Biochem. J. (1992) 284, 633–636 (Printed in Great Britain)

RESEARCH COMMUNICATION

Protein kinase-dependent effects of okadaic acid on hepatocytic autophagy and cytoskeletal integrity

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The protein phosphatase inhibitor okadaic acid suppressed autophagy completely in isolated rat hepatocytes, as measured by the sequestration of electroinjected [^{3}H]raffinose into sedimentable autophagic vacuoles. Okadaic acid was effectively antagonized by the general protein kinase inhibitors K-252a and KT-5926, the calmodulin antagonist W-7, and by KN-62, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II). These inhibitors also antagonized a cytoskeleton-disruptive effect of okadaic acid, manifested as the disintegration of cell corpses after breakage of the plasma membrane. CaMK-II, or a closely related enzyme, would thus seem to play a role in the control of autophagy as well as in the control of cytoskeletal organization.

INTRODUCTION

Autophagy is the non-selective process by which cells sequester and degrade portions of their cytoplasm in response to amino acid deprivation or growth-inhibitory signals [1]. In rat hepatocytes, autophagy is modulated by pancreatic and adrenergic hormones as well as by cyclic nucleotides [2], suggesting a regulatory role for protein kinases and protein phosphorylation. In the present study okadaic acid, a protein phosphatase inhibitor [3] that causes generalized hyperphosphorylation of hepatocytic proteins [4], was used along with various protein kinase inhibitors to investigate the role of protein phosphorylation in the control of hepatocytic autophagy.

MATERIALS AND METHODS

Hepatocytes were isolated from 18 h-starved male Wistar rats (250–300 g) by collagenase perfusion [5], electropermeabilized [6], loaded with [^{3}H]raffinose [7] (5 Ci/mmol; du Pont–New England Nuclear), washed and suspended in suspension buffer [5] containing 15 mM-pyruvate. The cells were incubated in 5 cm-diameter albumin-coated plastic Petri dishes [2 ml of cell suspension containing 15 mg of cells (wet wt.)/dish] for up to 3 h at 37 °C. After incubation the cells were electrodisrupted (i.e. the plasma membranes were broken by a high-voltage discharge in a non-ionic medium) and centrifuged through a density cushion to separate the organelle-containing cell corpses from cytosol [6]. Although okadaic acid (OA) treatment of the cells caused disintegration of the majority of the cell corpses, the autophagic vacuoles remained intact and sedimentable. The amount of [^{3}H]raffinose sedimenting with the cell corpse/vacuole pellet was measured and calculated as a percentage of the total cell-associated ^{3}H radioactivity. The net accumulation of sedimentable [^{3}H]raffinose during the incubation period was taken as a measure of autophagic sequestration and expressed as a percentage of the total or as the percentage/h.

All protein kinase inhibitors were tested over a wide dose range (10^{-6}–10^{-4} M, or wider if necessary) in the absence and presence of OA (10–15 nM). The dose of inhibitor capable of producing a 50% inhibition of autophagy, or of reducing the autophagy-inhibitory effect of OA (relative to samples with protein kinase inhibitor alone) by 50%, was estimated from the dose–response curves, sometimes by extrapolation to higher doses. Toxic effects were indicated by a significant loss of total cell-associated ^{3}H radioactivity above the control value, signalling plasma-membrane damage.

To assess the effects of OA and protein kinase inhibitors on the hepatocytic cytoskeleton, the number of structurally coherent cell corpses per unit volume of cell suspension, after electrodisruption, was counted microscopically and expressed as a percentage of the control value.

OA was purchased from Moana Bioproducts, Honolulu, HI, U.S.A. Dichlororibofuranosylbenzimidazole, 6-thioguanine, 2-aminopurine, 6-dimethylaminopurine, H-7, quercetin and W-7 were obtained from Sigma; genistein and tyrphostin from Gibco (Life Technologies, Uxbridge, Middx., U.K.; A-3 and the erbstatin analogue methyl-2,5-dihydroxycinnamate from Biomol Research Laboratories, Plymouth, PA, U.S.A., and KN-62 and CKI-7 from Seikagaku Corp., Chuo-Ku, Tokyo, Japan. K-252a and KT-5926 were kindly given by Dr. H. Kase, Kyowa Hakko Kogyo Co., Sunto-Gun, Japan.

RESULTS

Inhibition of autophagy by OA

In control cells, autophagically sequestered [^{3}H]raffinose was found to accumulate at a rate of 3.5–4.0%/h. Addition of OA to the medium caused a dose-dependent inhibition of autophagy, with essentially complete suppression at 30 nM (Fig. 1*a*). Complete inhibition of autophagy was also observed with the related protein phosphatase inhibitors microcystin and calyculin A, but at somewhat higher doses (results not shown). The inhibition by OA was well sustained during 3 h of incubation (Fig. 1*b*). Upon delayed addition of OA the amount of [^{3}H]raffinose already sequestered was completely retained, showing that OA did not interfere with the integrity of the autophagic vacuoles or with the assay procedure (results not shown). OA, furthermore, had no effect on cellular viability or ATP content (results not shown).

Abbreviations used: CaMK-II, Ca^{2+}/calmodulin-dependent protein kinase II; CK-I, casein kinase I; CK-II, casein kinase II; MLCK, myosin light-chain kinase; OA, okadaic acid; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PKG, cyclic GMP-dependent protein kinase; Tyr kinase, tyrosine protein kinase.

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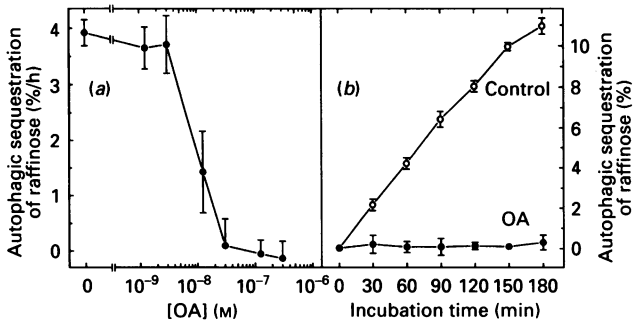

Fig. 1. Inhibition of autophagic sequestration by OA

Hepatocytes electroloaded with [^{3}H]raffinose were incubated at 37 °C in the absence (○) or presence (●) of OA. (*a*) Incubation for 3 h at the concentrations of OA indicated; (*b*) incubation with 30 nM-OA for the length of time indicated. The net amount of [^{3}H]raffinose sequestered into sedimentable autophagic vacuoles during the incubation was measured and expressed as a percentage of the total cell-associated ^{3}H radioactivity. Each value is the mean (±range/S.E.M.) for two to four experiments.

OA-antagonistic effects of protein kinase inhibitors

OA, by inhibiting the important protein phosphatases 1 and 2A, may serve to maintain phosphate groups added to cellular proteins by various protein kinases [8]. In an attempt to identify the protein kinase(s) involved in OA-induced inhibition of hepatocytic autophagy, we tested the ability of various protein kinase inhibitors to antagonize the effect of doses of OA (10–15 nM) which almost completely inhibited autophagy. As an indicator of specificity, the ability of the inhibitors to suppress autophagy on their own (in the absence of OA) was also measured.

Fig. 2 illustrates the dose-dependent effects of three protein kinase inhibitors: CKI-7, a specific inhibitor of casein kinase I (CK-I) [9] (Fig. 2*a*); KT-5926, an inhibitor of several protein kinases, in particular myosin light-chain kinase (MLCK) [10] (Fig. 2*b*); and KN-62, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) [11] (Fig. 2*c*). Whereas CKI-7 was neither autophagy-inhibitory nor OA-antagonistic, the other two antagonized the effect of OA at micromolar concentrations while having relatively little direct effect on autophagy in the absence of OA.

Similar dose–response studies were performed with a number of protein kinase inhibitors, the results being summarized in Table 1. Specific inhibitors of CK-I [9] and CK-II [12], protein kinase N, a nerve-growth-factor-activated enzyme present in PC12 pheochromocytoma cells [13], or haem-regulated and double-stranded RNA-dependent protein kinases [14], had no effect on either parameter, excluding these enzymes from further consideration. 6-Dimethylaminopurine, a meiotic inhibitor suspected to inhibit protein kinase p34^{cdc2} [15], inhibited autophagy at high concentrations, but did not antagonize the effect of OA. The isoquinolinesulphonamide H-7, an effective inhibitor of protein kinase C (PKC), cyclic AMP-dependent protein kinase (PKA) and cyclic GMP-dependent protein kinase (PKG) [16], had some effect on autophagy at very high concentrations, but no OA antagonism was observed. An involvement of any of the above-mentioned enzymes in either autophagy or the action of OA would thus seem unlikely.

Staurosporine, a very general protein kinase inhibitor that potently inhibits PKA and PKC, MLCK, CaMK-II and tyrosine protein kinases (Tyr kinases) [17,18], had a markedly suppressive effect on autophagy. No OA-antagonistic effect was seen, but, in the case of autophagy-inhibitory kinase inhibitors, such antagonism would only be observed if the agent in question were more potent as an antagonist than as an agonist. The same reservation would apply to the Tyr kinase inhibitors tyrphostin [19], erbstatin [19] and quercetin [20]. All of these were effective inhibitors of autophagy, possibly indicating the participation of a Tyr kinase in that process, but none of them showed any OA antagonism. Genistein, which like quercetin is a flavonoid with reported Tyr kinase-inhibitory activity [20], somewhat surprisingly presented the opposite pattern, with marked OA antagonism but little direct effect on autophagy. This deviant effect may indicate that, in hepatocytes, genistein is capable of inhibiting non-Tyr kinases, and suggests that a more extensive investigation of the protein kinase specificities of the flavonoids is warranted.

The naphthalenesulphonamide A-3 inhibits a variety of protein kinases, including PKA, PKG, PKC, MLCK and CK-II [21]. This general protein kinase inhibitor antagonized OA markedly, and also had a suppressive effect on autophagy at somewhat higher concentrations. Two other general protein kinase inhibitors, KT-5926 and K-252a, displayed significant autophagy-inhibitory activity, but they were about five times more potent as OA antagonists. K-252a is known as an inhibitor of PKA, PKG, PKC and MLCK [10] as well as of the Tyr kinase activity of the nerve-growth-factor receptor [22]. KT-5926 is more selective towards MLCK [10]. In addition, both K-252a and KT-5926 were recently shown to be potent inhibitors of CaMK-II [23], implicating the latter enzyme as a possible candidate with respect to autophagy regulation. In support of this contention, KN-62, a highly specific inhibitor of CaMK-II [11], completely reversed

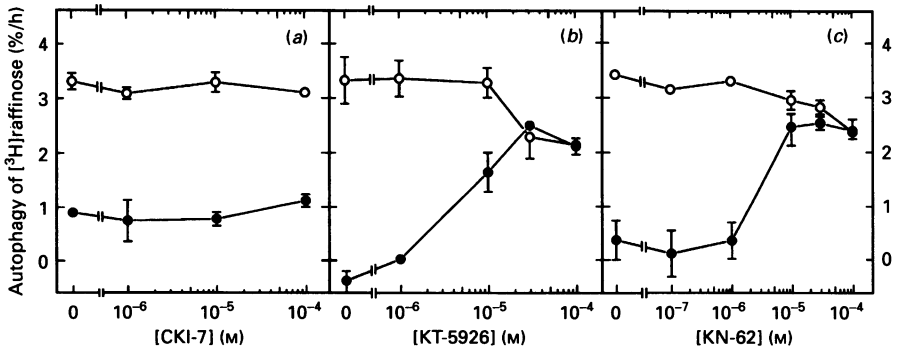

Fig. 2. Antagonistic effects of protein kinase inhibitors on inhibition of autophagy by OA

Hepatocytes electroloaded with [^{3}H]raffinose were incubated for 3 h at 37 °C with (●) or without (○) 10–15 nM-OA and protein kinase inhibitor CKI-7 (*a*), KT-5926 (*b*) or KN-62 (*c*) at the concentrations indicated. Autophagy was measured at the net rate of [^{3}H]raffinose accumulation (%/h) in sedimentable autophagic vacuoles. Each value is the mean (±range/S.E.M.) for two or three experiments. Some of the error bars are hidden by the symbols.

Table 1. OA-antagonistic and autophagy-inhibitory effects of protein kinase inhibitors

Hepatocytes electroloaded with [^{3}H]raffinose were incubated for 3 h at 37 °C with or without OA (10–15 nM) and protein kinase inhibitors at various concentrations. Autophagy was measured as the net rate of accumulation of sedimentable [^{3}H]raffinose during the incubation period. The dose of protein kinase inhibitor capable of producing a 50% suppression of autophagy, or of reducing the autophagy-inhibitory effect of OA by 50%, was estimated from dose–response curves. Each dose value given represents the mean (±range/S.E.M.) for the number of experiments given in parentheses. 'No effect' refers to concentrations up to 1 mM or to toxic levels. A, cyclic AMP-dependent protein kinase; C, protein kinase C; CaM, Ca^{2+}/calmodulin-dependent protein kinase II; G, cyclic GMP-dependent protein kinase; Tyr, Tyr kinase; ds, double-stranded; dep., dependent.

Inhibitor	Preferred protein kinase substrates	Ref(s).	Dose giving 50% antagonism or inhibition (μM)			
			OA antagonism		Inhibition of autophagy	
CKI-7	CK-I	[9]	No effect	(1)	No effect	(1)
5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole	CK-II	[12]	No effect	(1)	No effect	(1)
6-Thioguanine	Protein kinase N	[13]	No effect	(1)	No effect	(1)
2-Aminopurine	Haem-/dsRNA-dep. kinases	[14]	No effect	(3)	No effect	(4)
6-Dimethylaminopurine	Protein kinase p34^{cdc2}?	[15]	No effect	(1)	400±100	(2)
H-7	A, G, C	[16]	No effect	(4)	650±130	(4)
Staurosporine	A, G, C, MLCK, CaM, Tyr	[17,18]	No effect	(2)	60±15	(2)
Erbstatin analogue*	Tyr	[19]	No effect	(2)	60±0	(2)
Tyrphostin	Tyr	[19]	No effect	(1)	100	(1)
Quercetin*	Tyr	[20]	No effect	(1)	80	(1)
Genistein	Tyr	[20]	80±20	(3)	800±300	(3)
A-3*	A, G, C, MLCK, CK-II	[21]	100±50	(3)	300±100	(6)
KT-5926	A, G, C, MLCK, CaM	[10,23]	25±12	(3)	130±30	(3)
K-252a	A, G, C, MLCK, CaM, Tyr	[10,22,23]	7±0	(2)	25±5	(2)
KN-62	CaM	[11]	4±1	(3)	500±250	(3)
W-7 (calmodulin antagonist)*	MLCK, CaM	[21]	90±20	(4)	300±100	(2)

* Toxic effect (reduced plasma-membrane integrity) above 100 μM.

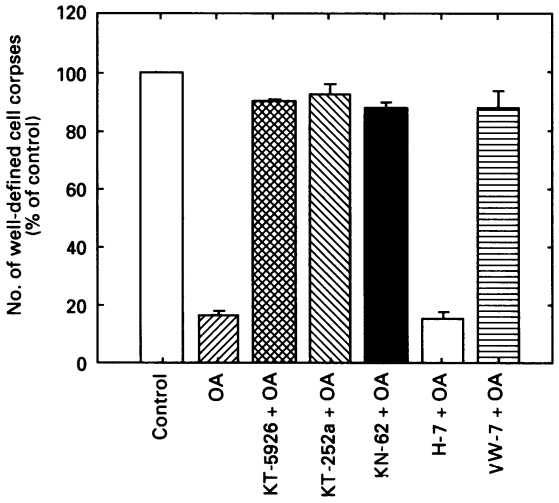

Fig. 3. Antagonistic effects of protein kinase inhibitors on OA-induced disruption of the hepatocytic cytoskeleton

Hepatocytes were incubated for 3 h at 37 °C with or without 15 nM-OA in combination with protein kinase inhibitors KN-62 (10 μM), K-252a (100 μM), KT-5926 (100 μM), H-7 (300 μM) or the calmodulin antagonist W-7 (150 μM). After electrodisruption the number of structurally recognizable cell corpses [6] per unit volume of cell suspension was counted microscopically and expressed as a percentage of the control value. Each value is the mean (±S.E.M.) for three to six experiments.

the effect of OA, while alone having only a moderate effect on autophagy (Fig. 2a and Table 1). Involvement of a calmodulin-dependent enzyme was further indicated by the OA-antagonistic effect of W-7, a calmodulin antagonist [21] that had only a moderate direct inhibitory effect on autophagy. These results would seem to favour CaMK-II [24,25], or a closely related enzyme, as the protein kinase most likely to be responsible for the autophagy-inhibitory phosphorylation potentiated by OA.

Effects of inhibitors on the hepatocytic cytoskeleton

During the course of these studies we observed that OA-treated hepatocytes, rather than forming coherent cell corpses on electrodisruption [6], tended to disintegrate and take on the appearance, when viewed in the optical microscope, of a homogenate containing only a few recognizable cell corpses. OA thus appears to induce a general disruption of the cytoskeletal network. To see if the latter effect, like the inhibition of autophagy, could be antagonized by protein kinase inhibitors, we quantified cytoskeleton disruption by counting the number of recognizable cell corpses per unit volume of electrodisrupted material after treatment of the intact cells with various combinations of OA and inhibitors. As shown in Fig. 3, K-252a, KT-5926, KN-62 and the calmodulin antagonist W-7 effectively prevented the cytoskeleton-disruptive effect of OA. H-7, which does not inhibit CaMK-II, and which failed to antagonize the autophagy-inhibitory effect of OA, was also unable to reverse the cytoskeleton-disruptive effect of the latter. On the basis of the known inhibitor specificities, these effects would seem to indicate that CaMK-II may be involved in regulation of the structural organization of the hepatocytic cytoskeleton.

DISCUSSION

The parallel effects of OA and the other inhibitors on hepatocytic autophagy and cytoskeletal integrity may indicate an involvement of cytoskeletal proteins in the autophagic process. It

is not unreasonable to assume that the initial spreading and folding of the sequestering organelle, the phagophore [26], may require the formation of a filamentous scaffold, and/or anchorage to the cytoskeleton. The putative cytoskeletal element involved is probably not the microtubules, since the microtubule poison vinblastine has only a moderate effect on autophagic sequestration [26]. The inability of the microfilament inhibitor cytochalasin B to affect autophagic-lysosomal protein degradation [27] would, furthermore, speak against a role for microfilaments, leaving intermediate filaments as the most likely candidate.

Previous studies have shown that that OA and related protein phosphatase inhibitors induce pronounced morphological alterations in hepatocytes, including reorganization of the microfilament and intermediate-filament networks, organelle segregation, surface blebbing and reduced attachment to the culture substratum [28–31]. These alterations could possibly reflect the documented ability of CaMK-II to phosphorylate cytoskeletal proteins like vimentin and myosin light chains as well as cross-linking proteins like microtubule-associated protein 2, tau and synapsin [24,32–34]. Whether OA acts by inhibiting dephosphorylation of CaMK-II itself [24] or of its substrate proteins is not clear, nor is it known whether CaMK-II is the exclusive mediator of ultrastructural changes induced by OA.

Several growth factors elicit intracellular Ca^{2+} release and activation of CaMK-II [35,36], and it is noteworthy that the structural changes initiating mitosis can be blocked by microinjection of CaMK-II-inhibitory peptides or antibodies [37]. CaMK-II may thus have a growth-regulatory function analogous to that of $p34^{cdc2}$ kinase, which upon microinjection induces the characteristic reorganization of the cytoskeletal network observed in mitotic cells [38].

The cellular properties induced by OA and CaMK-II (disassembled cytoskeleton and suppressed autophagy) resemble the characteristics of rapidly proliferating cells, and it should be borne in mind that OA and related compounds like microcystin are potent tumour promoters in many tissues, including the liver [39,40]. If CaMK-II plays a role in the abnormal growth of cancer cells, selective CaMK-II inhibitors may represent an interesting starting point for the development of new antineoplastic agents.

We thank Ms. Mona Birkeland and Ms. Margrete Fosse for expert technical assistance. This work was generously supported by The Norwegian Cancer Society.

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Received 18 March 1992/6 April 1992; accepted 7 April 1992