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The mechanism of action of cantharidin in smooth muscle

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1 The aim of this study was to investigate the mechanism(s) of the vasoconstrictor effect of cantharidin in bovine preparations.

2 Catalytic subunits of protein phosphatase type 1 (PP 1) and type 2A (PP 2A) were immunologically identified in coronary arteries, isolated smooth muscle cells and ventricular myocardium.

3 The mRNAs coding for catalytic subunits of PP 1α, PP 1β and PP 2Aα were identified by hybridization with specific cDNA-probes in total RNA from coronary arteries, isolated smooth muscle cells and ventricles.

4 The activities of catalytic subunits of PP 1 and PP 2A separated by column chromatography from coronary arteries, isolated smooth muscle cells and ventricles were inhibited by cantharidin in a concentration-dependent manner.

5 Cantharidin increased the phosphorylation state of smooth muscle proteins including the regulatory light chains of myosin in ^{32}P-labelled intact smooth muscle cells in a concentration-dependent manner.

6 Cantharidin did not affect cytosolic calcium concentrations in aortic smooth muscle cells.

7 It is suggested that cantharidin contracts smooth muscle preparations by increasing the phosphorylation state of regulatory proteins due to inhibition of phosphatase activities. Thus, cantharidin might be a useful tool to study the function of phosphatases in smooth muscle.

Keywords: Cantharidin; serine/threonine protein phosphatases; smooth muscle

Introduction

An important mechanism in the regulation of cellular processes is the reversible phosphorylation of proteins which can result in conformational changes and alterations of their activites and functions. The phosphorylation state of regulatory proteins depends on the relative activities of protein kinase(s) and protein phosphatase(s) that catalyse the phosphorylation and dephosphorylation reactions, respectively. Thus, increased phosphorylation can result from increased activity of protein kinases or inhibition of protein phosphatases. Here, we studied the role of serine/threonine protein phosphatases (in brief: phosphatases) in smooth muscle preparations. The discovery of cell membrane permeant phosphatase inhibitors like okadaic acid (Tachibana *et al*., 1981), calyculin A (Ishihara *et al*., 1989a, b) and cantharidin (Honkanen, 1993) has facilitated the study of the function of phosphatases (Shenolikar & Nairn, 1991; Wera & Hemmings, 1995; Hunter, 1995).

Phosphatase inhibitors like okadaic acid and/or cantharidin increased force of contraction in guinea-pig cardiac muscle (Kodama *et al*., 1986; Neumann *et al*., 1993; 1995) and human cardiac muscle (Linck *et al*., 1996). The positive inotropic effects were accompanied by inhibition of phosphatase activities in guinea-pig cardiac myocytes (Neumann *et al*., 1995) and inhibition of phosphatase activity in homogenates of human myocardium (Linck *et al*., 1996). Moreover, these drugs increased whole cell calcium currents in guinea-pig isolated cardiac myocytes and increased the phosphorylation state of cardiac regulatory proteins like e.g. phospholamban (Hescheler *et al*., 1988; Neumann *et al*., 1993; 1995). Thus, it is thought that by enhancing the phosphorylation state these compounds exert a positive inotropic effect in animal cardiac preparations.

On the other hand, it has been demonstrated that okadaic acid contracts intestinal and vascular smooth muscle preparations (Shibata *et al*., 1982; Ozaki *et al*., 1987; Hirano *et al*., 1989). The contractile effects of okadaic acid in the smooth muscle have been explained by inhibition of intracellular phosphatase activity (Takai *et al*., 1987) and an enhanced phosphorylation state of regulatory proteins (Obara *et al*., 1989; Gong *et al*., 1992).

Recently, we have demonstrated that the phosphatase inhibitor cantharidin increases force of contraction in bovine isolated coronary artery rings and – at the same time – inhibits phosphatase activity in homogenates from coronary arteries (Knapp *et al*., 1997). However, it is not known which types of phosphatases can be inhibited by cantharidin in vascular smooth muscle. Moreover, it has not been elucidated whether cantharidin actually increases the phosphorylation state of regulatory proteins in intact vascular smooth muscle cells.

In order to facilitate the comparison with previous work on the vasoconstrictor effects of cantharidin in smooth muscle preparations (Knapp *et al*., 1997), all experiments were performed on bovine tissue. Thus, the aim of this study was to identify the mechanism of action of cantharidin more thoroughly.

Methods

Contraction experiments

Experiments were performed similarly as described by Böhm *et al*. (1984) and Knapp *et al*. (1997). Bovine hearts were obtained from a local slaughterhouse.

*Dedicated to Prof. Dr. Husso Scholz on the occasion of his 60th birthday.

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Left coronary arteries (anterior descending branch) were cleaned of connective tissue and cut into rings with an approximate internal diameter of 4 mm. Rings were mounted in organ chambers containing Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 118, $NaHCO_3$ 25, $CaCl_{2.5}$, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.1, ethylenedinitrilotetraacetic acid 0.026, indomethacin 0.01 and atropine 0.01, continuously gassed with 95% O_2 and 5% CO_2. Rings were allowed to equilibrate in KHS for 60 min under a resting tension of about 20 mN and were then contracted twice with KCl (75 mM). After washout, cantharidin was added in a non-cumulative manner. Changes in isometric force of contraction were recorded for 180 min.

Trabeculae carneae were isolated from bovine right ventricles. The bathing solution contained (mM): NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA 0.05, ascorbic acid 0.28 and glucose 5.0, continuously gassed with 95% O_2 and 5% CO_2. Isometric force of contraction was measured after preloading each muscle to optimal length. Trabeculae carneae were electrically stimulated at 1 Hz with rectangular pulses of 5 ms duration, the voltage was about 10–20% greater than the threshold. Preparations were allowed to contract until equilibrium was reached (30 min). Thereafter, cantharidin was added cumulatively.

Culture of bovine vascular smooth muscle cells

Smooth muscle cells from bovine aortae and coronary arteries were obtained by using an explant method as described previously (Knapp *et al*., 1997). In brief, pieces of the arterial wall were transferred into tissue culture flasks and allowed to dry. Then smooth muscle culture medium composed of Dulbecco's modified Eagles medium containing 10% foetal calf serum, L-glutamine (584 μg ml^{-1}), amphotericin B (4.3 μg ml^{-1}), penicillin (100 iu ml^{-1}), streptomycin (100 μg ml^{-1}) and gentamicin (50 μg ml^{-1}) was carefully added. After cell growth was detected, cells were subcultured in trypsin (0.05%)/EDTA (0.02%)-solution. For experiments only cells between passages 2 and 6 were used. Identity and purity of cultured cells was confirmed by immunohistochemistry and Western blotting with a monoclonal antibody directed against α-smooth muscle actin.

Phosphatase activity

Preparation of homogenates Frozen bovine coronary arteries and frozen bovine right ventricular heart tissue were powdered in liquid nitrogen and homogenized in buffer A containing (mM) Tris-HCl (pH 7.4) 20, ethyleneglycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid 2, ethylenedinitrilotetraacetic acid 5, benzamidine 1, phenylmethylsulphonylfluoride 0.5 and β-mercaptoethanol (0.1%) three times for 30 s each with a Polytron PT-MR 3000 (Kinematica AG, Littau, Switzerland). Samples were centrifuged for 20 min at 14,000 × *g* (4°C). Bovine vascular cells were treated with trypsin and collected by centrifugation at 366 × *g* for 10 min (4°C). Cells were homogenized in buffer A by passing the cells through a needle (0.4 × 19 mm). Samples were centrifuged at 12,000 × *g* for 30 min. The supernatants are termed homogenate throughout this work. Aliquots of homogenates were used for determination of phosphatase activity.

Separation of phosphatases The separation was performed as described by Neumann *et al*. (1995). The dialysed sample was applied to a column containing heparin-Sepharose equilibrated in buffer A. Fractions of 3 ml were collected in the flow through (Peak 1, PP 2A) and in a linear gradient from 0 to 0.5 M NaCl (Peak 2, PP 1A) in buffer A. Aliquots of fractions obtained were used for determination of phosphatase activity.

Phosphatase assay Phosphatase activity was determined as described previously (Neumann *et al*., 1993) with [^{32}P]-phosphorylase a as substrate. The incubation mixture contained (mM) Tris-HCl (pH 7.0) 20.0, caffeine 5.0, ethylenediaminetetraacetic acid 0.1 and β-mercaptoethanol 0.1% (vol/vol). The reaction was started by adding aliquots of homogenates or aliquots of peak fractions. The reaction was stopped by addition of 50% trichloroacetic acid. Precipitated protein was sedimented by centrifugation and the supernatant was counted in a liquid scintillation counter.

Phosphorylation experiments

Labelling of bovine vascular smooth muscle cells Bovine aortic smooth muscle cells were harvested by trypsin (0.05%)/EDTA (0.02%)-solution and washed twice in phosphate-free solution (Na-HEPES buffer) consisting of (mM): NaCl 132.0, KCl 4.8, $MgSO_4$ 1.2, glucose 10.0, HEPES 10.0 and sodium pyruvate 2.5, pH 7.4 at 37°C. The cells were sedimented by centrifugation (366 × *g*; 4°C) for 10 min. Smooth muscle cells were resuspended in 10 ml of Na-HEPES buffer and incubated with 5 mCi of ^{32}P-labelled orthophosphate for 2 h at 37°C.

Protein phosphorylation Adenosine deaminase (20 u ml^{-1}) was added to avoid interference from endogenous adenosine upon treatment. The drug solution (100 μl) was preincubated at 37°C before being mixed with the final cell suspension of bovine smooth muscle cells (100 μl) and kept at 37°C. Reaction was stopped after 30 min by addition of 100 μl sodium dodecyl sulphate (SDS) stop solution (Laemmli, 1970) consisting of tris(hydroxymethyl) animomethane 62.5 mM, SDS 10% (w/v), glycerol 10% (v/v), DL-dithiothreitol 0.6% (w/v) and a trace of bromophenol blue, pH adjusted to 6.8. Protein was measured according to the method of Bradford (1976).

SDS–PAGE and autoradiography

Samples were heat treated (95°C) and aliquots of 100 μl corresponding to 20–30 μg protein were applied to each lane. Gels were run, dried and incorporated radioactivity was quantitated by PhosphorImager (Molecular Dynamics, Krefeld, Germany) with ImageQuant software.

Immunological identification of protein phosphatases types 1 and types 2A

Tissues and vascular cells were homogenized in buffer containing (mM) Tris (pH 7.4) 20, $MgCl_2$ 5, EDTA 10, dithiothreitol 1, phenylmethylsulphonylfluoride 1 and leupeptin 0.01. Following gel electrophoresis, separated proteins were transferred to nitrocellulose membranes in sodium phosphate buffer (50 mM, pH 7.4, 4°C). Nitrocellulose membranes were incubated with polyclonal anti-phosphatase 1α or polyclonal anti-phosphatase 2A. Proteins binding antibodies were visualized with alkaline phosphatase-conjugated goat anti rabbit IgG and colour reagents as described previously (Neumann *et al*., 1991).

Immunological identification of the regulatory light chains of myosin

^{32}P-labelled smooth muscle cells were incubated with cantharidin, okadaic acid or an appropriate amount of DMSO as control for 30 min at 37°C. The reaction was stopped and samples were subjected to electrophoresis. Separated proteins were transferred to nitrocellulose membranes and were incubated with monoclonal anti-myosin (light chains 20 kDa). Proteins binding the antibody were visualized with alkaline phosphatase-conjugated goat anti mouse IgM and colour reagents as described previously (Neumann *et al*., 1991).

Northern blotting

Coronary arteries, right ventricular tissue and vascular smooth muscle cells (from cattle) were homogenized using a microdismembrator (Braun Melsungen, Melsungen, Germany) in TriStar-Reagent (AGS, Heidelberg, Germany). Total RNA was extracted according to the manufacturer's instructions. First strand cDNA was reverse transcribed from 1 μg of total rat heart RNA in 10 μl of 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6.0 mM $MgCl_2$, 1.0 mM each dNTP (Pharmacia, Uppsala, Sweden), 5.0 mM DL-dithiothreitol, 50 $\mu g\ ml^{-1}$ bovine serum albumin (BSA), 10 units of human placental RNAse inhibitor (AGS, Heidelberg, Germany) and 30 units of TrueScript reverse transcriptase (AGS, Heidelberg, Germany) at 41°C for 60 min. Primers based on the published cDNA sequences of the rat for PP 1α (Sasaki *et al*., 1990), PP 1β (Sasaki *et al*., 1990) and PP 2Aα (Kitagawa *et al*., 1988) were employed to generate subtype-specific probes by RT–PCR.

The primers for PP 1α were 5′;-CCG CTG ATA AGA ATA AGG G-3′ and 5′-CAT GGC AGC ATG ATT TCT GT-3′, for PP 1β 5′-GCT GCG TTA GTT ACC CAG ATG AGA GC-3′ and 5′-ACT CAG, AGC TGA CAA TGT CCC ACT G-3′, for PP 2Aα 5′-GCC ATG AAC ACC GTC TGT TG-3′ and 5′-CCC TGA CTT CTC AGT CTC AAG-3′.

All PCR reactions were carried out in a total volume of 50 μl containing 20 mM Tris-HCl (pH 8.5, 25°C), 16 mM $(NH_4)_2SO_4$, 200 μM each dNTP, 1.5–2.0 mM $MgCl_2$ and 1.5 units Taq DNA polymerase (AGS, Heidelberg, Germany). An amount of cDNA equivalent to 100 ng RNA in each reaction was subjected to 30 cycles of denaturation (1 min, 94°C), annealing (2 min, 56°C for PP 1α, 62°C for PP 1β and 58°C for PP 2Aα), and extension (2 min, 72°C). All PCR reactions were performed in a thermal cycler (Omnigene, model TR3 CM220, MWG-Biotech, Ebersberg, Germany). Sizes of PCR-products were compared to DNA size markers (MBI Fermentas, Vilnius, Latvia). $MgCl_2$ titration curves were performed with each pair of primers to optimize amplification specificity. PCR-products were visualized on 2% agarose gels, cut out, purified with the GENECLEAN II kit (Bio 101, La Jolla, CA, U.S.A.), and used as probes in Northern blots. The PP 2Aα PCR product was cut by restriction enzyme digestion with the enzyme Dde I and the 305 bp fragment was used as a probe.

In order to confirm the identity of all PCR-products cycle sequencing with AmpliTaq-FS DNA polymerase (Applied Biosystems, Weiterstadt, Germany) and an ABI PRISM-310 automated sequencer (Applied Biosystems, Weiterstadt, Germany) was performed.

For Northern blots RNAs (20 μg) were separated on 1% denaturing agarose gels and transferred to nylon membranes (Amersham Buchler, Braunschweig, Germany) by capillary transfer in 20 × SSC. After prehybridization of the membranes in a solution containing 50% deionized formamide, 5 × Denhardt solution, 0.9 M NaCl, 60 mM NaH_2PO_4, 6.0 mM EDTA, 0.2 mg ml^{-1} tRNA from yeast and 0.1% sodium dodecyl sulphate (SDS) at pH 7.4, membranes were hybridized overnight at 42°C in the same buffer containing the probes, which were labelled with [α-^{32}P]-dCTP (NEN DuPont, Bad Homburg, Germany) by random priming (Megaprime-kit, Amersham Buchler, Braunschweig, Germany). Hybridized membranes were washed at a final stringency of 0.2 × SSC, 0.1% SDS at 55–60°C as appropriate, exposed to PhosphorImager-screens and visualized in a PhosphorImager (Molecular Dynamics, Krefeld, Germany).

Measurement of cytosolic calcium ($[Ca^{2+}]_i$)

Bovine aortic smooth muscle cells were seeded on to 15 mm diameter coverslips and used 3–5 days later at subconfluent density. Cells were washed twice with physiological saline solution (PSS) containing (in mM): NaCl 145, KCl 5.6, $MgSO_4$ 1, $CaCl_2$ 1, HEPES 20, glucose 10, pH 7.4 and then incubated with cell permeant indo-1-acetoxymethylester (5 μM) and 0.0025% (vol/vol) of the nonionic surfactant Pluronic F-127 (20% in DMSO; Molecular Probes, Eugene, Oregon, U.S.A.) for 60 min at 25°C. At the end of the loading period, the coverslips were washed twice with PSS and maintained for an additional 60 min to allow for complete deesertification of the indicator at room temperature. Cells were then placed in a perfusion chamber on the stage of a modified inverted microscope (Diaphot 200; NIKON, Tokio, Japan) and superfused with prewarmed PSS (1.2 ml min^{-1}). All experiments were performed at 25°C. $[Ca^{2+}]_i$ was recorded from a field of approximately 20 cells by use of a dual-emission microfluorescence system (PTI, Princeton, New Jersey, U.S.A.). The ratio of the two emission wavelengths (405 nm/495 nm) was used as an index of intracellular free calcium ion concentration. Data acquisition and processing were supported by a software (Felix Version 1.1, PTI, Princeton, New Jersey, U.S.A.) for intracellular calcium measurement.

Chemicals

Amphotericin B, atropine, benzamidine, cantharidin, indomethacin, leupeptin, phenylmethylsulphonylfluoride and DMEM were from Sigma (Deisenhofen, Germany), L-glutamine and penicillin/streptomycin solution were purchased from Serva (Heidelberg, Germany) and Gibco (Eggenstein, Germany), respectively. Gentamicin and trypsin/EDTA solution were obtained from Boehringer Mannheim (Mannheim, Germany). Foetal calf serum was from eurobio (Raunheim, Germany) and Boehringer Mannheim (Mannheim, Germany). Tissue culture flasks were from Becton Dickinson (Heidelberg, Germany) and Sarstedt (Nürnbrecht, Germany). Indo 1/AM and Pluronic were from Molecular Probes (Leiden, Netherlands). Antibodies against protein phosphatase 1α (rabbit polyclonal IgG) and protein phosphatase 2A (rabbit polyclonal IgG) were from BIOMOL (Hamburg, Germany). The antibody directed against the regulatory light chains of myosin (mouse monoclonal anti-myosin light chains 20 kDa) was obtained from Sigma (Deisenhofen, Germany). All other chemicals used were of analytical or best commercially available grade.

Statistics

Results are expressed as mean ± s.e.mean. Significance was estimated by Student's *t* test for paired and unpaired

observations as appropriate. A P value less than 0.05 was considered significant. pEC_{50} and pIC_{50} values are defined as $-\log mol^{-1}$ of EC_{50} and IC_{50} values respectively.

Results

Contraction experiments

Previous studies indicated a vasoconstrictor effect of cantharidin in coronary artery rings. In a comparative fashion the effects of cantharidin on both, force of contraction in isolated electrically driven bovine trabeculae carneae and coronary artery preparations from cattle, were studied. Cantharidin increased force of contraction in trabeculae carneae from cardiac ventricles in a concentration-depending manner. The maximal effect was reached at 30 μM (Figure 1). In isolated coronary artery preparations cantharidin concentration-dependently increased developed tension but did not reach plateau up to 100 μM (Knapp *et al*., 1997). These data indicate that vasoconstrictor and inotropic effects occur at comparable concentrations of cantharidin.

Phosphatase activity in homogenates from coronary arteries, ventricles and vascular cells

Previously, we showed that the vasoconstrictor effects of cantharidin are accompanied by inhibition of phosphatase activity in homogenates from coronary arteries, coronary artery smooth muscle cells and aortic smooth muscle cells (Knapp *et al*., 1997). Since okadaic acid is known to be more potent than cantharidin in inhibiting phosphatase activity (Honkanen, 1993; Neumann *et al*., 1995), the effects of okadaic acid were studied for comparison in the same experimental set-up.

In these experiments okadaic acid was about two orders of magnitude more potent than cantharidin in inhibiting phosphatase activity in homogenates from coronary arteries, coronary artery smooth muscle cells and aortic smooth muscle cells with pIC_{50} values of about 8.69, 8.73 and 8.35, respectively. For comparison, bovine cardiac phosphatases were also studied. Data are summarized in Table 1. Inhibition

Table 1 Inhibition of phosphatase activites in homogenates from coronary arteries, coronary artery smooth muscle cells (CASMCs), aortic smooth muscle cells (BASMCs) and right ventricles

	Cantharidin	*Okadaic acid*
Coronary arteries	6.10±0.03	8.69±0.16
CASMCs	6.13±0.03	8.73±0.60
BASMCs	6.51±0.13	8.35±0.24
Right ventricles	6.08±0.29	7.81±0.38

Values represent mean±s.e.mean of pIC_{50} values of at least three experiments.

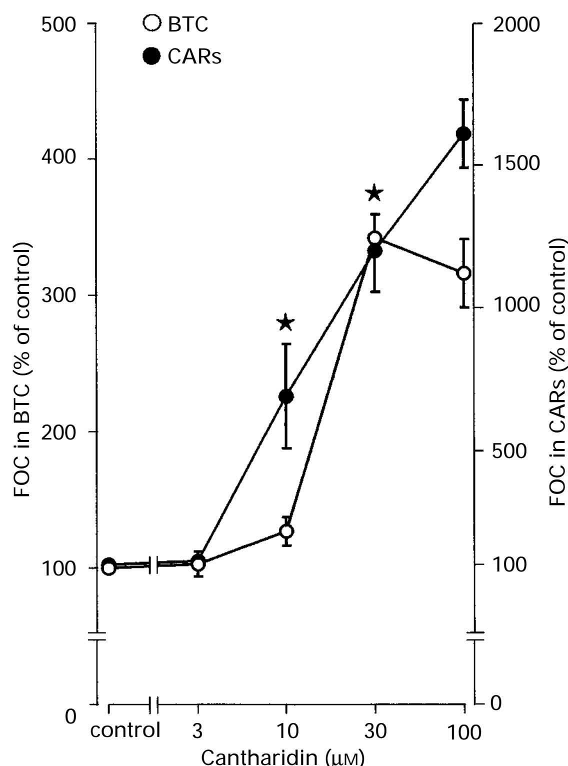

Figure 1 Effects of cantharidin on force of contraction (FOC) in isolated electrically driven trabeculae carneae (BTC) and isolated coronary artery rings (CARs). Values represent mean and vertical lines s.e.mean of 4 trabeculae carneae from 4 bovine hearts (right ventricle) and of at least 8 isolated coronary artery rings. Abscissa scale: concentrations of cantharidin. Ordinate scale: force of contraction as % of predrug value (Control=100%). The asterisks denote the first significant differences versus control.

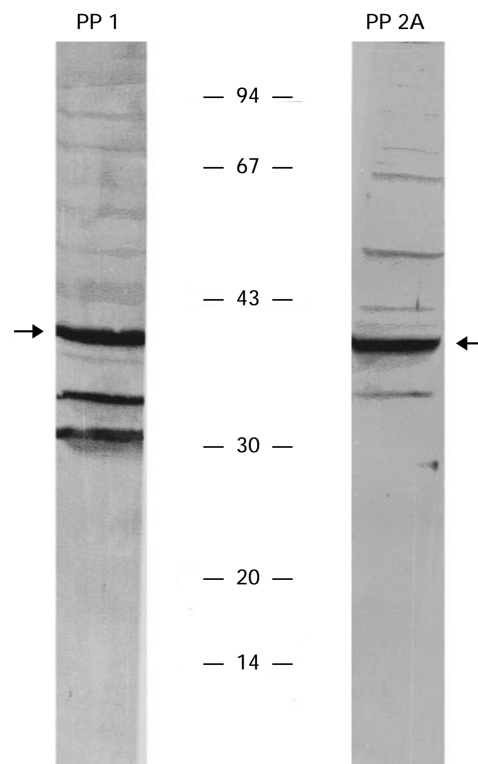

Figure 2 Immunological identification of phosphatase type 1 (PP 1) and type 2A (PP 2A). Bovine aortic smooth muscle cells were homogenized as described in Methods and subjected to electrophoresis. Separated proteins were transferred to nitrocellulose and were incubated with polyclonal anti-phosphatase 1α (PP 1) or anti-phosphatase 2A (PP 2A). Protein binding antibodies were visualized by use of alkaline phosphatase-conjugated goat anti rabbit IgG and colour reagents. The arrows indicate prominent bands at the expected molecular weight of about 36 kDa. In the middle, molecular weight standards are indicated. A representative experiment of 3 experiments is shown.

values for cantharidin were comparable to those obtained previously (Knapp *et al*., 1997).

Immunological identification of phosphatase type 1 (PP 1A) and 2A (PP 2A) catalytic subunits

Extracts from aortic smooth muscle cells were subjected to gel electrophoresis and transferred to nitrocellulose membranes. These blots were incubated with antibodies raised against the catalytic subunits of PP 1α and PP 2A (Figure 2). Prominent bands at the expected molecular weight of about 36 kDa were detectable, indicating the presence of catalytic subunits of PP 1α and PP 2A in these cells. Moreover, catalytic subunits of PP 1α and PP 2A could be identified in extracts from coronary arteries and heart ventricle, as well as in cultured coronary artery smooth muscle cells (data not shown). Next, we extended our study to the mRNA level by performing Northern blot experiments.

Northern blots

Total RNA was prepared from the tissues and isolated cells, separated by agarose electrophoresis and hybridized on membranes with specific cDNA probes. Major transcripts coding for PP 1α, PP 1β and PP 2Aα were detectable at the expected sizes of about 1.8 kb (Figure 3a), 3.2 kb (Figure 3b) and 2.0 kb (Figure 3c), respectively, with probes based on rat sequences. In addition, a minor transcript for PP 2Aα was detected at 1.1 kb. For PP 1γ and PP 2Aβ no specific signals were detectable in bovine tissues and vascular cells under our experimental conditions.

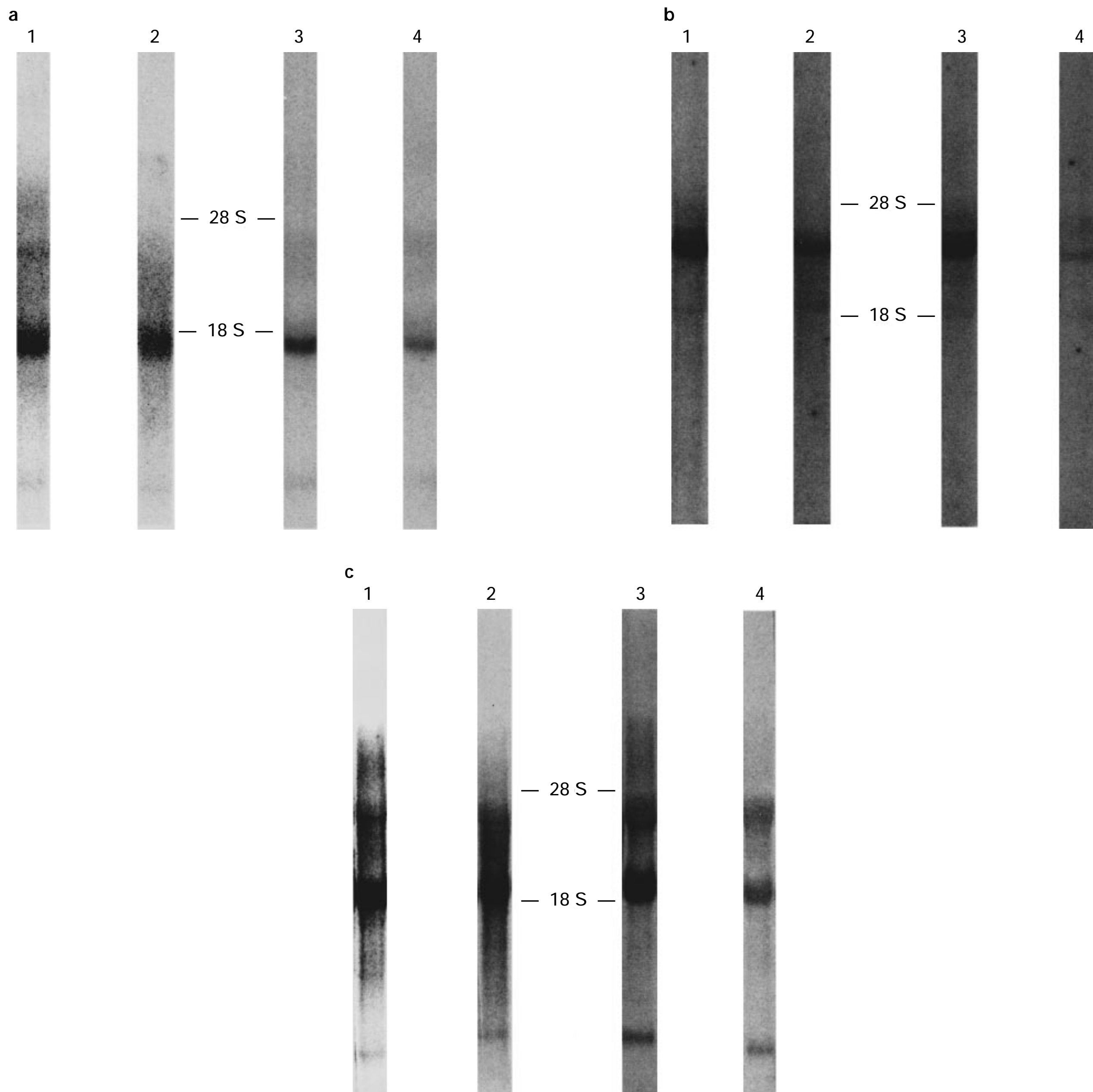

Figure 3 Expression of different isoforms of phosphatase catalytic subunits. Northern blot analysis for PP 1α (a), PP 1β (b) and PP 2Aα (c). Total RNA was extracted from right ventricle (1), coronary arteries (2), aortic smooth muscle cells (BASMCs, 3) and coronary artery smooth muscle cells (CASMCs, 4). RNA was separated on 1% agarose gels (20 μg per lane) and blotted onto nylon membranes which were hybridized overnight in buffer containing the probes. Hybridized membranes were washed, exposed to PhosphorImager-screens and visualized in a PhosphorImager. Representative experiments of at least 2 (PP 1β), 3 (PP 1α) or 4 (PP 2Aα) experiments are shown.

Effects of cantharidin and okadaic acid on separated phosphatase activities

Catalytic subunits of PP 1 and PP 2A were separated from bovine coronary arteries by column chromatography (data not shown). Cantharidin inhibited the activities of PP 1 and PP 2A with pIC_{50}s of 6.12±0.26 and 7.72±0.09, respectively (Figure 4a). Okadaic acid was equieffective as but more potent than cantharidin with pIC_{50}s of 7.55±0.10 for PP 1 and 10.20±0.11 for PP 2A (Figure 4b). The corresponding pIC_{50} values for aortic smooth muscle cells, coronary artery smooth muscle cells and bovine right ventricle are depicted in Table 2.

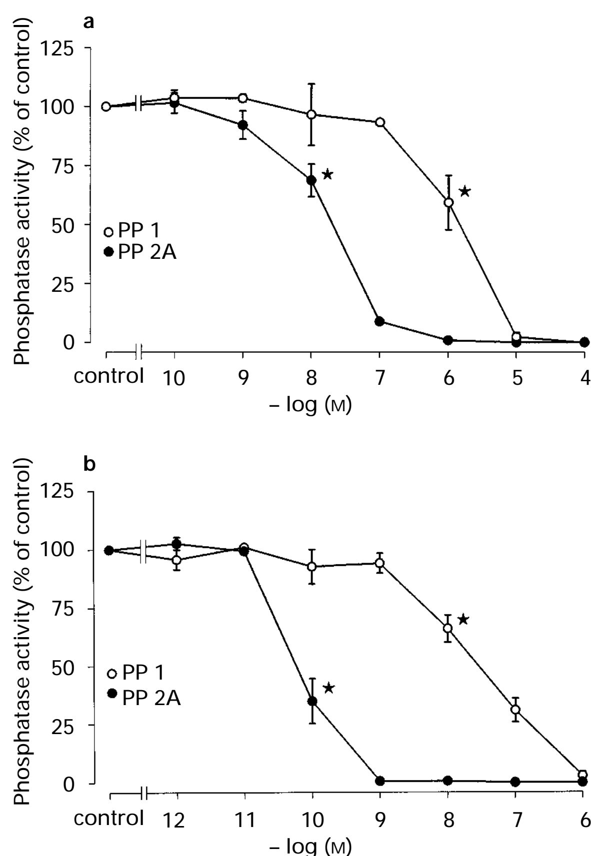

Figure 4 Inhibition of purified PP 1 and PP 2A activities from bovine coronary arteries by cantharidin (a) and okadaic acid (b). Phosphorylase a phosphatase activity is expressed as % of solvent control (DMSO). The concentration of solvent was constant under all experimental conditions. Symbols represent mean and vertical lines s.e.mean of at least 3 experiments. Abscissae: concentrations of cantharidin or okadaic acid. Ordinates: phosphorylase a phosphatase activity as % of control. The asterisks denote the first significant differences versus control.

Protein phosphorylation in bovine aortic smooth muscle cells

The effects of cantharidin and – for comparison – okadaic acid on protein phosphorylation were studied in ^{32}P-labelled intact aortic smooth muscle cells. Cantharidin and okadaic acid concentration-dependently increased the phosphorylation state of various smooth muscle proteins. A representative autoradiogram for cantharidin is depicted in Figure 5. Specifically, cantharidin and okadaic acid increased the phosphorylation state of the regulatory light chains of myosin (MLC) which were tentatively identified by immunoblot analysis in aortic smooth muscle cells (Figure 6). The effects of cantharidin and okadaic acid on MLC - phosphorylation were quantified in Figure 7. Numerous additional phosphoproteins were recognizable (Figure 5) but their identity was not addressed in this study.

Cytosolic calcium concentrations ($[Ca^{2+}]_i$)

To determine the effect of cantharidin on aortic smooth muscle $[Ca^{2+}]_i$, indol/AM-loaded monolayers of aortic smooth

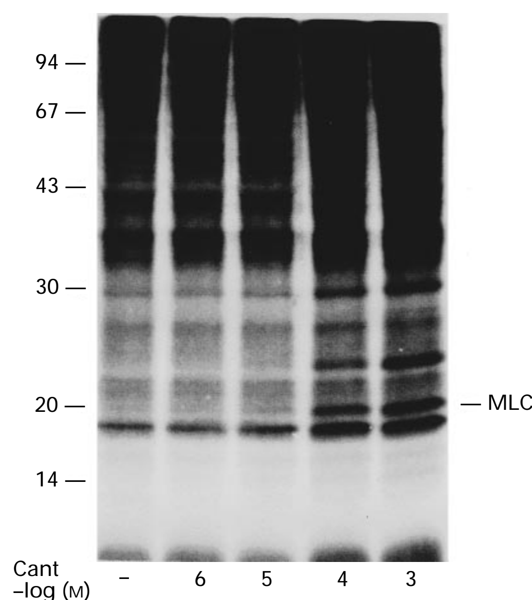

Figure 5 Concentration-dependent effect of cantharidin on protein phosphorylation in ^{32}P-labelled bovine aortic smooth muscle cells. ^{32}P-labelled smooth muscle cells were incubated with cantharidin (Cant) for 30 min at 37°C. The reaction was stopped by addition of SDS stop solution and samples were subjected to electrophoresis and autoradiography. Incorporated radioactivity was quantified as described in Methods. A representative autoradiogram is shown. On the left side, molecular weight standards are indicated.

Table 2 Inhibition of phosphatase type 1 (PP 1) and type 2 (PP 2A) activities purified from coronary arteries, coronary artery smooth muscles cells (CASMCs), aortic smooth muscle cells (BASMCs) and right ventricles

	Cantharidin		*Okadaic acid*	
	PP 1	*PP 2A*	*PP 1*	*PP 2A*
Coronary arteries	6.12±0.26	7.72±0.09	7.55±0.10	10.20±0.11
CASMCs	5.85±0.06	7.56±0.08	7.55±0.04	10.37±0.03
BASMCs	5.76±0.04	7.40±0.15	7.49±0.03	10.53±0.13
Right ventricles	5.81±0.08	7.43±0.12	7.54±0.01	9.96±0.16

Values represent mean±s.e.mean of pIC_{50} values of at least three experiments.

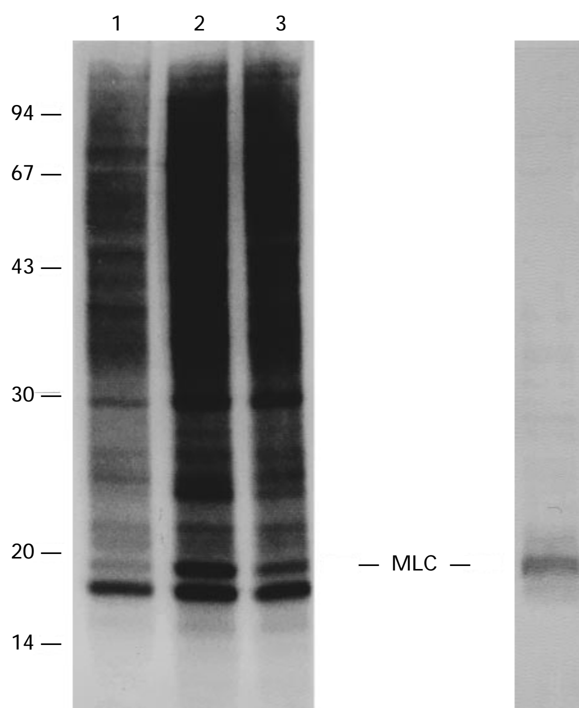

Figure 6 Immunological identification of the regulatory light chains of myosin (MLC) in ^{32}P-labelled bovine aortic smooth muscle cells. ^{32}P-labelled smooth muscle cells were incubated with 10 μM okadaic acid (3), 1 mM cantharidin (2) or an appropriate amount of DMSO (1) as control for 30 min at 37°C. The reaction was stopped and samples were subjected to electrophoresis. Separated proteins were transferred to nitrocellulose membranes and were incubated with monoclonal anti-myosin (light chains 20 kDa). Proteins binding the antibody were visualized by use of alkaline phosphatase-conjugated goat anti mouse IgM and colour reagents. A corresponding immunoblot is depicted on the right hand side. Incorporated radioactivity of nitrocellulose membranes was quantitated by PhosphorImager with ImageQuant software (Autoradiogram). On the left hand side, molecular weight standards are indicated. A representative experiment of 3 experiments is shown.

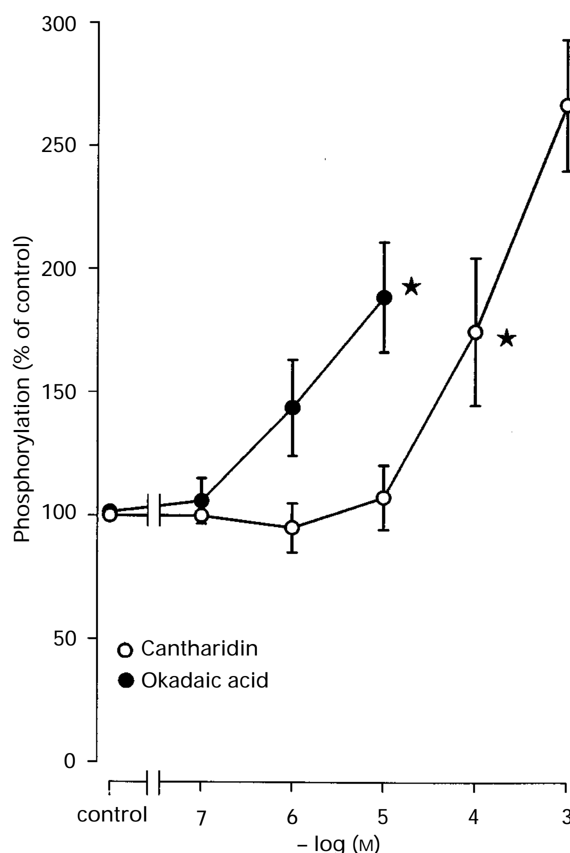

Figure 7 Concentration-dependent effects of cantharidin and okadaic acid on protein phosphorylation in ^{32}P-labelled bovine aortic smooth muscle cells. ^{32}P-labelled smooth muscle cells were incubated with cantharidin or okadaic acid for 30 min at 37°C. The reaction was stopped by addition of SDS stop solution and samples were subjected to electrophoresis and autoradiography. Incorporated radioactivity was quantified as described in Methods. Symbols represent mean and vertical lines s.e.mean of at least 6 experiments. Abscissa scale: concentrations of cantharidin and okadaic acid. Ordinate scale: phosphorylation as % of control. The asterisks denote the first significant differences versus control (DMSO).

muscle cells were exposed to 100 μM cantharidin for 30 min. This concentration of cantharidin was chosen based on the phosphorylation data (Figure 7). Cantharidin did not affect $[Ca^{2+}]_i$ within 30 min ($99.5 \pm 4.3\%$ of predrug value, $n=5$). Similar results were obtained in control cells treated with an appropriate amount of the solvent DMSO ($101 \pm 3.8\%$ of predrug value, $n=5$). However, the cells responded to 10 μM ATP at the end of the incubation period with a rapid increase in $[Ca^{2+}]_i$ ($199 \pm 45\%$ of predrug value, $n=10$). Furthermore, the cells responded to 75 mM potassium chloride with a sustained and to 5-hydroxytryptamine (10 μM) with a rapid transient elevation in free calcium levels (data not shown). Thus, cantharidin did not affect $[Ca^{2+}]_i$ under resting conditions in smooth muscle cells.

Discussion

We studied the mechanism of action of cantharidin in smooth muscle preparations. Cantharidin increased basal tone of bovine isolated coronary artery rings. This increase could not be explained by reductions of cyclic AMP levels in smooth muscle cells (Knapp *et al.*, 1997). However, initial work indicated that cantharidin might act via inhibition of phosphatase activity (Knapp *et al.*, 1997). In guinea-pig myocytes the positive inotropic effect of cantharidin was accompanied by phosphatase inhibition and by enhanced protein phosphorylation (Neumann *et al.*, 1995). Moreover, cantharidin inhibited phosphatase activity in homogenates from bovine coronary arteries (Knapp *et al.*, 1997).

However, it was speculative which phosphatase subtype(s) is/are inhibited by cantharidin and whether the same subtype(s) is/are inhibited in myocardial tissue and smooth muscle cells. If the vasoconstrictor effect of cantharidin is due to phosphatase inhibition we hypothesized that cantharidin should increase the phosphorylation state of proteins which regulate smooth muscle contractility.

The present study extends our previous work by presenting evidence that several subtypes of phosphatases are present in bovine smooth muscle preparations. These data indicate that type 1 and type 2A phosphatases, which are the main serine/threonine phosphatases in mammalian cells (Shenolikar & Nairn, 1991), can be inhibited by cantharidin.

By use of specific probes the mRNAs coding for phosphatase type 1 (1α, 1β) and type $2A\alpha$ were detectable in smooth muscle preparations. Others have identified type $2A\alpha$ in total bovine cardiac RNA (Green *et al.*, 1987). The identification of mRNA for phosphatases in smooth muscle preparations from cattle has hitherto not been revealed. However, the expression of phosphatases was detectable in

all tissues studied. This might indicate that their functional presence is essential in all cardiovascular tissues.

Moreover, we extended previous studies by identifying phosphatases type 1 and type 2A on the protein level by use of specific antibodies. Others have detected type 2A phosphatase by antibodies in bovine cardiac muscle (Mumby *et al*., 1987). Data on bovine smooth muscle cells have not yet been presented.

The next task ahead was to test whether these smooth muscle phosphatases can actually be inhibited by cantharidin. Therefore, we modified a column chromatographic procedure for smooth muscle preparations (coronary arteries and vascular cells). This procedure has been used by several groups to purify phosphatases in liver, myocardium and cardiomyocytes (Erdödi *et al*., 1985; Neumann *et al*., 1994). The identification of the catalytic subunits of phosphatases in column chromatography was based on their order of elution from the affinity column and their different sensitivity to okadaic acid. Phosphatase type 2A elutes first because it does not interact with heparin which is immobilized to the column, whereas type 1 remains on the column and can be eluted by a salt gradient. The pattern of elution and the inhibitory potency of okadaic acid were very similar in all smooth muscle preparations studied. This fits well with our Western and Northern blot data which also presented evidence for the ubiquitous expression of these phosphatases.

These data on phosphatase inhibition indicate that cantharidin should exert strong effects in all tissues examined as all contain phosphatases inhibitable by cantharidin.

Therefore, an additional test for the mechanism of action of cantharidin in smooth muscle was to study its effect on protein phosphorylation. If phosphatase inhibition occurs in the intact cells (not just in broken cell preparations), the phosphorylation state of all proteins that are physiologically dephosphorylated by phosphatases type 1 and/or type 2A should be greatly increased. This is exactly what was noted in smooth muscle cells. Cantharidin concentration-dependently increased the phosphorylation state of numerous smooth muscle proteins including the regulatory light chains of myosin. The phosphorylation of myosin light chains is thought to be largely responsible for contraction of smooth muscle (Kamm *et al*., 1989; Somlyo & Somlyo, 1992). Hence, these data support the view that cantharidin enhances contractility in the smooth muscle mainly by phosphatase inhibition and subsequent phosphorylation of regulatory proteins. The low potency of cantharidin in enhancing phosphorylation state, compared to inhibition of protein phosphatase activity, might be due to differences in permeability between intact smooth muscle cells and broken cell preparations (homogenates). Furthermore, the lack of protein kinase activity in the phosphatase assay system may contribute to these differences.

The lack of effect of cantharidin on cytosolic calcium concentrations in smooth muscle cells also strongly supports the notion that cantharidin acts almost exclusively via inhibition of phosphatases. Other vasoconstrictor stimuli (e.g. KCl, 5-hydroxytryptamine) can elevate intracellular Ca^{2+}. This is thought to activate the myosin light chain kinase and thus increase the phosphorylation state of the regulatory light chains of myosin (Miller-Hance & Kamm, 1991). However, pure inhibitors of protein phosphatases increase tension without increasing Ca^{2+}. This has been clearly demonstrated for okadaic acid in smooth muscle preparations from the intestine (Ozaki *et al*., 1987) and from porcine coronary arteries (Hirano *et al*., 1989). Thus we conclude that cantharidin acts distally from Ca^{2+} in the signal transduction cascade of the smooth muscle.

In summary, we present evidence that the vasoconstrictor effect of cantharidin is mainly if not solely due to phosphatase inhibition. This conclusion is based on the findings that cantharidin inhibits isolated phosphatases, inhibits phosphatases in cell homogenates, increases the phosphorylation state of contractile proteins in intact cells and does not act via elevation of free cytosolic Ca^{2+} levels. Due to its toxic effects (Li & Casida, 1992; Li *et al*., 1993; Polettini *et al*., 1992), cantharidin cannot be used therapeutically for the treatment of e.g. septic shock, where positive inotropy and vasoconstriction might be desired. However, cantharidin may be a useful lead compound for the development of new drugs that selectively inhibit smooth muscle phosphatases.

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