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European Journal of Pharmacology 294 (1995) 693–701

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Selectivity of action of staurosporine on Ca^{2+} movements and contractions in vascular smooth muscles

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Received 15 June 1995; revised 14 September 1995; accepted 26 September 1995

Abstract

We examined the effects of staurosporine, a protein kinase inhibitor, on Ca^{2+} movements and contractions due to KCl and 12-deoxyphorbol 13-isobutyrate (DPB), which are thought to activate myosin light chain kinase and protein kinase C, respectively. In rabbit aortae, staurosporine inhibited contractions due to KCl (65.4 mM) and DPB (1 μM) with IC_{50} values of 140.5 $\pm$ 1.3 nM and 13.3 $\pm$ 1.3 nM, respectively. Calphostin C, a putative inhibitor of protein kinase C, inhibited DPB-induced contraction with much less effect on the KCl-induced one. On the other hand, wortmannin, an inhibitor of myosin light chain kinase, was 4 times more potent on KCl-induced contraction than the DPB-induced one. Staurosporine at 100 nM decreased the rise in cytosolic Ca^{2+} due to KCl, whereas wortmannin did not affect it. In rabbit cerebral arteries permeabilized with β-escin, staurosporine at 100 nM, but not 30 nM, inhibited Ca^{2+}-induced contraction in the presence of 1 mM ATP. The results indicate that staurosporine preferentially inhibits a contraction dependent on protein kinase C than that dependent on myosin light chain kinase in vascular smooth muscles. Its ability to inhibit KCl-induced contraction involves inhibition of voltage-dependent Ca^{2+} channels.

Keywords: Staurosporine; Calphostin C; Wortmannin; Protein kinase C; Myosin light chain kinase; Aorta; (Rabbit)

1. Introduction

It is widely accepted that Ca^{2+}-calmodulin-dependent myosin light chain kinase, which phosphorylates myosin light chain, has a pivotal role in the initiation of contraction of vascular smooth muscles stimulated by substances which elevate the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). In addition, protein kinase C is also assumed to be involved in certain types of contraction, e.g. in receptor agonists-induced contractions. This assumption has been derived from the facts that many receptor agonists which induce a contraction produce diacylglycerol, an endogenous activator of protein kinase C, and that phorbol esters, exogenous protein kinase C activators, cause tension development. To clarify the roles of myosin light chain kinase and protein kinase C in various types of contraction, selective inhibitors of these kinases are awaited. Recently several kinds of protein kinase inhibitor have come into use for study. Staurosporine was shown to be a potent inhibitor of protein kinases since it inhibits protein kinase C and cyclic AMP-dependent protein kinase at nanomolar level (Tamaoki et al., 1986). Staurosporine has been reported to inhibit contractions of vascular smooth muscles caused by KCl, receptor agonists and phorbol esters (Boonen and De Mey, 1991; Merkel et al., 1991; Sasaki et al., 1991; Henrion and Laher, 1993). Nevertheless, some groups claim that this substance is not a useful tool because of lack of selectivity (Henrion and Laher, 1993; Shimamoto et al., 1993). On the other hand, calphostin C and wortmannin have been reported to be highly selective inhibitors of protein ki-

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SSDI 0014-2999(95)00616-8

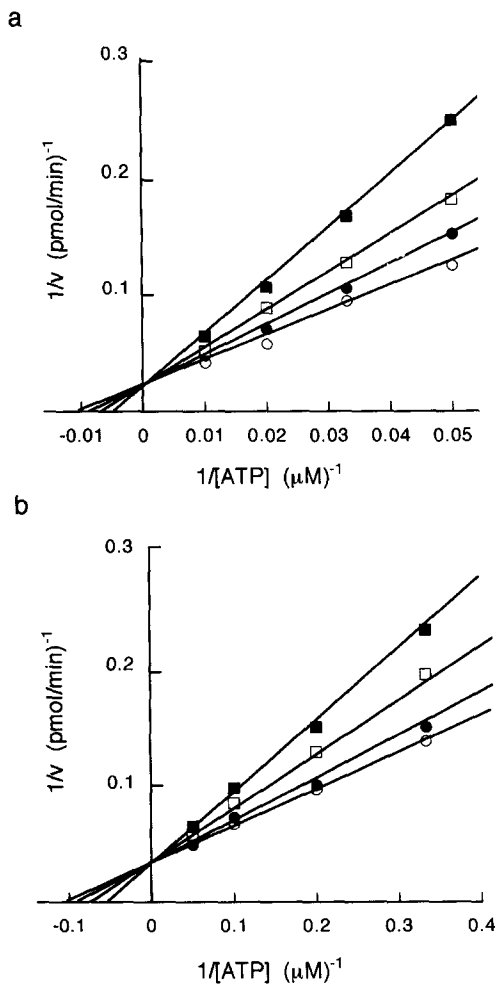

Fig. 1. Lineweaver-Burk plots for the inhibition by staurosporine of protein kinase C and myosin light chain kinase. (a) Myosin light chain kinase. Staurosporine at 0 nM (○), 5 nM (●), 10 nM (□) and 20 nM (■) was used. (b) Protein kinase C. Staurosporine at 0 nM (○), 1 nM (●), 3 nM (□) and 5 nM (■) was used.

nase C (Kobayashi et al., 1989) and myosin light chain kinase (Nakanishi et al., 1992), respectively. However, there have been few comparative studies to define the selectivity of these inhibitors under the same experimental conditions. In this study we aimed to delineate the profile of action of staurosporine, calphostin C and wortmannin on $[Ca^{2+}]_i$ movements and contractions stimulated by KCl and a phorbol ester, which are thought to activate myosin light chain kinase and protein kinase C, respectively.

2. Materials and methods

2.1. Assay of protein kinase C and myosin light chain kinase activity

Myosin light chain kinase and myosin light chain were prepared from chicken gizzard as described (Walsh et al., 1983; Yoshida and Yagi, 1988, respectively). Calmodulin was prepared from porcine brain by the method of Yazawa et al. (1980). Protein kinase C was prepared from rat brain by the method of Inagaki et al. (1985). Calponin was prepared from chicken gizzard by the method of Takahashi et al. (1986).

The basic reaction mixture (pH 7.0, final volume 50 μl) contained: Tris-HCl 25 mM, $CaCl_2$ 0.1 mM, $MgCl_2$ 1 mM, bovine serum albumin 0.5 mg/ml, 2-mercaptoethanol 14 mM. For myosin light chain kinase assay [γ-^{32}P]ATP 20–100 μM (specific activity 370 MBq/ml), calmodulin 10 μg/ml, myosin light chain 400 μg/ml and myosin light chain kinase 0.5 μg/ml were added to the mixture, and for protein kinase C assay phosphatidylserine 50 μg/ml, [γ-^{32}P]ATP 3–20 μM, calponin

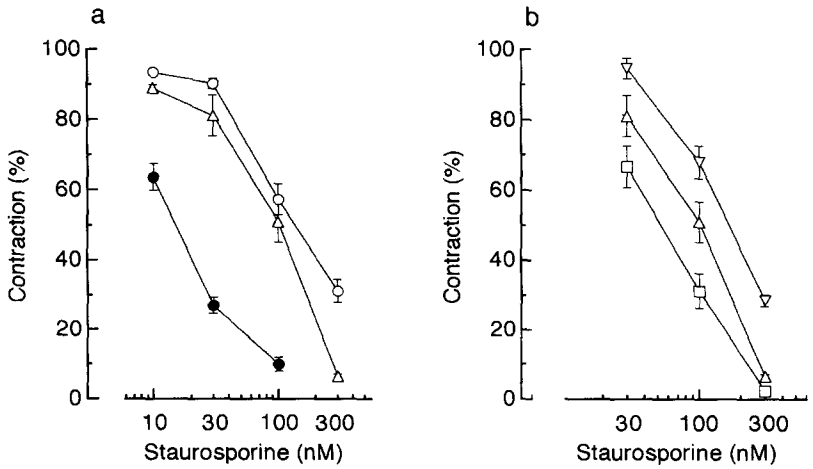

Fig. 2. Concentration-dependent inhibition by staurosporine of KCl-, DPB- and noradrenaline-induced contractions in rabbit aortae. In (a), each concentration of staurosporine was present for 60 min before addition of KCl (65.4 mM, ○), DPB (1 μM, ●) or noradrenaline (1 μM, △). In (b), the time-dependent effect of staurosporine is shown. Staurosporine was present for 30 (▽), 60 (△) or 90 (□) min before the addition of 1 μM noradrenaline. In the ordinate, 100% represents the maximum control contraction induced by each agonist. Each point represents the mean ± S.E.M. of 5–15 preparations.

100 μg/ml and protein kinase C 1 μg/ml were added. Assays were performed at 25°C for 3 min in the presence of 0–20 nM staurosporine and the reaction was terminated by the addition of 5 μl of 50% trichloroacetic acid. The sample was centrifuged at 3000 rpm for 15 min, the pellet was resuspended in 5% trichloroacetic acid solution, and this operation was repeated 3 times. The final pellet was dissolved in 1 ml of 1 N NaOH, and the radioactivity was measured using a liquid scintillation counter (Wallac 1400, Pharmacia, Tokyo, Japan). K_i values were calculated from Lineweaver-Burk plot.

2.2. Vascular smooth muscle preparations

Thoracic aortae and cerebral arteries were isolated from male rabbits (2–3 kg) anaesthetized with pentobarbital-Na (40 mg/kg i.v.). The endothelium and adventitia were removed from the aorta and helical strips were made. Small rings of the cerebral artery were cut open and the rectangular strips in a transverse direction of 800–1000 μm length, 400 μm width and less than 30 μm thickness were made.

2.3. Measurement of tension of aortic strips

For the concentration-response study, aortic strips were suspended in an organ bath containing 5 ml physiological saline solution (PSS) of the following composition (mM): NaCl 136.8, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 11.9 and glucose 5.5 (pH 7.2–7.4 when gassed with 95% O_2 and 5% CO_2). The strips were allowed to equilibrate for 1 h, maintaining the resting tension of 1 g at 37°C. Isometric tension was measured with a force-displacement transducer (SB-1T, Nihon-Kohden, Tokyo, Japan) and recorded on a pen-writing recorder (R-10, Rika-Denki, Tokyo, Japan).

2.4. Cytosolic Ca^{2+} measurements

$[Ca^{2+}]_i$ in aortae was measured with the fluorescent indicator fura-PE3 as described (Seto et al., 1995). We used fura-PE3 rather than fura-2 because fura-PE3 was retained better in cells (Dr. H. Karaki, personal communication). Briefly, the aortic strips were loaded with 2.5 μM fura-PE3/AM dissolved in normal PSS containing 0.03% cremophor EL for 15–18 h. After loading, one end of the strip was fixed in an organ bath (37°C) constructed in a fluorimeter (CAF-100, JASCO, Tokyo, Japan) and the other end was connected to a force-displacement transducer. The tension and $[Ca^{2+}]_i$ were measured simultaneously.

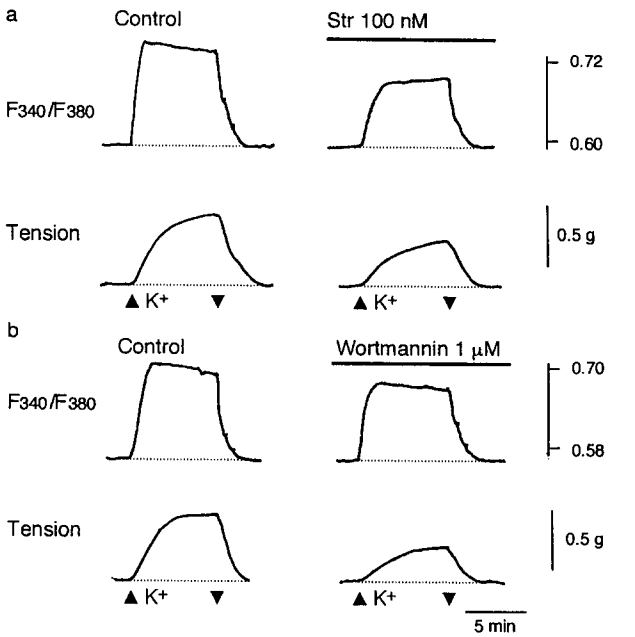

Fig. 3. Example records of effects of staurosporine and wortmannin on the 65.4 mM KCl-induced changes in $[Ca^{2+}]_i$ and tension in rabbit aortae. After observing the control response to KCl, preparations were treated with staurosporine (Str, 100 nM) or wortmannin (1 μM) for 30 min until a second addition of KCl. Upper traces: ratio of fluorescence excited at 340 nm to that at 380 nm. Upward triangle represents the application of KCl and downward triangle washing out high KCl.

Table 1
IC_{50} values of staurosporine, calphostin C and wortmannin for inhibition of KCl- and DPB-induced contractions in rabbit aortae

Drugs	KCl-induced contraction		DPB-induced contraction	
	IC_{50}	n	IC_{50}	n
Staurosporine	140.5 ± 1.3 nM	15	13.3 ± 1.3 nM	28
Calphostin C	> 100 μM	16	10.1 ± 1.6 μM	19
Wortmannin	0.62 ± 0.19 μM	11	2.53 ± 0.56 μM	19

A single dose of inhibitor was applied to a preparation 60 min before the addition of 65.4 mM KCl or 1 μM DPB. Data are expressed as mean ± S.E.M., and n indicates the number of experiments.

2.5. Tension measurement in β-escin permeabilized cerebral arteries

One end of the cerebral artery strip was placed in a superfusion bath (fluid volume 0.2 ml) with a small pin (diameter 100 μm). The other end of the strip was tied with a fine silk thread, connected to a strain gage transducer (UL-2GR, Minebea, Tokyo, Japan) and 30 mg of resting tension was applied. Before permeabilization, the muscle was superfused for 1 h with Tris-buffered PSS of the following composition (mM): NaCl 136.8, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2.5, glucose 5.5 and Tris-HCl 5.0 (pH 7.4), and the responses to isotonic 130 mM KCl were observed twice. Thereafter, the muscle was superfused with a rigor solution (in mM:

K-propionate 115.0, Tris-maleate 20.0, $MgCl_2$ 1.8, $CaCl_2$ 0.01, EGTA 10.0, pCa > 8, pH 6.8) then permeabilized with 80 μM β-escin dissolved in the rigor solution for 30–35 min. A23187 (10 μM) was added to the β-escin solution to remove functional Ca^{2+} stores. After the permeabilization, the muscle was incubated in a relaxing solution (in mM: K-propionate 130.0, Tris-maleate 20.0, $MgCl_2$ 4.0, Na_2ATP 3.75 (or 1.0), creatine phosphate 10.0, creatine phosphokinase 72 units/ml, calmodulin 500 ng/ml, EGTA 10.0, pCa > 8 with 10 mM EGTA, pH 6.84) for 14 min, then 10 μM Ca^{2+} was added (activating solution). pCa in the activating solution was calculated assuming the apparent binding constant of EGTA-Ca as 1×10^6 M^{-1} at pH 6.8. First, the control response to cumulatively added Ca^{2+} was observed. After washing with the relaxing solution, staurosporine was applied to a test preparation or no drug to a control preparation. Staurosporine was present for 10 min until the next Ca^{2+}-induced contraction was elicited. Experiments were performed at room temperature (23–24°C).

2.6. Reagents

Reagents used were phosphatidylserine (Funakoshi, Tokyo, Japan), [γ-^{32}P]ATP (Du Pont, Wilmington, DE, USA), staurosporine (Asahi Chemical Industry, Shizuoka, Japan), wortmannin, calphostin C (Kyowa-Medex, Tokyo, Japan), fura-PE3/AM (Wako Pure Chemicals, Osaka, Japan), noradrenaline (Tokyo-Kasei, Tokyo, Japan), 12-deoxyphorbol 13-isobutyrate (DPB, Sigma Chemicals, St. Louis, MO, USA). Staurosporine, wortmannin, calphostin C and DPB were dissolved in dimethyl sulfoxide at 1 or 10 mM. The solution containing calphostin C was exposed to a fluorescent light before use to activate the substance (Bruns et al., 1991).

2.7. Statistics

Data are expressed as means ± S.E.M. Student's t-test was performed for comparison and the significance was considered at the level of $P < 0.05$. IC_{50} values of inhibitors were calculated by Professor I. Takayanagi's program based on the method of Tallarida et al. (1979).

3. Results

3.1. Inhibition by staurosporine of protein kinase C and myosin light chain kinase

Fig. 1 shows the inhibition by staurosporine of myosin light chain kinase and protein kinase C activi-

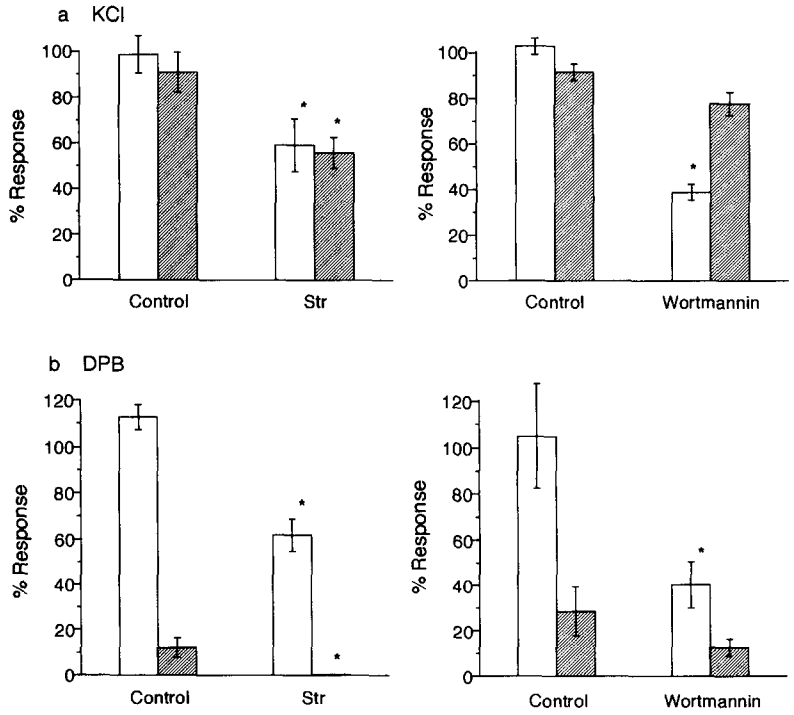

Fig. 4. Effects of staurosporine (Str) and wortmannin on rise in $[Ca^{2+}]_i$ and contraction induced by 65.4 mM KCl (a) or 1 μM DPB (b) in rabbit aortae. The protocol is given in the legends for Fig. 3 and Fig. 5. The response of tension and $[Ca^{2+}]_i$ to first KCl is expressed as 100% in the ordinate. Open columns: contraction. Hatched columns: $[Ca^{2+}]_i$. Left: effect of staurosporine (Str, 100 nM for KCl, 30 nM for DPB). Right: effect of 1 μM wortmannin. Each column represents the mean ± S.E.M. of 7–15 preparations. * Significantly different from the second control ($P < 0.05$, paired t-test).

ties of purified enzymes. Lineweaver-Burk plot analysis revealed that inhibition was competitive for both myosin light chain kinase and protein kinase C with respect to ATP concentrations. The K_i values were 18.4 and 4.4 nM for myosin light chain kinase and protein kinase C, respectively.

3.2. Effects of protein kinase inhibitors on contractions due to contractile stimulants

In rabbit aortae high KCl (65.4 mM) and noradrenaline (1 μM) caused sustained contractions with a relatively rapid upstroke, while DPB (1 μM) caused a slowly developing contraction, attaining a peak at 20–30 min. The magnitude of the maximum contraction induced by each stimulant was $115.2 \pm 3.8\%$ ($n = 16$) for noradrenaline and $75.7 \pm 4.8\%$ ($n = 16$) for DPB (KCl-induced contraction was taken as 100%). Fig. 2a shows the concentration-dependent inhibition by staurosporine of contractions induced by KCl (65.4 mM), noradrenaline (1 μM) and DPB (1 μM). Each concentration of staurosporine was applied 60 min before the addition of each agonist. Staurosporine inhibited the DPB-induced contraction ($IC_{50} = 13.3 \pm 1.3$ nM) more potently than the KCl- or noradrenaline-induced contraction ($IC_{50} = 140.5 \pm 1.3$ nM or 85.1 ± 1.3 nM, respectively). Fig. 2b shows the dose-effect relationship of staurosporine after varied pretreatment periods. The inhibitory effects of staurosporine on the noradrenaline-induced contraction developed with the period of pretreatment, suggesting that the penetration of staurosporine into cells is slow.

Table 1 summarizes the IC_{50} values of staurosporine, calphostin C and wortmannin against KCl- or DPB-induced contractions. Calphostin C inhibited the DPB-induced contraction at above 1 μM, while it only slightly inhibited the KCl-induced contraction even at 10 μM. On the other hand, wortmannin inhibited the KCl-induced contraction more potently than that induced by DPB. None of inhibitors affected the resting tension in aortae.

3.3. Changes in cytosolic Ca^{2+} during KCl- and DPB-induced contractions and the effects of staurosporine and wortmannin

In fura-PE3-loaded aortae, isotonic 65.4 mM KCl-PSS induced a sustained rise in $[Ca^{2+}]_i$ concomitantly with tension development (Fig. 3). When the application of KCl was repeated with an interval of 30 min, the second $[Ca^{2+}]_i$ response decreased by about 10%, although the mechanical response was reproducible (Fig. 4a). Therefore, we compared the $[Ca^{2+}]_i$ response in the presence of staurosporine with the time-matched control, which was not given staurosporine. The rise in $[Ca^{2+}]_i$ and the contraction in response to KCl in the presence of 100 nM staurosporine for 30 min were significantly depressed as compared with those in control muscles. On the other hand, wortmannin (1 μM) did not significantly affect the $[Ca^{2+}]_i$ response to KCl, while it significantly inhibited the contraction (Fig. 4a). We could not test the effect of calphostin C on the $[Ca^{2+}]_i$ response because this substance quenched the fluorescence dye.

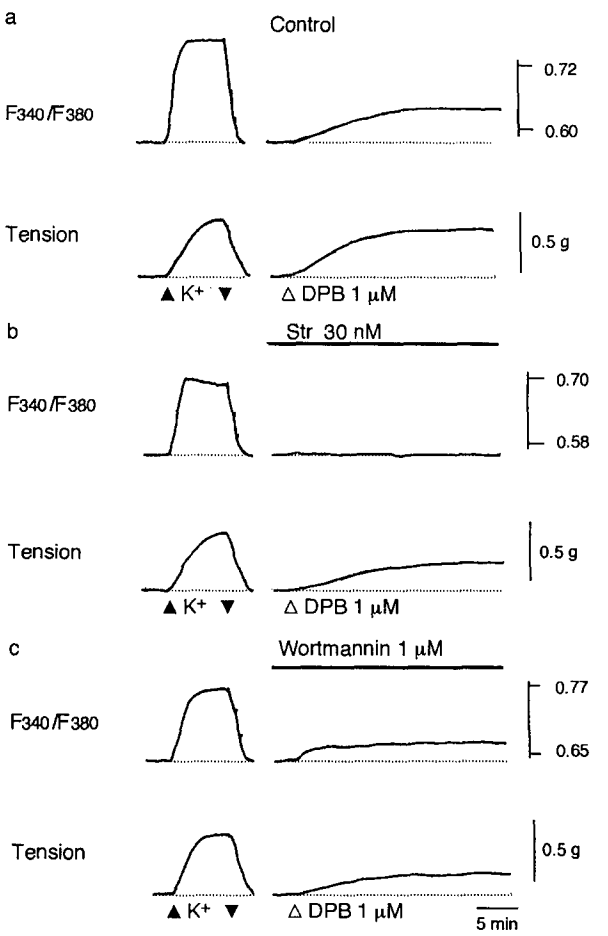

Fig. 5. Example records of effects of staurosporine and wortmannin on the DPB-induced changes in $[Ca^{2+}]_i$ and tension in rabbit aortae. (a) Control responses to isotonic 65.4 mM KCl and 1 μM DPB. In this trace, an example from a preparation which exhibited a rise in $[Ca^{2+}]_i$ in response to DPB is shown. DPB was applied 40 min after washing out high KCl. When tested, 30 nM staurosporine (b, Str) or 1 μM wortmannin (c) was applied 30 min before the addition of DPB.

In contrast to KCl, the effect of DPB on $[Ca^{2+}]_i$ was quite variable. In 8 of 18 preparations DPB did not change the level of $[Ca^{2+}]_i$ while it increased the tension to $109.1 \pm 11.7\%$ (contraction due to 65.4 mM KCl was taken as 100%). In 10 other preparations it slightly increased the $[Ca^{2+}]_i$ level, some showing a transient increase while others a small and sustained increase (Fig. 5). The average of maximum increase of $[Ca^{2+}]_i$ was $12.0 \pm 6.3\%$ of the rise induced by 65.4

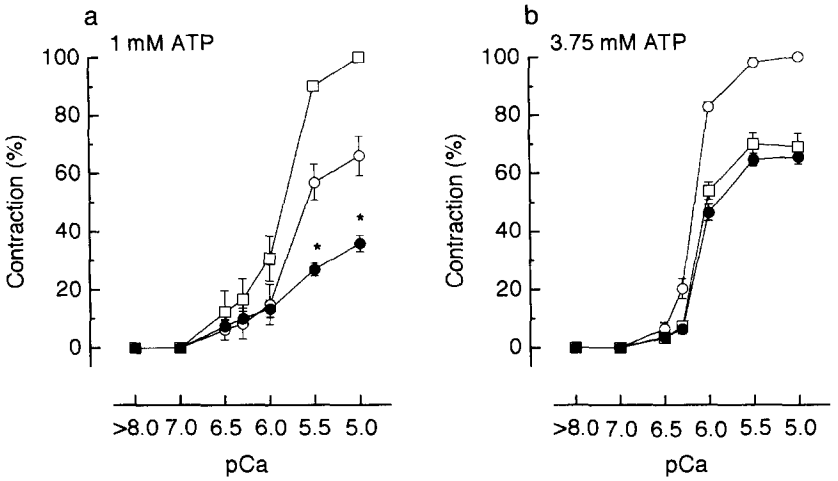

Fig. 6. Effect of staurosporine on Ca^{2+}-induced contraction of β-escin-permeabilized cerebral artery. (a) In the presence of 1 mM ATP, (b) in the presence of 3.75 mM ATP. (□) First response to cumulatively added Ca^{2+}. The responses to the second application of Ca^{2+} were observed in the presence (●) or absence (○, second control) of 100 nM staurosporine. In the ordinate, 100% represents the maximum contraction in the first control. Each point represents the mean ± S.E.M. of 6 preparations. * Significantly different from the second control ($P < 0.05$, paired t-test).

mM KCl and the maximum tension was 115.9 ± 4.3% (Fig. 4b). In these preparations the rate of rise in tension was slightly greater than those which did not show an increase in $[Ca^{2+}]_i$ but the peak amplitude of the contraction did not depend on whether a preparation exhibited a rise in $[Ca^{2+}]_i$. In the presence of 30 nM staurosporine for 30 min, $[Ca^{2+}]_i$ did not increase upon application of DPB and the contraction was depressed to 62.2 ± 7.0% ($n = 13$, Fig. 4b). When 1 μM wortmannin was present, $[Ca^{2+}]_i$ increased following application of DPB in some preparations. The average response to DPB of $[Ca^{2+}]_i$ in the presence of wortmannin was not different from that in its absence whereas the contraction was significantly smaller in wortmannin-treated preparations (Fig. 4b).

In Ca^{2+}-free PSS containing 0.5 mM EGTA, DPB induced a sustained contraction but it never increased $[Ca^{2+}]_i$. The rate of rise in the contraction was slightly slower than and the peak of contraction was slightly smaller than those observed in normal PSS (82.0 ± 3.8% of the response observed in normal PSS, $n = 15$). Staurosporine (30 nM) inhibited the DPB-induced contraction in Ca^{2+}-free PSS by 69.7 ± 2.7% ($n = 16$).

3.4. Effect of staurosporine on Ca^{2+}-induced contraction in β-escin permeabilized cerebral arteries

Next, we tested the effect of staurosporine on Ca^{2+}-induced contraction, which depends on myosin light chain kinase, in β-escin-permeabilized cerebral arteries. On cumulative application of Ca^{2+}, the muscle developed the tension in a concentration-dependent manner at from less than pCa 6.5, and attaining maximum tension at pCa 5.5–5.0 (Fig. 6). With 1 mM ATP, the maximum tension induced by Ca^{2+} was equal to or slightly larger than that induced by isotonic KCl observed before the permeabilization. When the induction of Ca^{2+} contraction was repeated with an interval of 10 min, the second contraction was decreased by about 25%. Treatment with staurosporine (100 nM) for 10 min prior to constructing the second pCa tension curve led to a decrease in the contraction stimulated by pCa 5.5 and 5.0 (Fig. 6). However, staurosporine at 30 nM did not inhibit the contraction (data not shown).

When the ATP concentration was increased to 3.5 mM, the rate of rise in tension at each Ca^{2+} concentration was faster than that with 1.0 mM ATP, although the deterioration of the pCa tension curve was the same as with the lower concentration of ATP. With 3.75 mM ATP, staurosporine (100 nM) did not significantly affect the pCa tension relationship (Fig. 6).

4. Discussion

Our data showed that KCl-induced contraction was accompanied with an increase in $[Ca^{2+}]_i$, which would in turn be expected to activate myosin light chain kinase (Kamm and Stull, 1989). On the other hand, DPB did not or only slightly increased $[Ca^{2+}]_i$. In a preparation where $[Ca^{2+}]_i$ was increased by DPB, the time-course of change in $[Ca^{2+}]_i$ did not coincide with that of tension development and the amplitude of the maximum contraction did not depend on whether a preparation exhibited a rise in $[Ca^{2+}]_i$ in response to DPB. Furthermore, in Ca^{2+}-free PSS, DPB induced a

contraction, which was slightly smaller than that observed in Ca^{2+}-containing PSS, without any increase in $[Ca^{2+}]_i$. Accordingly, it is clear that DPB could induce a contraction independent of a change in $[Ca^{2+}]_i$. It seems that a slight increase in $[Ca^{2+}]_i$ due to DPB contributed mainly to the rising phase of the contraction and only slightly to the maximum contraction. Such a small dependence of the DPB effect on $[Ca^{2+}]_i$ was also observed in other smooth muscles (ferret aorta, Jiang and Morgan, 1987; rabbit mesenteric artery, Sato et al., 1992; guinea-pig taenia caeci, Mitsui and Karaki, 1993). There have been some observations that stimulation of protein kinase C causes Ca^{2+} influx in vascular smooth muscle cells (Gleason and Flaim, 1986; Rembold and Murphy, 1988; Nakajima et al., 1993). Since DPB did not increase $[Ca^{2+}]_i$ in any preparation treated with staurosporine, a small increase in $[Ca^{2+}]_i$ due to DPB could be a consequence of protein kinase C activation.

In purified enzymes the K_i value of staurosporine against protein kinase C (4.4 nM) was about one-fourth of that against myosin light chain kinase (18.4 nM). The IC_{50} of staurosporine for DPB-induced contraction in rabbit aortae was about one-tenth of that for KCl-induced contraction (13.3 nM and 140.5 nM, respectively). It is believed that myosin light chain kinase is responsible for a depolarization-dependent contraction and protein kinase C is responsible for a phorbol ester-induced contraction (Rasmussen et al., 1987), so that it is likely that this substance more potently inhibits the protein kinase C-dependent mechanism than the myosin light chain kinase-dependent one. In addition, inhibition by staurosporine of the KCl-induced contraction was accompanied by a decrease of the $[Ca^{2+}]_i$ response. This means that the inhibition of voltage-dependent Ca^{2+} channels is at least partly responsible for the inhibition by staurosporine of the contraction to KCl. Therefore, the proportion of the action of staurosporine attributable to the direct inhibition of myosin light chain kinase in its depression of the KCl contraction should be smaller than that predicted from the IC_{50}. Thus, the effect of staurosporine appears far more selective for protein kinase C than myosin light chain kinase, when this substance is used on intact tissues (Kageyama et al., 1991).

In order to know how effectively staurosporine inhibits myosin light chain kinase-dependent contraction, we observed the effect of staurosporine on Ca^{2+}-induced contraction in β-escin-permeabilized arteries, where $[Ca^{2+}]_i$ was fixed. Staurosporine at 100 nM partially inhibited the contraction, while it at 30 nM had no effect. This rather weak activity supports the view that this substance is not so effective on the myosin light chain kinase-dependent contraction.

As mentioned above, there was a gap between concentrations of staurosporine to inhibit purified enzymes and contractions of intact muscles. One cause for the gap could be due to slow access of staurosporine to the cell interior as shown in Fig. 2b. In β-escin permeabilized cerebral arteries staurosporine at 130 nM did not inhibit Ca^{2+}-induced contraction. Thus, this compound was still not potent on the myosin light chain kinase-dependent contraction in muscles where easy access of the inhibitor to the enzyme was permitted. Therefore, another cause may also be involved in the weak potency on tissues. Staurosporine may non-specifically bind to some cell constituents or its action is neutralized by some unknown mechanism in tissues. Staurosporine did not significantly affect the Ca^{2+}-induced contraction in permeabilized arteries when ATP was 3.75 mM. The ineffectiveness at high ATP can be explained by the result that staurosporine competitively inhibits the ATP binding to purified kinases. For purified enzymes ATP between 20–100 μM was used, while 1 mM or higher concentration of ATP was present for permeabilized muscles. Provided that staurosporine inhibits kinases by antagonism to ATP, 10–50 times higher concentrations are assumed to be necessary to inhibit the enzymes in permeabilized tissues or intact tissues. This could be another cause for the gap of concentrations used in the biochemical assay and in tissues.

Concerning the inhibition by staurosporine of $[Ca^{2+}]_i$ response to KCl, it is unlikely that protein kinase C or myosin light chain kinase is involved in the Ca^{2+} influx stimulated by KCl, since calphostin C was virtually ineffective on the KCl-induced contraction and wortmannin did not affect the $[Ca^{2+}]_i$ response to KCl. Therefore, the inhibition by staurosporine of the $[Ca^{2+}]_i$ response to KCl could not be ascribed to its action on protein kinase C or myosin light chain kinase but probably to the action on another kinase, such as tyrosine kinase (Badwey et al., 1991; Wijetunge et al., 1992), or some non-specific action.

Calphostin C effectively inhibited the DPB-induced contraction, while it only slightly inhibited the KCl-induced one. Although we could not observe the effect of calphostin C on the $[Ca^{2+}]_i$ response to KCl, this substance may not have a significant effect on the $[Ca^{2+}]_i$ response because the inhibitory effect of calphostin C on the KCl-induced contraction was very small. In contrast, wortmannin was more effective on the KCl contraction than the DPB contraction. The fact that it did not significantly affect the $[Ca^{2+}]_i$ response to KCl indicates that the inhibition by wortmannin of KCl-induced contraction was a result of direct inhibition of myosin light chain kinase. These features are consistent with the reported actions of these substances (Nakanishi et al., 1992; Henrion and Laher, 1993; Shimamoto et al., 1993). Wortmannin was reported to be 100 times more selective for myosin light chain kinase over protein kinase C (Nakanishi et

al., 1992). In this study, however, wortmannin was only 4 times more potent on the KCl-induced contraction than the DPB-induced one, when calculated from IC_{50}. In our preliminary study (Ito et al., 1994), DPB caused phosphorylation of myosin light chain in rabbit aortae incubated in Ca^{2+}-containing medium and wortmannin significantly inhibited the phosphorylation. Phosphorylation of vascular smooth muscle myosin light chain by phorbol esters was also observed by others (Fujiwara et al., 1988; Rembold and Murphy, 1988; Singer, 1990). From these, a possibility arises that crosstalk between protein kinase C and myosin light chain kinase is involved in the DPB-induced contraction, in ways such that an increase in $[Ca^{2+}]_i$ induced by a phorbol ester activates myosin light chain kinase, and protein kinase C activation may increase the affinity of myosin light chain kinase to Ca^{2+}-calmodulin or inhibit myosin light chain phosphatase activity (Somlyo et al., 1989; Itoh et al., 1993). If this is the case, a myosin light chain kinase inhibitor would partly inhibit a protein kinase C-dependent contraction. This issue is presently under investigation.

Although calphostin C was selective for protein kinase C-dependent contraction, a much higher concentration was needed for the inhibition compared with staurosporine, e.g. 10 μM calphostin C inhibited the DPB-induced contraction by $53.6 \pm 3.5\%$ and the KCl-induced contraction by $27.3 \pm 4.5\%$ while staurosporine at 30 nM inhibited the DPB-induced contraction by $73.0 \pm 1.3\%$ but slightly inhibited the KCl-contraction (by $9.7 \pm 1.4\%$). In this sense, staurosporine at low concentrations can be regarded as selective as calphostin C to protein kinase C-dependent contraction. On the other hand, wortmannin has been shown to inhibit phosphatidylinositol 3-kinase (Arcaro and Wymann, 1993). The present data on wortmannin are consistent with its expected effect on myosin light chain kinase. At present, it is difficult to speculate whether an action related to phosphatidylinositol 3-kinase was involved in the wortmannin depression of contractions. To assess it, the role of phosphatidylinositol 3-kinase in smooth muscle contraction must be clarified. Although none of these inhibitors is ideal, these substances can be used to clarify the role of protein kinases in receptor-mediated contractions, if an appropriate concentration is used.

Acknowledgements

A part of this study was supported by a grant from the Ministry of Education, Science and Culture of Japan (No. 07660404).

References

Arcaro, A. and M.P. Wymann, 1993, Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses, Biochem. J. 296, 297.

Badwey, J.A., R.W. Erickson and J.T. Curnutte, 1991, Staurosporine inhibits the soluble and membrane-bound protein tyrosine kinases of human neutrophils, Biochem. Biophys. Res. Commun. 178, 423.

Boonen, H.C.M. and J.G.R. De Mey, 1991, Effects of a phorbol ester and staurosporine on electro- and pharmacomechanical coupling in a resistance artery, Eur. J. Pharmacol. 202, 25.

Bruns, R.F., F.D. Miller, R.L. Merriman, J.J. Howbert, W.F. Heath, E. Kobayashi, I. Takahashi, T. Tamaoki and H. Nakano, 1991, Inhibition of protein kinase C by calphostin C is light-dependent, Biochem. Biophys. Res. Commun. 176, 288.

Fujiwara, T., T. Itoh, Y. Kubota and H. Kuriyama, 1988, Actions of a phorbol ester on factors regulating contraction in rabbit mesenteric artery, Circ. Res. 63, 893.

Gleason, M.M. and S.F. Flaim, 1986, Phorbol ester contracts rabbit aorta by increasing intracellular calcium and by activating calcium influx, Biochem. Biophys. Res. Commun. 138, 1362.

Henrion, D. and I. Laher, 1993, Effects of staurosporine and calphostin C, two structurally unrelated inhibitors of protein kinase C, on vascular tone, Can. J. Physiol. Pharmacol. 71, 521.

Inagaki, M., M. Watanabe and H. Hidaka, 1985, *N*-(2-Aminoethyl)-5-isoquinolinesulfonamide, a newly synthesized protein kinase inhibitor, functions as a ligand in affinity chromatography. Purification of Ca^{2+}-activated, phospholipid-dependent and other protein kinases, J. Biol. Chem. 260, 2922.

Itoh, H., A. Shimomura, S. Okubo, K. Ichikawa, M. Ito, T. Konishi and T. Nakano, 1993, Inhibition of myosin light chain phosphatase during Ca^{2+}-independent vasocontraction, Am. J. Physiol. 265, C1319.

Ito, K.M., M. Asano, M. Seto, Y. Sasaki and K. Ito, 1994, Effects of protein kinase inhibitors on changes in cytosolic Ca^{2+}, contraction and myosin light chain phosphorylation caused by phorbol ester and KCl in rabbit aortas, Jpn. J. Pharmacol. 64 (Suppl. 1), 242P.

Jiang, M.J. and K.G. Morgan, 1987, Intracellular calcium levels in phorbol ester-induced contractions of vascular muscle, Am. J. Physiol. 253, H1365.

Kageyama, M., T. Mori, T. Yanagisawa and N. Taira, 1991, Is staurosporine a specific inhibitor of protein kinase C in intact porcine coronary arteries?, J. Pharmacol. Exp. Ther. 259, 1019.

Kamm, K.E. and J.T. Stull, 1989, Regulation of smooth muscle contractile elements by second messengers, Annu. Rev. Physiol. 51, 299.

Kobayashi, E., H. Nakano, M. Morimoto and T. Tamaoki, 1989, Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C, Biochem. Biophys. Res. Commun. 159, 548.

Merkel, L.A., L.M. Rivera, D.J. Colussi and M.H. Perrone, 1991, Protein kinase C and vascular smooth muscle contractility: effects of inhibitors and down-regulation, J. Pharmacol. Exp. Ther. 257, 134.

Mitsui, M. and H. Karaki, 1993, Contractile and relaxant effects of phorbol ester in the intestinal smooth muscle of guinea-pig taenia caeci, Br. J. Pharmacol. 109, 229.

Nakajima, S., M. Fujimoto and M. Ueda, 1993, Spatial changes of $[Ca^{2+}]_i$ and contraction caused by phorbol esters in vascular smooth muscle cells, Am. J. Physiol. 265, C1138.

Nakanishi, S., S. Kakita, I. Takahashi, K. Kawahara, E. Tsukuda, T. Sano, K. Yamada, M. Yoshida, H. Kase, Y. Matsuda, Y. Hashimoto and Y. Nonomura, 1992, Wortmannin, a microbial

product inhibitor of myosin light chain kinase, J. Biol. Chem. 267, 2157.

Rasmussen, H., Y. Takuwa and S. Park, 1987, Protein kinase C in the regulation of smooth muscle contraction, FASEB J. 1, 177.

Rembold, C.M. and R.A. Murphy, 1988, [Ca^{2+}]-dependent myosin phosphorylation in phorbol diester stimulated smooth muscle contraction, Am. J. Physiol. 255, C719.

Sasaki, Y., M. Seto, K. Komatsu and S. Omura, 1991, Staurosporine, a protein kinase inhibitor, attenuates intracellular Ca^{2+}-dependent contractions of strips of rabbit aorta, Eur. J. Pharmacol. 202, 367.

Sato, K., M. Hori, H. Ozaki, H. Takano-Ohmuro, T. Tsuchiya, H. Sugi and H. Karaki, 1992, Myosin phosphorylation-independent contraction induced by phorbol ester in vascular smooth muscle, J. Pharmacol. Exp. Ther. 261, 497.

Seto, M., K. Shindo, K. Ito and Y. Sasaki, 1995, Selective inhibition of myosin phosphorylation and tension of hyperplastic arteries by the kinase inhibitor HA1077, Eur. J. Pharmacol. 276, 27.

Shimamoto, Y., H. Shimamoto, C.-Y. Kwan and E.E. Daniel, 1993, Differential effects of putative protein kinase C inhibitors on contraction of rat aortic smooth muscle, Am. J. Physiol. 264, H1300.

Singer, H.A. 1990, Protein kinase C activation and myosin light chain phosphorylation in ^{32}P-labeled arterial smooth muscle, Am. J. Physiol. 259, C631.

Somlyo, A.P., T. Kitazawa, S. Himpens, G. Matthijs, K. Horiuti, S. Kobayashi, Y.E. Goldman and A.V. Somlyo, 1989, Modulation of Ca^{2+}-sensitivity and of the time course of contraction in smooth muscle: a major role of protein phosphatases?, Adv. Prot. Phosphatase 5, 181.

Takahashi, K., K. Hiwada and T. Kokubu, 1986, Isolation and characterization of a 34000-dalton calmodulin- and F-actin-binding protein from chicken gizzard smooth muscle, Biochem. Biophys. Res. Commun. 141, 20.

Tallarida, R.J., A. Cowan and M.W. Adler, 1979, pA_2 and receptor differentiation: a statistical analysis of competitive antagonism, Life Sci. 25, 637.

Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto and F. Tomita, 1986, Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase, Biochem. Biophys. Res. Commun. 135, 397.

Walsh, M.P., S. Hinkins, R. Dabrowska and D.J. Hartshorne, 1983, Smooth muscle myosin light chain kinase, Methods Enzymol. 99, 279.

Wijetunge, S., C. Aalkjaer, M. Schachter and A.D. Hughes, 1992, Tyrosine kinase inhibitors block calcium channel currents in vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 189, 1620.

Yazawa, M., M. Sakuma and K. Yagi, 1980, Calmodulin from muscle of marine invertebrates, scallop and sea anemone, J. Biochem. 87, 1313.

Yoshida, M. and K. Yagi, 1988, Two kinds of myosin phosphatases with different enzymatic properties from fresh chicken gizzard smooth muscle. Purification and characterization, J. Biochem. 103, 380.