Protein phosphatase composition in the smooth muscle of guinea-pig ileum studied with okadaic acid and inhibitor 2

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Using okadaic acid, a potent inhibitor of type 2A and type 1 protein phosphatases, and inhibitor 2, an intrinsic inhibitory factor of type 1 phosphatase, we characterized the phosphorylated myosin light-chain (PMLC) phosphatase activity in the smooth-muscle extracts of guinea-pig ileum. In the intact fibres the control activity was 254 ± 13 nmol of P_i /min per g wet wt. ($n = 15$) against ^{32}P -labelled PMLC ($4 \mu M$) from chicken gizzard. The following phosphatase fractions were identified: an inhibitor-2-sensitive (type 1) fraction (fractional activity = 35%), a Mg^{2+} -dependent and okadaic acid-insensitive (type 2C) fraction (17%), and two type 2A-like fractions that had different susceptibility to okadaic acid. The type 2A-like fraction with lower affinity to okadaic acid accounted for 30% of the control activity. After the cell membrane was permeabilized by Triton X-100, more than 60% of this fraction remained and accounted for about 90% of the total activity, whereas the other fractions were nearly abolished. The type 2A-like fraction may be bound to some intracellular structure such as contractile proteins.

INTRODUCTION

According to the current theory of smooth-muscle activation, contractile force depends on the phosphorylation state of myosin light chains, which is determined by the activity balance between phosphorylated myosin light-chain (PMLC) phosphatase and Ca^{2+} -calmodulin-dependent myosin light-chain kinase (for review see, e.g., Hartshorne & Siemankowski, 1981). Phosphorylase, the rate-limiting enzyme of glycogenolysis, is also phosphorylated during contraction in a variety of smooth muscles, suggesting a co-ordinated regulation of contractility and metabolism by phosphorylation (Diamond & Brody, 1966; Namm, 1971; Silver & Stull, 1982). In this scheme, the intracellular activity of protein phosphatase(s) plays an essential role in dephosphorylating the regulatory proteins to turn off the contractile and metabolic systems. Several protein phosphatases which dephosphorylate myosin, phosphorylase *a* and/or phosphorylase kinase have been isolated from some restricted types of smooth-muscle tissue such as avian gizzard (Pato & Adelstein, 1980; Onishi *et al.*, 1982; Pato & Kerc, 1985) and bovine aorta (Werth *et al.*, 1982; DiSalvo *et al.*, 1982). For most mammalian smooth muscles, however, little is known about the phosphatase composition, mainly because of the limited amount of tissue available for biochemical analyses.

Recently a marine sponge toxin, okadaic acid, was reported to have a potent inhibitory action on the PMLC phosphatase activity of the smooth muscle tissues (Takai *et al.*, 1987; Bialojan *et al.*, 1988; see Takai, 1988, for a review). The specificity and kinetics of the inhibitory

action have been studied by using purified protein phosphatases (Hescheler *et al.*, 1988; Bialojan & Takai, 1988; Haystead *et al.*, 1989). The toxin has an especially high affinity to the catalytic subunits of type 2A and type 1 phosphatase (for classification of protein phosphatases see Ingebritsen & Cohen, 1983). Type 2B phosphatase (= calcineurin) is inhibited to a lesser extent, whereas type 2C phosphatase is not affected by okadaic acid. Type 1 phosphatase, which is thought to be responsible for dephosphorylation of the regulatory subunit of phosphorylase kinase (Ingebritsen *et al.*, 1983), is specifically inhibited by nanomolar concentrations of two heat-stable proteins, termed inhibitor 1 and inhibitor 2 (Huang & Glinzmann, 1976; Cohen *et al.*, 1977). Type 2B phosphatase is strictly dependent on Ca^{2+} and calmodulin for activity, and is unaffected by Mg^{2+} (Stewart *et al.*, 1983), whereas type 2C phosphatase has an absolute requirement for Mg^{2+} and is not affected by either Ca^{2+} or calmodulin (Ingebritsen *et al.*, 1983). Neither type 1 nor type 2A phosphatase is affected by these bivalent cations and calmodulin. These characteristics of the protein phosphatases allow us to inhibit each of them selectively, and thereby we can identify them when they are present as a mixture in a relatively small amount of tissue extract.

In the present experiments we have examined the effect of applying the phosphatase inhibitors and/or removing the bivalent cations on the PMLC phosphatase activity in the smooth-muscle extract of guinea-pig ileum. The results indicate that the ileal extract contains at least four distinct phosphatase activities: an inhibitor 2-sensitive (type 1) phosphatase activity, a Mg^{2+} -dependent and okadaic acid-insensitive (type 2C) phosphatase activity

Abbreviation used: PMLC, phosphorylated myosin light chain.

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and two type 2A-like phosphatase activities that have different susceptibilities to okadaic acid.

EXPERIMENTAL

Materials

Okadaic acid, isolated from the black sponge *Halichondria okadai*, was kindly given by Dr. Y. Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan). [γ - 32 P]ATP was obtained from NEN. ATP and calmodulin (from pig brain) were purchased from Boehringer Mannheim. All other chemical reagents were products of Sigma Chemical Co.

Preparation of protein phosphatases

The catalytic subunits of type 1 phosphatase and type 2A phosphatase (type 2A_c) were prepared from rabbit skeletal muscle as described by Tung *et al.* (1984). The aortic type 2A phosphatase, which has a subunit structure comprising three polypeptides, was isolated from bovine aorta by the procedure described previously (DiSalvo *et al.*, 1982; Bialojan & Takai, 1988). [This enzyme has been referred to as a PCM (polycation modulable) phosphatase, because it is markedly stimulated by polycations such as polylysine.] The type 2C phosphatase was purified from rabbit liver by the method of McGowan & Cohen (1987).

Preparation of substrates

Phosphorylatable myosin light chains were isolated from chicken gizzard and bovine heart as described by Cummins & Lambert (1986). They were [32 P]phosphorylated (DiSalvo *et al.*, 1982) by using myosin light-chain kinase from chicken gizzard (Ngai *et al.*, 1984).

Preparation of muscle extracts

Guinea pigs of either sex, weighing about 450 g, were stunned and bled, and the ileum was isolated. The longitudinal and circular smooth-muscle layers were carefully dissected out under a binocular microscope. The muscles were incubated for 1 h at 35 °C in the Hepes-Tyrod solution containing 136 mM-NaCl, 5.9 mM-KCl, 1.2 mM-CaCl₂, 1.2 mM-MgCl₂, 11.6 mM-Hepes and 11.6 mM-dextrose (pH 7.4). After incubation they were blotted and weighed (wet weight). The PMLC phosphatase activities of the muscle extracts were normalized to this weight.

Some longitudinal muscle fibres were treated for 1 h at 4 °C with a skinning solution containing 30 mM-imidazole, 4 mM-EGTA, 5 mM-KCl, 50 mM-sucrose, 2 mM-dithioerythritol and 1% (v/v) Triton X-100 (pH 7.4 at 25 °C; adjusted with KOH). The fibres were incubated at -20 °C in 500 vol. of a glycerol solution of the following composition: 30 mM-imidazole, 4 mM-EGTA, 10 mM-MgCl₂, 7.5 mM-ATP, 1 mM-NaN₃, 2 mM-dithioerythritol and 50% (v/v) glycerol (pH 6.7 at 25 °C). They were used 4–5 days after skinning for phosphatase assays. After the skinning treatments, the plasma membranes of the muscle fibres are permeabilized to large molecules such as calmodulin or protein phosphatases (DiSalvo *et al.*, 1983a). The skinned fibres contract reversibly in response to Ca²⁺ application in the presence of calmodulin and MgATP (see, e.g., Bialojan *et al.*, 1988). The purified protein phosphatases could be preserved in the glycerol solution at -20 °C for at least 1 week with minimal loss of activity.

For the phosphatase assays, the muscle fibres were homogenized in 5 vol. (v/w) of an ice-cold solution containing 40 mM-Tris/HCl, 60 mM-KCl, 5 mM-EGTA, 1 mM-dithioerythritol and 0.1 mM-phenylmethanesulphonyl fluoride (pH 7.4 at 30 °C). The homogenates were centrifuged for 15 min at 2 °C and 17 500 g, and resulting supernatants were used for the assay of phosphatase activities.

Assay of protein phosphatase activities

All assays were carried out at 30 °C. Unless otherwise mentioned, reaction mixtures contained 40 mM-Tris/HCl, 60 mM-KCl, 10 mM-MgCl₂, 5 mM-EGTA, 1 mM-dithioerythritol and 1 mg of bovine serum albumin/ml (pH 7.4). Reactions were started by injecting 32 P-labelled myosin light chains (PMLC; 4 μ M) into the reaction mixture, and terminated by addition of ice-cooled trichloroacetic acid (20%, w/v) and 6 mg of bovine serum albumin/ml. Released P_i was measured by a standard procedure (DiSalvo *et al.*, 1982). The PMLC phosphatase activities in the reaction mixture were such that less than 5% of the radioactive substrate was consumed during the reaction time. Under such conditions the reactions were linear against time.

Okadaic acid was dissolved in pure dimethyl sulphoxide and added to the experimental solutions. The maximal concentration of dimethyl sulphoxide was 0.1% (v/v). The control activities were not changed by addition of this amount of the solvent.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Dose-inhibition relationships

Let a solution contain n different types of protein phosphatase, each of which has a definite affinity to the inhibitor (okadaic acid). If the enzymes have no interaction with each other, the total protein phosphatase activity of the solution in the presence of the inhibitor, V , is given as the sum of the Hill functions, i.e.:

$$V = V(I) = \sum_{k=1}^n \frac{v_k}{1 + (I/K_k)^{h_k}} \quad (1)$$

where I is the concentration of the inhibitor, v_k is the partial activity, K_k is the concentration required to obtain 50% inhibition (ID₅₀), h_k is the Hill coefficient, and the integer k indicates the k th phosphatase. Note that the values of the Hill coefficient are not necessarily equal to 1 for the inhibition of protein phosphatase by okadaic acid (see Bialojan & Takai, 1988). In the present results, the values of the constants were estimated by fitting the dose-inhibition relationships to eqn. (1) by a non-linear least-squares method with the use of a computer program (Snedecor & Cochran, 1980). It should be noted that the fitting procedure itself does not determine the number of phosphatases, n . It must be specified before application of the method, on the basis of experimental observations.

Statistics

The numerical values were presented as means $\pm$ S.E.M., and differences were assessed by Student's t test (Snedecor & Cochran, 1980). Differences were evaluated as statistically significant when a two-sided probability of less than 0.05 was obtained.

Table 1. PMLC phosphatase activity of the purified protein phosphatases

The gizzard PMLC phosphatase activity and the cardiac PMLC phosphatase activity of the purified protein phosphatases were measured. The concentrations of okadaic acid required to inhibit the cardiac PMLC phosphatase activity by 50% (ID_{50}) are also listed for reference (see Bialojan & Takai, 1988). See the text for further details: $n = 3$.

Phosphatase	$10^{-3} \times$ PMLC phosphatase activity (units/mg of protein)		G/H	ID_{50} (nM)
	Gizzard (G)	Cardiac (H)		
Type 1	0.47 ± 0.02	0.050 ± 0.002	9.4	315
Type 2A _c (skeletal muscle)	9.72 ± 0.11	8.10 ± 0.15	1.2	1.2
Type 2A (aorta)	15.10 ± 0.13	1.14 ± 0.05	10.8	205
Type 2C	3.78 ± 0.08	0.096 ± 0.002	39.4	$\geq 10 \mu M$

RESULTS

PMLC phosphatase activity of the purified protein phosphatases

Table 1 gives the PMLC phosphatase activity of the purified protein phosphatases relevant to the present results. The measurements were made with PMLC ($4 \mu M$) prepared from chicken gizzard and bovine heart as substrate. The list includes previous results concerning the affinity of the enzymes to okadaic acid for a reference (Bialojan & Takai, 1988). The activities against gizzard PMLC were markedly higher than those against cardiac PMLC, except for the catalytic subunit of type 2A phosphatase, which dephosphorylated either type of PMLC at a similar rate.

Inhibition of the PMLC phosphatase activity in the ileal extracts by okadaic acid

As shown in Fig. 1(a), okadaic acid (0.1 pM – $10 \mu M$) had a dose-dependent inhibitory effect on the PMLC phosphatase activity in the crude extract of the longitudinal smooth-muscle layer of guinea-pig ileum. When gizzard PMLC ($4 \mu M$) was used as substrate ($\circ$), the dose-inhibition curve appeared to have three components (I, II and III; see also Fig. 2). The partial activities of components I, II and III are given in Table 2 as v_1 , $v_2 + v_3$ and v_4 respectively. (From the reasons described below, the fitting procedure to determine the parameters of the dose-inhibition curves was performed on the assumption that there are four rather than three different types of protein phosphatase in the muscle extract.) A consider-

Fig. 1. Inhibition of the PMLC phosphatase activity in ileal extracts by okadaic acid

The inhibitory effect of okadaic acid on the protein phosphatase activity in the extracts of ileal smooth muscle was examined with gizzard PMLC ($\circ$; $4 \mu M$) and cardiac PMLC ($\bullet$; $4 \mu M$) as substrates. (a) Longitudinal muscle layer. (b) Circular muscle layer. A Ca^{2+} -free Tris buffer containing 4 mM-EGTA and 10 mM- Mg^{2+} (pH 7.4) was used. The dose-inhibition relationship appeared to have three components (I, II, III and I', II', III'). The curves are least-squares fits to eqn. (1). The parameters characterizing the curves are given in Table 2. The broken lines indicate the partial activities of the components (Table 2). See the text for further explanation.

Table 2. Parameters of the dose-inhibition relationships

The values characterizing the dose-inhibition curves illustrated in Figs. 1 and 2 were determined by least-squares fitting of the data to eqn. (1) (see the Experimental section), assuming the presence of four distinct phosphatase activities in the extracts (see the Discussion section). I (I'), II (II') and III (III') indicate the components of the curves (Figs. 1 and 2). v_k , K_k and h_k denote the partial activity (units/g wet wt.), the ID_{50} (pM or nM) and the Hill coefficient of the k th phosphatase activity respectively. The type 1 activity was assumed to be abolished (i.e. $v_3 = 0$) in the presence of 100 nM inhibitor 2 (see the text). K_4 was assumed to be infinite. $V(0)$ stands for the total activity in the absence of okadaic acid. Abbreviation: n.d., not determined.

Muscle layer	Substrate and inhibitor	I (I')			II (II')						III (III')	V(0) (units/g wet wt.)
		v ₁	K ₁ (pM)	h ₁	v ₂	K ₂ (nM)	h ₂	v ₃	K ₃ (nM)	h ₃	v ₄	
Longitudinal	Gizzard PMLC	44	40	1.04	77	18	0.92	90	232	0.86	43	254 ± 13 (n = 15)
	+inhibitor 2	45	36	1.01	73	15	0.89	0	—	—	43	161 ± 17 (n = 4)
	Cardiac PMLC	37	52	0.96	6	13	0.88	9	260	0.82	1.4	54 ± 7 (n = 5)
	+inhibitor 2		n.d.			n.d.		0	—	—	1.4	45 ± 3 (n = 3)
Circular	Gizzard PMLC	74	40	1.01	115	19	0.90	133	217	0.89	43	364 ± 19 (n = 10)
	+inhibitor 2	70	45	1.03	123	15	0.85	0	—	—	42	235 ± 22 (n = 3)
	Cardiac PMLC	64	45	1.00	12	12	0.93	15	229	0.84	2	93 ± 7 (n = 7)
	+inhibitor 2		n.d.			n.d.		0	—	—	2	78 ± 8 (n = 3)
	Category of activity...	type 2A-like			type 2A-like			type 1			type 2C	

able portion of the control activity [Table 2; $V(0)$] was inhibited by okadaic acid in the concentration range 1 pM–1 nM (component I). The major part of the curve appeared in the range 1 nM–10 μ M (component II). More than 15 % of the control activity was not inhibited by up to 10 μ M-okadaic acid (component III).

When the substrate was PMLC (4 μ M) from bovine heart (Fig. 1a; ●), the control activity was about one-fifth of that obtained with gizzard PMLC (Table 2). In this case, too, the dose-inhibition relationship appeared to have three components (I', II' and III'). Components I', II' and III' were located in approximately the same concentration ranges of okadaic acid as components I, II and III respectively. However, the fractional activities of the components, as well as the control activities, differed markedly between these two dose-inhibition relationships. The values of v_1 were similar for both types of PMLC, whereas the values of $v_2 + v_3$ and v_4 for cardiac PMLC were about 9 % and 3 % of those for gizzard PMLC respectively (Table 2).

Qualitatively similar dose-inhibition relationships with three components were obtained for the extract of the circular muscle layer of the ileum (Fig. 1b; see also Table 2).

It has been shown that the affinities of the purified protein phosphatases are nearly constant for different types of substrate (Bialojan & Takai, 1988). Accordingly, the dose-inhibition relationships with multiple components may suggest that the smooth-muscle extracts contain several types of protein phosphatase which have different affinities for okadaic acid and dephosphorylate gizzard PMLC and cardiac PMLC at different rates (cf. Table 1). This possibility has been further examined in the following experiments.

Effects of inhibitor 2

As shown in Table 2, inhibitor 2 partially suppressed the PMLC phosphatase activity in the ileal extracts [see the values of $V(0)$]. For the longitudinal muscle layer, the

phosphatase activities against gizzard PMLC and cardiac PMLC were decreased by 37 % and 17 % respectively in the presence of 100 nM-inhibitor 2 (Table 2). At this concentration, inhibitor 2 suppressed more than 95 % of the purified type 1 phosphatase activity measured with

Fig. 2. Change of the dose-inhibition relationship by inhibitor 2

The experiments are similar to those illustrated in Fig. 1. The protein phosphatase activity in the extract of ileum (longitudinal muscle layer) was measured against gizzard PMLC (4 μ M). The inhibitory action of okadaic acid was examined either in the absence (○) or in the presence (●) of inhibitor 2 (100 nM). Inhibitor 2 decreased a part of component II, whereas it had little effect on components I and III. The parameters characterizing the dose-inhibition curves are included in Table 2. See the text for further explanation.

either type of PMLC ($4 \mu\text{M}$), but it had little effect on the catalytic subunit of type 2A phosphatase. [High concentrations of inhibitor 2 reportedly inhibit type 2A phosphatase also (see Brautigan *et al.*, 1986).] Similar inhibitory action was observed for the circular muscle layer (Table 2). These findings indicate the presence of type 1 phosphatase in the muscle extracts. The results were not significantly changed when the concentration of inhibitor 2 was increased to 300 nM, suggesting that the type 1 phosphatase activity in the extract was nearly abolished by inhibitor 2. Therefore the partial activity of the type 1 fraction (Table 2; v_3) was estimated as the difference between the control activity and the activity in the presence of 100 nM inhibitor 2 (see 'Fitting procedure' below).

Fig. 2 shows the change in the dose-inhibition relationship in the presence of inhibitor 2. The parameters of the curves are included in Table 2 (for the fitting procedure to determine the parameters see below). Gizzard PMLC ($4 \mu\text{M}$) was used as substrate. Inhibitor 2 (100 nM) decreased the partial activity of component II, whereas it had little effect on components I and III. Essentially similar results were obtained for the circular muscle extract (Table 2). These observations support the idea that the inhibitor-2-sensitive (type 1) phosphatase activity is related to a part of component II in the dose-inhibition relation.

The effect of inhibitor 2 was also examined in the skinned preparations of the longitudinal fibres (Table 3). The phosphatase activities against gizzard PMLC and cardiac PMLC both decreased by only about 10% in the presence of inhibitor 2 (100 nM). Thus the inhibitor-2-

sensitive fraction of the PMLC phosphatase activity was smaller in the skinned fibres than in the intact fibres [cf. Table 2; $V(0)$].

Fig. 3 shows the dose-inhibition relationships for the skinned preparations. The data were well fitted by a single Hill function. For the curve obtained with gizzard PMLC the value of the Hill coefficient (h) was 0.87, and the concentration of okadaic acid required to obtain 50% inhibition (ID_{50}) was 15 nM. The parameters had nearly the same values ($h = 0.88$ and $ID_{50} = 20$ nM) for cardiac PMLC. These values are very similar to those reported for the cardiac PMLC phosphatase activity in the skinned fibres of the guinea-pig taenia coli (Takai *et al.*, 1987). At concentrations of lower than 0.1 nM, okadaic acid had little inhibitor effect on the PMLC phosphatase activity, which was almost abolished by $10 \mu\text{M}$ -okadaic acid (Fig. 3). Thus component I (I') and component III (III') as observed in the intact fibres (see Fig. 1) disappeared after skinning. These observations support the idea that components I, II and III of the dose-inhibition relationship correspond to different phosphatase activities. In the skinned fibre, nearly 90% of the PMLC phosphatase activity was insensitive to inhibitor 2 (Table 3), and the dose-inhibition relationship was located in almost the same concentration range of okadaic acid as was component II (cf. Fig. 1 and Table 2). Therefore the main part of the phosphatase activity remaining after skinning is likely to be the same phosphatase activity that gave rise to the inhibitor-2-insensitive fraction of component II (see Fig. 2).

Mg²⁺ requirement of the okadaic acid-insensitive fraction

Table 4 shows the effect of Mg²⁺ removal of the okadaic acid-resistant fraction of the PMLC phosphatase activity in the longitudinal smooth muscle. In the solution containing 10 mM-Mg²⁺, a considerable portion of the PMLC phosphatase activity was not inhibited by $10 \mu\text{M}$ -okadaic acid (see also Figs. 1 and 2; components III and III'). Under this condition, gizzard PMLC gave 30-fold higher activity than cardiac PMLC. The okadaic acid-resistant fraction was strikingly decreased by removing Mg²⁺ from the reaction mixture (Table 4).

Very similar results were obtained with the purified type 2C phosphatase, which is not affected by up to $10 \mu\text{M}$ -okadaic acid (Bialojan & Takai, 1988). In the presence of Mg²⁺ the gizzard PMLC activity was about 40-fold higher than the cardiac PMLC phosphatase activity (see Table 1). The PMLC phosphatase activities

Fig. 3. Inhibition of the PMLC phosphatase activity in the extract of the skinned ileal smooth muscle

The experiments are similar to those illustrated in Fig. 1. Gizzard PMLC ($4 \mu\text{M}$; ○) and cardiac PMLC ($4 \mu\text{M}$; ●) were used as substrates. Note the disappearance of components I and III after the skinning procedure (cf. Fig. 1a). See the text for further explanation.

Table 3. Effect of inhibitor 2 on the PMLC phosphatase activity in the skinned fibre

The phosphatase activity in the extract of the skinned ileal smooth muscle (longitudinal layer) were examined in the absence (control) and presence of 100 nM inhibitor 2 ($n = 3$). The concentration of substrate (PMLC) was $4 \mu\text{M}$.

	PMLC phosphatase activity (units/g wet wt.)	
	Gizzard	Cardiac
Control	52 ± 4	5.4 ± 1.4
Inhibitor 2 (100 nM)	47 ± 4	4.8 ± 1.0

Table 4. Effect of removal of Mg^{2+} on the okadaic acid-insensitive fraction

The PMLC phosphatase activity in the extract of the ileal smooth muscle (longitudinal layer) was measured in the presence of $10 \mu M$ -okadaic acid ($n = 3$). The concentration of substrate (PMLC) was $4 \mu M$. Note the marked decrease in the activity by Mg^{2+} removal.

[Mg^{2+}] (mM)	PMLC phosphatase activity (units/g wet wt.)	
	Gizzard	Cardiac
10	42 ± 3	1.4 ± 0.1
0	3.6 ± 0.2	0.26 ± 0.03

were decreased to less than 1% of the control values by removal of Mg^{2+} , confirming the previous report (Ingebritsen *et al.*, 1983). In the muscle extract, some activity remained in Mg^{2+} -free conditions (Table 4). This is probably due to the residual activity of the component II fraction (see Figs. 1 and 2). These observations indicate that the ileal smooth muscle contains a phosphatase activity which belongs to the category of type 2C phosphatase.

No Ca^{2+} + calmodulin-dependent phosphatase activity in the smooth-muscle extracts

All the experiments described above were done with Ca^{2+} -free and calmodulin-free solution containing 5 mM-EGTA (see the Experimental section). Neither the gizzard PMLC phosphatase activity nor the cardiac PMLC phosphatase activity in the ileal extracts was significantly increased by adding Ca^{2+} and calmodulin. For example, the gizzard PMLC ($4 \mu M$) activity of the longitudinal muscle extract in the absence and presence of 0.2 mM- Ca^{2+} and 0.2 μM -calmodulin was 262 ± 18 and 265 ± 19 units (nmol of P_i /min)/g wet wt. ($n = 4$) respectively. Therefore the smooth muscle of the ileum appeared to have little, if any, type 2B phosphatase (=calcineurin) activity.

The PMLC activities in the ileal extracts were also unaffected when either 0.1 mM- $MnCl_2$ (0 EGTA) or 0.1 mM-ATP was added to the reaction mixture.

Fitting procedure

The values of the parameters of the dose-inhibition relationships listed in Table 2 were determined mostly by least-squares fitting of the dose-inhibition relationships to eqn. (1) (see the Experimental section). The following parameters were specified or assumed before application of the fitting procedure, based on the experimental results.

- (1) The number of phosphatases, $n = 4$.
- (2) For the inhibitor-2-sensitive (type 1) fraction and the okadaic acid-insensitive (type 2C) fraction we assigned the index numbers ($= k$) 3 and 4 respectively. The indices 1 and 2 were assigned so that $K_1 < K_2$. (Such an arbitrary assignment of k does not essentially alter the results of the fitting procedure.)
- (3) According to the assignment of k , K_4 was assumed to be infinite.
- (4) The partial activity of the type 1 activity ($= v_3$) was determined as the difference between the control activity

and the activity in the presence of 100 nM inhibitor 2, assuming that the type 1 activity was specifically abolished by inhibitor 2 (see above); i.e. $v_3 = 0$ for the results with inhibitor 2. This assumption seems to be adequate because the dose-inhibition relationships were well fitted to eqn. (1), and the values of v_k and K_k ($k = 1, 2$ and 4) in the presence of inhibitor 2 agreed well with those in the absence of inhibitor 2 (Table 2).

DISCUSSION

The present results suggest the presence of the following four protein phosphatase activities in the ileal extracts (see Table 2). (1) A phosphatase activity that has a very high affinity (i.e. a very low value of ID_{50} , K_1) to okadaic acid, but is not affected by inhibitor 2. This phosphatase activity, which dephosphorylates gizzard PMLC and cardiac PMLC at similar rates, accounts for component I (I') of the dose-inhibition curve and has similarities to the catalytic subunit of the type 2A phosphatase from rabbit skeletal muscle (cf. Table 1). (2) A phosphatase activity that has a high susceptibility to okadaic acid, but is not affected by inhibitor 2. The inhibitor-2-insensitive fraction of component II (Fig. 2) is attributed to this enzyme activity, for which gizzard PMLC is a better substrate than cardiac PMLC (Table 2). This phosphatase activity has some resemblance to the aortic type 2A phosphatase, but the values of ID_{50} are different by a factor of 10 (cf. Table 1). (3) A type 1 phosphatase activity that has a relatively high affinity to okadaic acid ($ID_{50} = K_3$), and accounts for the inhibitor-2-sensitive fraction of component II (Fig. 2). (4) A type 2C phosphatase activity that is not inhibited by up to $10 \mu M$ -okadaic acid and gives rise to component III in the dose-inhibition curve. This enzyme activity is characterized by a strict dependence on Mg^{2+} for activation (Table 4). These four activities account for virtually all the PMLC phosphatase activity in the ileal extracts. No Ca^{2+} + calmodulin-dependent activity is detected in the ileal extract (see the Results section). In bovine aorta a type 1 phosphatase which is activated by MgATP (DiSalvo *et al.*, 1982) and an inhibitor-2-insensitive phosphatase which requires Mn^{2+} for activity (Werth *et al.*, 1982) have been described. In the ileal extracts, neither MgATP-stimulated activity nor Mn^{2+} -dependent activity has been identified (see the Results section).

An advantageous feature of the procedure described in this paper is that it can be applied to a relatively small amount of tissue extract. However, it should be noted that this procedure does not distinguish isoenzymes which have similar susceptibility to the inhibitors and similar dependence on the bivalent cations. Further studies are necessary to establish whether each of the multiple phosphatase activities is ascribable to a single enzyme or to separate distinct enzymes.

In the ileal extract the relation between the concentration of okadaic acid and its inhibitory effect on the PMLC phosphatase activity is given as a dose-inhibition curve with three components. Essentially similar dose-inhibition relationships have been obtained for the smooth-muscle extracts of guinea-pig taenia coli and rabbit portal vein (A. Takai, M. Troschka & A. V.

Somlyo, unpublished work). In the bovine aorta DiSalvo *et al.* (1983b) have identified an inhibitor-2-sensitive (type 1) phosphatase activity as well as Mg^{2+} -dependent (type 2C) phosphatase activity, which is present also in turkey gizzard (Pato *et al.*, 1983). Thus smooth-muscle tissues have similarities in their phosphatase composition.

A considerable phosphatase activity remains in the smooth-muscle extract after the skinning procedure (Table 3). The type 2A-like fraction with a very high affinity to okadaic acid and the type 2C fraction are lost by skinning (Fig. 3). The skinning procedure decreases the type 1 phosphatase activity by about 95% (see the Results section). The activity remaining after skinning is similar to the type 2A-like fraction with lower affinity to okadaic acid in the ratio of gizzard PMLC phosphatase activity to cardiac PMLC phosphatase activity as well as in the susceptibility to okadaic acid. Therefore it is reasonable to think that the phosphatase activity in the skinned fibre mostly consists of the type 2A-like phosphatase activity. The PMLC phosphatase activity remaining after skinning amounts to more than 60% of the type 2A-like activity in the intact fibres (cf. Tables 2 and 3). The reason why only this activity is so resistant to the skinning procedure is not clear from the present experiments. It is unlikely to be due to destruction of type 1 and type 2C phosphatases during the skinning procedure, because the purified protein phosphatases are relatively stable in the glycerol solution (see the Experimental section). During purification of myosin light-chain kinase from chicken gizzard, a PMLC phosphatase activity is co-purified together with the $MgATPase$ activity of myosin through several purification steps (see Sobieszek & Barylko, 1984). The major form of type 1 phosphatase in skeletal muscle is associated with the glycogen-protein particles (Cohen, 1985). Therefore an interesting possibility is that the type 2A-like phosphatase activity may be bound to contractile protein.

Skinned smooth muscles contract reversibly in response to Ca^{2+} application in the presence of $MgATP$ and calmodulin, accompanied by a reversible increase in myosin phosphorylation (Bialojan *et al.*, 1988). Inhibition of the PMLC phosphatase activity by okadaic acid produces a marked increase in tension and myosin phosphorylation, and slows down relaxation and myosin dephosphorylation induced by Ca^{2+} removal after prior activation with Ca^{2+} (Bialojan *et al.*, 1988). The present results, together with the previous observations, indicate that the type 2A-like activity may function *in vivo* in dephosphorylating smooth-muscle myosin to induce relaxation.

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REFERENCES

- Bialojan, C. & Takai, A. (1988) *Biochem. J.* **256**, 283–290
- Bialojan, C., Rüegg, J. C. & Takai, A. (1988) *J. Physiol. (London)* **398**, 81–95
- Brautigan, D. L., Gruppuso, P. A. & Mumby, M. (1986) *J. Biol. Chem.* **261**, 14924–14928
- Cohen, P. (1985) *Eur. J. Biochem.* **151**, 439–448
- Cohen, P., Nimmo, G. A. & Antoniow, J. F. (1977) *Biochem. J.* **162**, 435–444
- Cummins, P. & Lambert, S. J. (1986) *Circ. Res.* **58**, 846–868
- Diamond, J. & Brody, T. (1966) *J. Pharmacol. Exp. Ther.* **152**, 212–220
- DiSalvo, J., Jiang, M. J., Vandenheede, J. R. & Merlevede, W. (1982) *Biochem. Biophys. Res. Commun.* **108**, 534–540
- DiSalvo, J., Gifford, D., Bialojan, C. & Rüegg, J. C. (1983a) *Biochem. Biophys. Res. Commun.* **111**, 906–911
- DiSalvo, J., Gifford, D. & Jiang, M. J. (1983b) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 67–71
- Hartshorne, D. J. & Siemankowski, R. F. (1981) *Annu. Rev. Physiol.* **43**, 519–530
- Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P. & Hardie, D. G. (1989) *Nature (London)* **337**, 78–81
- Hescheler, J., Mieskes, G., Rüegg, J. C., Takai, A. & Trautwein, W. (1988) *Pflügers Arch.* **412**, 248–252
- Huang, F. L. & Glinsmann, W. H. (1976) *Eur. J. Biochem.* **70**, 419–426
- Ingebritsen, T. S. & Cohen, P. (1983) *Eur. J. Biochem.* **132**, 255–261
- Ingebritsen, T. S., Foulkes, J. G. & Cohen, P. (1983) *Eur. J. Biochem.* **132**, 263–274
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McGowan, C. G. & Cohen, P. (1987) *Eur. J. Biochem.* **166**, 713–722
- Namm, D. H. (1971) *J. Pharmacol. Exp. Ther.* **178**, 299–310
- Ngai, P. K., Carruthers, C. A. & Walsh, M. P. (1984) *Biochem. J.* **218**, 863–870
- Onishi, H., Umeda, J., Uchiwa, H. & Watanabe, S. (1982) *J. Biochem. (Tokyo)* **91**, 265–271
- Pato, M. D. & Adelstein, R. S. (1980) *J. Biol. Chem.* **255**, 6535–6538
- Pato, M. D. & Kerc, E. J. (1985) *Biol. Chem.* **260**, 12359–12366
- Pato, M. D., Adelstein, R. S., Crouch, D., Safer, B., Ingebritsen, T. S. & Cohen, P. (1983) *Eur. J. Biochem.* **132**, 283–287
- Silver, P. J. & Stull, J. T. (1982) *J. Biol. Chem.* **257**, 6145–6150
- Snedecor, G. W. & Cochran, W. G. (1980) *Statistical Methods*, 7th edn., pp. 143–160, 175, 191, 385–388 and 407–409, Iowa State University Press, Ames
- Sobieszek, A. & Barylko, B. (1984) *Smooth Muscle Contraction*, pp. 283–316, Marcel Dekker, New York
- Stewart, A. A., Ingebritsen, T. S. & Cohen, P. (1983) *Eur. J. Biochem.* **132**, 289–295
- Takai, A. (1988) *J. Muscle Res. Cell Motil.* **9**, 563–565
- Takai, A., Bialojan, C., Troschka, M. & Rüegg, J. C. (1987) *FEBS Lett.* **217**, 81–84
- Tung, H. Y. L., Resink, T. J., Shenolikar, S. & Cohen, P. (1984) *Eur. J. Biochem.* **138**, 635–641
- Werth, D. K., Haeberle, J. R. & Hatherway, D. R. (1982) *J. Biol. Chem.* **257**, 7306–7309