(1,4,5)P_3 injection in oocyte injected with wild-type PKR cDNA. (**c**) Response elicited by Ins(1,4,5)P_3 injection in oocyte injected with K296P cDNA. The arrows indicate the time of Ins(1,4,5)P_3 injection and the bars indicate the responses were all obtained in 2 mM Ca^{2+} OR2. (**d**) Current responses were elicited following the same protocol described in Figure 2 (**f**) except that oocytes were incubated in 1 μM thapsigargin in Ca^{2+}-free OR2 for only 15 min before challenge in high Ca^{2+} OR2. Wild-type PKR injected oocytes, like the control, failed to respond.

Current responses were tested after stores had been depleted by prolonged treatment (> 2 h) with the SERCA inhibitor thapsigargin [9,23,25]. Control oocytes exposed to thapsigargin (3 h) and challenged with high (10 mM) extracellular [Ca^{2+}] responded with the activation of an entry current (54 ± 12 nA, $n = 18$; Figure 2f) that rapidly decayed in Ca^{2+}-free OR2 (results not shown) [9,25]. Overexpression of PKR inhibited (10 of 16 oocytes: 13 ± 10 nA, $n = 16$, $P < 0.001$) or blocked (6 of 16 oocytes) thapsigargin-induced current responses (Figure 2f).

A dominant-negative PKR mutant enhances capacitative Ca^{2+}-entry dependent currents

To explore roles of endogenous PKR in regulating Ca^{2+} signalling, current responses were tested in oocytes expressing a dominant-negative PKR mutant (K296P) (Figure 1). SPR-activated Ca^{2+} discharge-dependent currents were significantly attenuated in the K296P expressing oocytes (201 ± 36 nA, $n = 15$ versus 1510 ± 405 nA, $n = 18$, $P < 0.001$ compared with controls; Figure 3a) and similar results were observed using LPA stimulation (106 ± 41 nA, $n = 7$ versus 769 ± 222 nA, $n = 12$, $P < 0.001$ compared with controls; Figure 3c).

Despite the pronounced attenuation of the Ca^{2+} discharge-dependent current responses evoked by receptor stimulation, K296P-expressing oocytes gave enhanced Ca^{2+} entry-dependent current responses (SP induced Ca^{2+} entry-dependent current responses: 234 ± 62 nA, $n = 15$ versus 99 ± 16 nA, $n = 18$, $P < 0.001$ compared with controls; Figure 3b; LPA induced Ca^{2+} entry-dependent current responses: 117 ± 29 nA, $n = 5$ versus 73 ± 26 nA, $n = 7$, $P < 0.05$ compared with controls; Figure 3d). Thus expression of the dominant-negative PKR mutant resulted in two actions; reduction of early Ca^{2+} discharge-dependent currents and enhancement of late Ca^{2+} entry-dependent currents.

Because agonist-activated Ca^{2+} release from intracellular stores was diminished in the dominant-negative PKR-expressing oocytes, Ins(1,4,5)P_3 production or its effects might be altered by endogenous PKR. When Ins(1,4,5)P_3 (1 μM) was micro-injected directly into oocytes, by-passing receptor activation, current responses in control oocytes (238 ± 42 nA, $n = 35$; Figure 4a) [9,28,29] were not significantly affected by PKR overexpression (246 ± 52 nA, $n = 6$, $P > 0.05$ compared with controls; Figure 4b). However, Ins(1,4,5)P_3-induced current responses were significantly diminished in oocytes expressing the dominant negative K296P mutant (60 ± 22 nA, $n = 6$, $P < 0.001$ compared with controls; Figure 4c). These results indicate that the perturbation of Ca^{2+} discharge-induced currents is not at the level of Ins(1,4,5)P_3 formation.

PKR regulates Ca^{2+} storage in *Xenopus* oocytes

The agonist-evoked current responses observed for the dominant-negative PKR mutant K296P suggested that oocytes expressing this clone contained relatively depleted intracellular Ca^{2+} stores. Thus if Ca^{2+} stores were partially depleted in K296P overexpressing oocytes, thapsigargin treatment should reveal a shorter latency to the detection of depletion-induced Ca^{2+} entry-dependent currents. In control oocytes, thapsigargin treatment

requires 1–2 h before depletion-induced currents are measurable [9,23,25,30,31]. Control oocytes, or oocytes expressing PKR, were exposed to thapsigargin (1 μM) for 15 min and invariably failed to elicit currents when perfused with high external Ca^{2+} (10 mM). In contrast, the K296P-expressing oocytes mounted premature current responses in every case after 15 min exposure to thapsigargin (52 ± 18 nA, $n = 7$; Figure 4d).

Overall, the observations reported here suggest that PKR appears to play a novel role in maintaining releasable Ca^{2+} levels in the Ins(1,4,5)P_3-sensitive stores. Thus overexpression of PKR perturbs the Ca^{2+} store balance toward the more filled state, which results in attenuated capacitative Ca^{2+} entry responses. Similarly, overexpression of a dominant negative PKR mutant perturbs the Ca^{2+} store balance toward the more depleted state, which results in large Ca^{2+} entry responses associated with attenuated discharge responses. PKR may function, therefore, as an upstream regulator of the maintenance of Ca^{2+} stores, which may be relevant to cellular stresses, including viral infection, that trigger the activity or increase the level of expression of PKR. Future studies will focus on candidates for PKR protein substrates, such as SERCA isoforms which can alter releasable Ca^{2+} stores.

We are indebted to Dr. Randal J. Kaufman for provision of the PKR clones and the gift of the PKR antibody used in this study. D.T. was supported as a postdoctoral trainee by NIH grant T32HL07682.

REFERENCES

1 Berridge, M. J. (1993) Nature (London) **361**, 315–325
2 Putney, J. W. and Bird, G. S. (1993) Endocrine Rev. **14**, 610–631
3 Putney, J. W. (1990) Cell Calcium **11**, 611–624
4 Berridge, M. J. (1995) Biochem. J. **312**, 1–11
5 Putney, J. W. and Bird, G. S. (1993) Cell **75**, 199–201
6 Hallett, M. B., Pettit, E. J. and Davies, E. V. (1996) Biochem. J. **314**, 1054–1056
7 Randriamampita, C. and Tsien, R. Y. (1993) Nature (London) **364**, 809–814
8 Randriamampita, C. and Tsien, R. Y. (1995) J. Biol. Chem. **270**, 29–32
9 Thomas, D., Kim, H. Y. and Hanley, M. R. (1996) Biochem. J. **318**, 649–656
10 Kim, H. Y., Thomas, D. and Hanley, M. R. (1995) J. Biol. Chem. **270**, 9706–9708
11 Thomas, D. and Hanley, M. R. (1995) J. Biol. Chem. **270**, 6429–6432
12 Zweifach, A. and Lewis, R. S. (1995) J. Biol. Chem. **270**, 1445–14451
13 Parekh, A. B., Terlau, H. and Stuhmer, W. (1993) Nature (London) **364**, 814–818
14 Prostko, C. R., Dholakia, J. N., Brostrom, M. A. and Brostrom, C. O. (1995) J. Biol. Chem. **270**, 6211–6215
15 Srivastava, S. P., Davies, M. V. and Kaufman, R. J. (1995) J. Biol. Chem. **270**, 16619–16624
16 Brostrom, C. O., Prostko, C. R., Kaufman, R. J. and Brostrom, M. A. (1996) J. Biol. Chem. **271**, 24995–25002
17 Jaconi, M., Pyle, J., Bortolon, R., Ou, J. and Clapham, D. (1997) Curr. Biol. **7**, 599–602
18 Wu, S. and Kaufman, R. J. (1996) J. Biol. Chem. **271**, 1756–1763
19 Wu, S. and Kaufman, R. J. (1997) J. Biol. Chem. **272**, 1291–1296
20 Sasakawa, N., Ferguson, J. E., Sharif, M. and Hanley, M. R. (1994) Mol. Pharmacol. **46**, 380–385
21 Evans, J. P. and Kay, B. K. (1991) Methods Cell Biol. **36**, 133–148
22 Sasakawa, N., Sharif, M. and Hanley, M. R. (1994) FEBS Lett. **347**, 181–184
23 Petersen, C. C. and Berridge, M. J. (1994) J. Biol. Chem. **269**, 32246–32253
24 Ferguson, J. E. and Hanley, M. R. (1992) Arch. Biochem. Biophys. **297**, 388–392
25 Petersen, C. C. and Berridge, M. J. (1995) Biochem. J. **307**, 663–668
26 Bravo, R. and Allende, J. E. (1996) Arch. Biochem. Biophys. **172**, 648–653
27 Allende, C. C., Hinrichs, M. V., Santos, E. and Allende, J. E. (1988) FEBS Lett. **234**, 426–430
28 DeLisle, S., Blondel, O., Longo, F. J., Schnabel, W. E., Bell, G. I. and Welsh, M. J. (1996) Am. J. Physiol. **270**, C1255–1261
29 DeLisle, S., Marksberry, E. W., Bonnett, C., Jenkins, D. J., Potter, B. V., Takahashi, M. and Tanzawa, K. (1997) J. Biol. Chem. **272**, 9956–9961
30 Lupu-Meiri, M., Beit-Or, A., Christensen, S. B. and Oron, Y. (1993) Cell Calcium **14**, 101–110
31 Petersen, C. C., Berridge, M. J., Borgese, M. F. and Bennett, D. L. (1995) Biochem. J. **311**, 41–44

Received 15 September 1997/29 October 1997; accepted 22 December 1997