Volume 296, number 2, 225–230 FEBS 10603 January 1992

Okadaic acid inhibits angiotensin II stimulation of Ins(1,4,5)P_3 and calcium signalling in rat hepatocytes

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Received 14 October 1991; revised version received 22 November 1991

OKA[2] and CL-A significantly inhibit the ability of angiotensin II, ATP and vasopressin to raise $[Ca^{2+}]_i$ in rat hepatocytes, with a partial inhibition of the initial spike, and a complete inhibition of the following plateau. In contrast, the $[Ca^{2+}]_i$ response to thapsigargin, which releases intracellular calcium stores through a mechanism independent of inositol phosphates, is much less affected. The ability of angiotensin II to stimulate Ins(1,4,5)P_3 production is also reduced by OKA, with kinetics consistent with the inhibited $[Ca^{2+}]_i$ response. Since OKA and CL-A are potent and selective inhibitors of phosphoprotein phosphatases, these results provide further evidence that agonist-stimulated Ins(1,4,5)P_3 signalling can be inhibited by protein phosphorylation.

Okadaic acid; Angiotensin II; Calcium ion concentration; Intracellular; Inositol phosphate; Inositol 1,4,5-trisphosphate 3-kinase; Rat hepatocyte

1. INTRODUCTION

Stimulation of hepatocytes with angiotensin II or vasopressin leads to production of Ins(1,4,5)P_3 [1,2], increased $[Ca^{2+}]_i$ [1–4], and increases in the phosphorylation state of a number of proteins [3,5], many of which have been identified as putative substrates for either protein kinase C [5] or calmodulin-dependent protein kinases [6]. In many systems, the ability of agonists to induce such responses is inhibited by prior activation of protein kinase C, leading to the hypothesis that there may be a feedback inhibition of this signalling pathway [7]. In hepatocytes, for example, responses to both epidermal growth factor [1,3] and phenylephrine [4,8–11] are completely blocked by pretreatment with phorbol diesters. The mechanism for this phorbol ester-induced non-responsiveness may well be desensitization of the relevant receptors through a protein kinase C-dependent phosphorylation event [9,10,12,13]. The ability of angiotensin II and vasopressin to stimulate Ins(1,4,5)P_3 production and increases in $[Ca^{2+}]_i$ is, however, largely resistant to the prior activation of protein kinase C by phorbol esters [1,3,4,9,11], although a small degree of inhibition can be revealed at low concentrations of these agonists [1,4,9,11].

OKA and CL-A are two potent and selective inhibitors of phosphoprotein phosphatases types 1 and 2A [14,15]. OKA treatment has been shown to increase the phosphorylation state of many hepatocyte phosphoproteins [16]. In our preliminary experiments (J.C. Garrison, unpublished observations), OKA and CL-A clearly increase the phosphorylation of two phosphoproteins identified as substrates for protein kinase C beyond that which occurs with maximal phorbol diester treatment. In particular, the phosphorylation state of the protein previously designated as "spot b" [5], with a pI = 5.7 and M_r = 56 000, is much greater following OKA or CL-A treatment than has been observed with agonists or phorbol diesters. This result suggested that OKA and CL-A may be able to mimic certain effects of phorbol esters, but with greater efficacy. In view of the very slight effects of phorbol esters upon angiotensin II-dependent Ca^{2+} signalling [1,9], it was hypothesized that OKA and CL-A may induce significant inhibition of these responses. In support of this hypothesis, Garcia-Sainz et al., have recently reported that 1 μM OKA is able to inhibit angiotensin II-stimulated phosphoinositide turnover in rat hepatocytes [17], although effects on $[Ca^{2+}]_i$ levels were not reported. Our results show that both OKA and CL-A, at nanomolar concentrations, are able to inhibit the ability of angiotensin II to raise $[Ca^{2+}]_i$, and that OKA is also able to inhibit angiotensin II-stimulated increases in Ins(1,4,5)P_3 with kinetics appropriate to its inhibition of the $[Ca^{2+}]_i$ response. These results provide further evidence that this signalling pathway can be inhibited by protein phosphorylation.

Abbreviations: OKA, okadaic acid; CL-A, calyculin A; $[Ca^{2+}]_i$, intracellular calcium concentration; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; cBSA, crystalline bovine serum albumin; KRB, Krebs-Ringer bicarbonate, pH 7.4; DMSO, dimethylsulphoxide; HPLC, high-pressure liquid chromatography.

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2. MATERIALS AND METHODS

Rat hepatocytes were isolated as described [18] and maintained under 95% O_2/5% CO_2 for all subsequent procedures. For measurement of $[Ca^{2+}]_i$, hepatocytes at 2×10^6 cells/ml were preincubated in the dark in KRB containing 0.5 mM Ca^{2+} and supplemented with 20 mM HEPES, 1 mg/ml cBSA, 5 mM glucose, 5 mM pyruvate, and 20 mM lactate for 15 min at 37°C. Aliquots (2.5 ml) were withdrawn onto ice for autofluorescence controls and kept in the dark. The remaining cells were loaded with indo-1 by addition of 10 μM indo-1 tetrakisacetoxymethylester for a further 30 min incubation at 37°C. The cells were pelleted (5 min, $50 \times g$), the medium aspirated and the hepatocytes resuspended in supplemented KRB and aliquoted as before. Aliquots of cells were warmed to 37°C with gentle shaking in the presence of OKA, CL-A or the DMSO vehicle control for 30 min. The cells were then gently pelleted, resuspended in fresh, supplemented KRB and placed into a stirred cuvette maintained at 37°C in an SLM-8000 spectrofluorometer. The dye-loaded cells were excited at 332 nm, and fluorescence was recorded at 1 s intervals at both 400 nm and 485 nm. Agonists were added from 100-fold concentrated stocks in either 20 mM Tris-HCl, pH 7.4, plus 1 mg/ml cBSA (angiotensin II, vasopressin, ATP) or in 2% (v/v) DMSO in Tris-HCl/BSA (thapsigargin) to give the final concentrations shown in Table I. Maximal and minimal fluorescence were defined by addition of 30 μg/ml digitonin and 5 mM EGTA, pH 7.5, respectively. Since the autofluorescence of the cells is not negated during the calculation procedure for dual wavelength dye responses [19], the autofluorescence of cells not loaded with dye but otherwise treated equivalently was subtracted at both wavelengths prior to conversion of the data to apparent $[Ca^{2+}]_i$ through the use of the formula given by Grynkiewicz et al. [19].

To quantitatively compare response, basal $[Ca^{2+}]_i$ was defined as the mean of the calculated values for the 30 s prior to agonist addition, and the plateau increase in $[Ca^{2+}]_i$ was defined as the mean calculated value between 60 and 90 s after agonist addition minus the basal level. To analyse the $[Ca^{2+}]_i$ transient, the release of Ca^{2+} into the cytosol was fit to a model originally developed to analyse the episodic secretion of pituitary hormones that underlies the pulsatile concentrations of these hormones in plasma [20]. The analysis fitted the data to both the release of Ca^{2+} and its simultaneous removal by developing a convolution integral that was solved by iterative nonlinear least-squares parameter estimation, in which all values of $[Ca^{2+}]_i$ and their variances were considered simultaneously [20]. The peak increase in $[Ca^{2+}]_i$ was defined as the mean increase over the basal level achieved for the 5 s around the time point predicted for the peak by the program. OKA did not change the position of the peak responses. For example, the mean time to peak following angiotensin II stimulation (Fig. 1, Table I) was 12 ± 2 s in the control, and 14 ± 2 s in OKA-treated cells; following thapsigargin stimulation, mean time to peak was 36 ± 4 s in the control, and 40 ± 6 s in OKA-treated cells. Therefore, for the few records that could not be fit with the program, the peak could be defined by inspection of the data at these predicted positions.

Measurements of inositol phosphates were performed as previously described [1,21]. Suspensions of hepatocytes at $3–5 \times 10^6$ cells/ml were labelled with 30 μCi/ml [1,2-^{3}H]-*myo*-inositol (35 Ci/mmol) in KRB supplemented with 2 mg/ml cBSA, 10 mM glucose, 16 mM lactate, 4 mM pyruvate, 10 μM *myo*-inositol for 90 min at 37°C. Cells were pelleted, rinsed in KRB supplemented with 5 μM *myo*-inositol, and then resuspended in KRB and aliquoted on ice. Aliquots were warmed to 37°C with gentle shaking in the presence of 300 nM OKA or its DMSO vehicle for 30 min. The cells were then stimulated with angiotensin II and acid extracts prepared for HPLC analysis as described [1]. Separation of inositol phosphates by strong anion-exchange HPLC was performed as described [21], with monitoring of the eluate either by collection of fractions followed by scintillation counting [1], or by an on-line radioactivity detector (Radiomatic Flo-One β A250) [21]. For most experiments the on-line detector was used, allowing the full spectrum of inositol metabolites to be recorded [21]. In these experiments, presented in Fig. 3, the yield of inositol trisphophates was normalized to 100 000 cpm total content to control for slight variations in the cell counts, labelling intensity and recovery. The use of the on-line detector does, however, lead to a loss of absolute sensitivity, since the apparent background is higher and the efficiency of counting is lower than can be attained in a conventional scintillation counter (RRM and JGG, unpublished observations). Thus for the dose-response experiments (Fig. 2), where sub-maximal responses were quantitated, fractions were collected and counted in a liquid scintillation counter.

To assay for Ins(1,4,5)P_3 3-kinase activity, samples of cytosol from stimulated hepatocytes were prepared by a modification of the method of Biden et al. [22]. Briefly, hepatocytes were treated with 300 nM OKA for 13 min at 37°C, rapidly pelleted, and the supernatant aspirated. The pellet was suspended in 1 ml of 10 mM KCl/10 mM HEPES and left on ice for 10 min. Saturated KCl was added to restore iso-osmolarity, and the cells homogenized in a tight-fit glass Dounce homogenizer. The suspension was cleared at $100\,000 \times g$ for 1 h, and the supernatant used as a cytosolic extract. Ins(1,4,5)P_3 3-kinase activity was assessed in reactions of 25–50 μg cytosolic protein with 1–10 μM Ins(1,4,5)P_3 for 60 s at 25°C in the manner described [23].

Statistical significance was assessed by the one-tailed, paired *t*-test. Sigmoid curves were fitted to the data in Fig. 2 by non-linear regression using the software GraphPad InPlot.

Tumour promoters (OKA, CL-A and thapsigargin) were obtained from LC Service Corporation, Woburn MA. DMSO (puriss. p.a. grade) was from Fluka Chemical Corporation, Ronkonkoma, NY. The sources of all other reagents have previously been given [1,21,24].

3. RESULTS

In order to test the hypothesis that OKA may cause significant inhibition of angiotensin II-dependent signalling, the ability of angiotensin II to increase $[Ca^{2+}]_i$ in hepatocytes was assessed following pretreatment with OKA. Fig. 1A demonstrates that 100 nM angiotensin II is able to produce a similar spike and plateau increase in $[Ca^{2+}]_i$ in hepatocytes loaded with indo-1 as compared to those reported to occur in cells loaded with either quin-2 [1,3] or fluo-3 [25]. It is clear from panels B and C of Fig. 1 that OKA is able to produce a dose-dependent inhibition of the response to this maximal dose of angiotensin II. This result thus distinguishes the inhibitory action of OKA from that of phorbol esters, since we have previously shown that phorbol esters are only able to block the $[Ca^{2+}]_i$ responses to low doses (≤ 0.1 nM) of angiotensin II [1].

To further investigate the inhibitory effect of OKA upon angiotensin II-stimulated increases in $[Ca^{2+}]_i$, this effect was quantitated from several experiments, and compared with the effects of OKA and CL-A upon the ability of other agonists known to raise $[Ca^{2+}]_i$ in hepatocytes (Table I). Both OKA and CL-A produce significant partial inhibition of the magnitude of the initial spike response to angiotensin II, but there is a more dramatic effect upon the later plateau response, which is completely abolished. A very similar pattern is seen in cells stimulated with vasopressin or ATP, two other agonists that also raise $[Ca^{2+}]_i$ in hepatocytes through the generation of inositol phosphates [2,26].

Thapsigargin, which inhibits the Ca^{2+}-ATPase of sarcoplasmic reticulum [27] and the endoplasmic reticulum of hepatocytes [28], causes a slow, sustained increase in

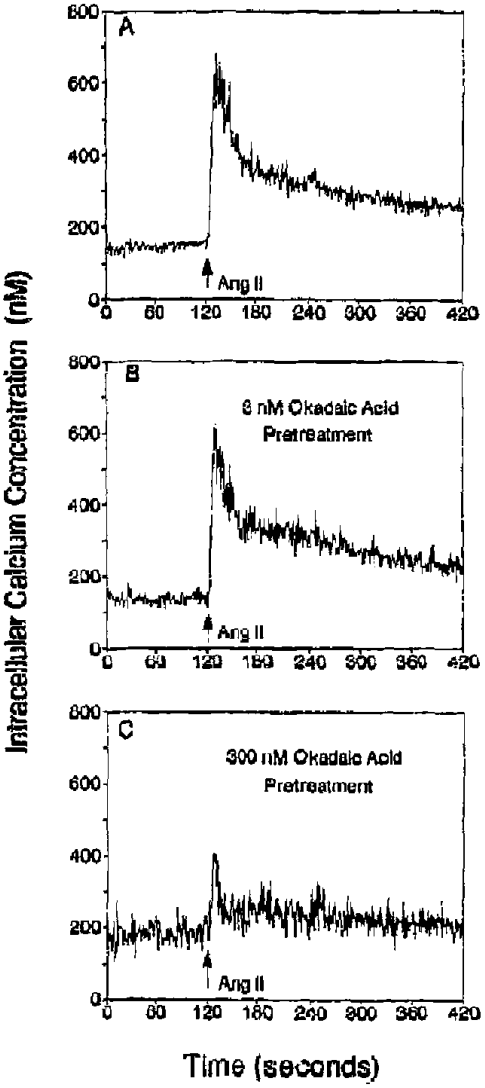

Fig. 1. Okadaic acid pretreatment inhibits angiotensin II-stimulated increases in $[Ca^{2+}]_i$. Representative traces from 3 (panel B) or 7 (panels A and C) independent experiments of indo-1 loaded hepatocytes stimulated with 100 nM angiotensin II. (A) DMSO treated control; (B) 30 min of 3 nM OKA treatment; and (C) 30 min of 300 nM OKA treatment.

hepatocyte $[Ca^{2+}]_i$ through mobilization of intracellular stores that include those mobilized by Ins(1,4,5)P_3 [28]. In contrast to the responses to those agonists that release calcium from these stores subsequent to Ins(1,4,5)P_3 production, the sustained increase in $[Ca^{2+}]_i$ induced by thapsigargin is much less sensitive to pretreatment with OKA of CL-A (Table 1).

Since OKA and CL-A appear more able to inhibit the increases in $[Ca^{2+}]_i$ induced by agents acting through Ins(1,4,5)P_3 production, this suggested that the mechanism might involve an inhibition of the production or action of Ins(1,4,5)P_3. The ability of angiotensin II to stimulate the production of Ins(1,4,5)P_3 following pretreatment with OKA is shown in Fig. 2. OKA induces a decrease in the stimulated accumulation of Ins(1,4,5)P_3 in response to the full range of angiotensin II concentrations. To further investigate the connection between the OKA-induced inhibition of the Ins(1,4,5)P_3 response to angiotensin II and the inhibition of the $[Ca^{2+}]_i$ response to angiotensin II, the effects of OKA on the kinetics of inositol phosphate production were investigated (Fig. 3). Fig. 3A shows that the kinetics of the Ins(1,4,5)P_3 response to angiotensin II demonstrate the same initial spike and later plateau characteristics as are seen in the $[Ca^{2+}]_i$ response (compare Fig. 1A with Fig. 3A). OKA pretreatment is able to reduce the initial spike of Ins(1,4,5)P_3 and to almost completely abolish the later plateau response (Fig. 3A), again consistent with the OKA-inhibited $[Ca^{2+}]_i$ response to angiotensin II (Fig. 1C). The fact that OKA treatment is able to reduce the total accumulation of inositol phosphates in response to angiotensin II (Fig. 3C) is consistent with an OKA-dependent inhibition of the production of Ins(1,4,5)P_3.

The ability of angiotensin II to stimulate the accumulation of Ins(1,3,4)P_3 is shown in Fig. 3B. It is apparent that, at least at the early time points of stimulation, the appearance of Ins(1,3,4)P_3 is largely insensitive to pretreatment of the cells with OKA. This is surprising since Ins(1,3,4)P_3 is produced from Ins(1,4,5)P_3, through the sequential actions of 3-kinase

Table 1

OKA and CL-A inhibition of agonist-induced increases in $[Ca^{2+}]_i$. Experiments such as those shown in Fig. 1 were quantitated as described in Section 2. Data are mean ± S.E.M. (= S.D. when $n = 2$) from the number of independent rat hepatocyte preparations shown in parentheses. Basal $[Ca^{2+}]_i$ levels were: 201 ± 16 nM in the control hepatocytes; 266 ± 19 nM following OKA treatment; 273 ± 31 nM following CL-A treatment

Agent	Peak increase in $[Ca^{2+}]_i$ over basal (nM)			Plateau increase in $[Ca^{2+}]_i$ over basal (nM)		
	Control (DMSO) pretreatment	300 nM OKA pretreatment	100 nM CL-A pretreatment	Control (DMSO) pretreatment	300 nM OKA pretreatment	100 nM CL-A pretreatment
100 nM Angiotensin II	199 ± 46 (8)	95 ± 22[a] (7)	19 ± 10[a] (3)	72 ± 20 (8)	−0.6 ± 17[a] (7)	−45 ± 20[a] (3)
100 nM Vasopressin	229 ± 54 (3)	38 ± 30[a] (3)	39 ± 2[a] (3)	66 ± 25 (3)	3 ± 16[b] (3)	−44 ± 25[a] (3)
100 μM ATP	169 ± 49 (3)	40 ± 15[a] (3)	45 ± 20[b] (3)	52 ± 22 (3)	−22 ± 16[a] (3)	−29 ± 9[a] (3)
200 nM Thapsigargin	173 ± 37 (6)	98 ± 31[a] (6)	109 ± 6 (2)	130 ± 39 (6)	83 ± 33[a] (6)	75 ± 17[b] (2)

[a]Reduced from control (DMSO) response ($P < 0.05$); [b]reduced from control (DMSO) response ($0.10 > P > 0.05$).

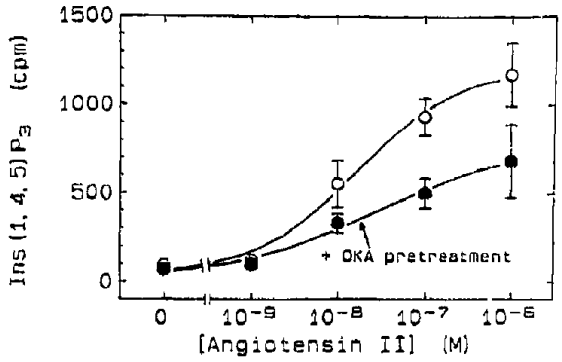

Fig. 2. Okadaic acid pretreatment inhibits angiotensin-stimulated production of Ins(1,4,5)P_3. Angiotensin II stimulates the production of Ins(1,4,5)P_3 with varying kinetics at different agonist concentrations [1]. For these experiments, angiotensin stimulation was terminated at 30 s, since this protocol gives close to the maximum response throughout the concentration range. Data are mean ± S.E.M. from 4 independent experiments. The reduction induced by OKA (30 min at 300 nM) was significant ($P < 0.05$) at all angiotensin II concentrations, including the zero angiotensin control (data: reduction from 91 ± 8 cpm to 68 ± 10 cpm).

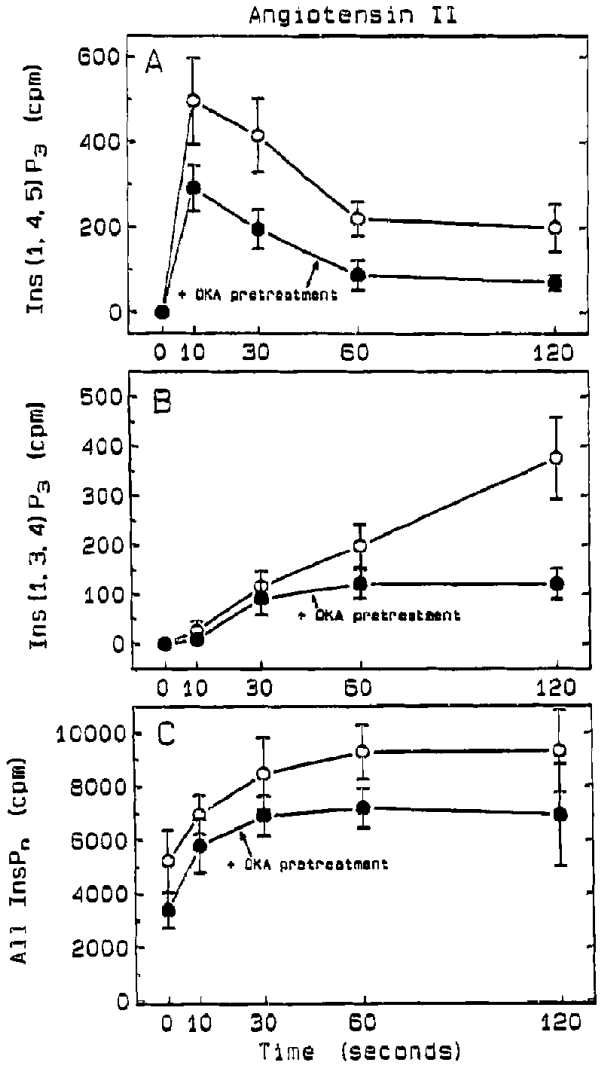

Fig. 3. Okadaic acid inhibition of angiotensin II-stimulated inositol phosphate accumulation. Data are mean ± S.E.M. from 5 independent experiments with 30 min of 300 nM OKA pretreatment and 100 nM angiotensin II stimulation at zero time. (A) Ins(1,4,5)P_3 accumulation. Ins(1,4,5)P_3 was reduced significantly ($P < 0.05$) by OKA pretreatment at 10, 30, 60 and 120 s time points. (B) Ins(1,3,4)P_3 accumulation. Ins(1,3,4)P_3 was reduced significantly ($P < 0.05$) only at 60 and 120 s time-points. (C) accumulation of all inositol phosphates (> 90% inositol monophosphate and bisphosphate, < 10% $InsP_3$ and $InsP_4$); reduced significantly ($P < 0.05$) at 10, 60 and 120 s time-points.

and 5-phosphatase enzymes [29], and thus would be expected to show a similar pattern of reduced accumulation as does Ins(1,4,5)P_3 following OKA pretreatment. Yet at these early time points, when Ins(1,4,5)P_3 accumulation is significantly reduced (Fig. 3A), Ins(1,3,4)P_3 accumulation is not (Fig. 3B). This suggests that there may be stimulated metabolism of Ins(1,4,5)P_3 through the 3-kinase pathway to maintain the levels of Ins(1,3,4)P_3 despite reduced amounts of its precursor, Ins(1,4,5)P_3. Further, OKA-induced inhibition of Ins(1,3,4)P_3 accumulation is only seen at later time points (Fig. 3B) when production of its precursor Ins(1,4,5)P_3 is almost back to basal levels (Fig. 3A). Interestingly, rat liver Ins(1,4,5)P_3 3-kinase has previously been reported to be stimulated following activation of protein kinase C and cAMP-dependent protein kinase [22]. Since OKA is able to increase the phosphorylation state of many of the same phosphoproteins as seen following the activation of such kinases [16], OKA treatment might also be expected to activate this enzyme. Indeed, preparation of cytosolic extracts of hepatocytes from control and 300 nM OKA-treated cells demonstrated a 3.4-fold increase in Ins(1,4,5)P_3 3-kinase activity (control = 354 pmol/min/mg protein; OKA-treated = 1214 pmol/min/mg protein). This would suggest that in addition to a decrease in Ins(1,4,5)P_3 production, there may also be increased metabolism of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 and thence Ins(1,3,4)P_3 in the OKA-treated cells.

4. DISCUSSION

The role of protein phosphorylation in the control of agonist-stimulated Ins(1,4,5)P_3 production and signalling has been further investigated in this study. The striking inhibition of angiotensin II-dependent second-messenger responses induced by OKA is far greater than the modest effects of phorbol diesters on these responses [1,9]. The observation that both OKA and CL-A blunt the $[Ca^{2+}]_i$ response to angiotensin II confirms that the inhibition is likely to be due to increased protein phosphorylation, since these agents share the ability to inhibit phosphoprotein phosphatases 1 and 2A [14,15]. Further, the lack of effect of 3 nM OKA upon angiotensin II-dependent responses, compared with the maximal effect of 300 nM OKA, corresponds with the dose-dependence of increases in protein phos-

phorylation induced by OKA [16]. Since there are phosphoproteins that show increased phosphorylation in response to OKA and CL-A beyond that seen in response to phorbol diesters (J.C. Garrison, unpublished), exemplified by the phosphoprotein previously designated as "spot b" [5], the identification of these phosphoproteins may reveal the sites at which agonist-stimulated Ins(1,4,5)P_3 signalling can be inhibited by phosphorylation.

In rat hepatocytes, there are two classes of agonists that can couple to Ins(1,4,5)P_3 production and increased $[Ca^{2+}]_i$; first, agonists such as epidermal growth factor and phenylephrine produce characteristically small Ins(1,4,5)P_3 responses that can be completely inhibited by pretreatment with phorbol diesters [1,3,4,9–11], putatively through phosphorylation-induced receptor desensitization [9,10,12,13]; second, agonists such as angiotensin II and vasopressin produce characteristically large Ins(1,4,5)P_3 responses which are largely refractory to the action of phorbol diesters, at least at maximal agonist doses [1,3,4,9,11].

OKA and CL-A are able to dramatically inhibit the ability of angiotensin II to raise $[Ca^{2+}]_i$. Several results of this study suggest that the primary effect of OKA on the angiotensin II signalling pathway may lie within the receptor/G protein/phospholipase C system that transduces the angiotensin II stimulus into production of Ins(1,4,5)P_3. For example, OKA and CL-A seem more able to inhibit the increases in $[Ca^{2+}]_i$ induced by agonists that couple through Ins(1,4,5)P_3 production, and, indeed, angiotensin II-stimulated Ins(1,4,5)P_3 accumulation is reduced by OKA with kinetics consistent with the inhibited $[Ca^{2+}]_i$ response. The site in the signalling machinery that is principally inhibited following OKA treatment is unclear, however. The angiotensin receptor in rat hepatocytes which putatively couples to phospholipase C has the characteristics of the AT_1 type [25,30] that has recently been cloned [31,32]. The predicted amino acid sequence reveals a number of potential phosphorylation sites that could be consistent with regulation of receptor function [31,32]. Since there is similar inhibition of responses to vasopressin and ATP as there is to angiotensin II in OKA-treated cells, a receptor-specific inhibitory mechanism would imply that there are equivalent means to inhibit each of these receptor systems. An alternative mechanism that would account for the ability of OKA to induce similar inhibitions of the responses to these agonists would be the phosphorylation of a common component of the signal transduction pathway subsequent to the receptor. A similar conclusion was recently drawn by Garcia-Sainz et al., who showed that OKA was able to inhibit phosphoinositide turnover stimulated by angiotensin II, vasopressin, epinephrine or mastoparan [17]. As yet a precise molecular description of the signalling pathway, specifying the G protein and phospholipase C isozymes concerned, is not available for any of these agonists, and thus the site at which OKA acts to inhibit the production of Ins(1,4,5)P_3 cannot yet be defined.

In addition to the ability of OKA to reduce angiotensin II-stimulated Ins(1,4,5)P_3 production, it seems possible that OKA and CL-A may be able to inhibit agonist-induced calcium signalling in at least two other ways. First, there may be a change in the intracellular calcium pools in hepatocytes treated with OKA or CL-A. Such an alteration may be reflected in the small increase in basal $[Ca^{2+}]_i$ seen in OKA- and CL-A-treated hepatocytes and may partly explain the reduced response to subsequent agonist addition, including the partial reduction seen following stimulation with thapsigargin (Table I). The increase in basal $[Ca^{2+}]_i$ is not likely to be caused by Ins(1,4,5)P_3 since the basal level of Ins(1,4,5)P_3 is apparently suppressed in OKA-treated hepatocytes (Fig. 2). Second, the potential increase in Ins(1,4,5)P_3 3-kinase activity in OKA-treated cells may also reduce the accumulation of Ins(1,4,5)P_3. The net effect of such an increase in 3-kinase activity upon the ability of agonists to stimulate increases in $[Ca^{2+}]_i$ would be difficult to predict, as the product of the 3-kinase, Ins(1,3,4,5)P_4, has also been proposed to play a role in calcium signalling [33].

In conclusion, we have demonstrated that angiotensin-stimulated Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ signalling can be inhibited by OKA and CL-A. These agents increase the phosphorylation state of numerous phosphoproteins in rat hepatocytes by virtue of their ability to inhibit the basal actions of phosphoprotein phosphatases. These results thus provide further evidence that a phosphoprotein may be a component of the signalling machinery that transduces the agonist stimulus into production of Ins(1,4,5)P_3 and subsequent increases in $[Ca^{2+}]_i$. The observation that the inhibition of the angiotensin response is greater than we have previously been able to demonstrate with phorbol diester pretreatment would indicate that this phosphoprotein is a substrate for both protein kinase C and either phosphoprotein phosphatase 1 or 2A, and that increases in the phosphorylation state of this protein produce desensitization of the signalling pathway.

Acknowledgements: We would like to thank Gwendolyn S. Harris and Dr. Cynthia M. Yoshida for preparation of hepatocytes, and Dr. Michael L. Johnson for his invaluable help with data quantitation. OKA for initial experiments was generously provided by Dr. Joseph Di Salvo (Univ. of Minnesota, Duluth) and Dr. Philip Cohen (Univ. of Dundee). This work was supported by DK-19952. RRM is a Howard Hughes Medical Institute Predoctoral Fellow.

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