A selective effect of protein kinase C activators on noradrenaline release compared with subsequent contraction in canine isolated saphenous veins

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1 Effects of protein kinase C (PKC) activators and inhibitors on both tritium overflow and subsequent contraction evoked by transmural nerve stimulation (TNS) were investigated in canine saphenous veins prelabelled with [³H]-noradrenaline.

2 Activation of PKC by stepwise increasing concentrations (0.01 nM–1 μ M) of 12-O-tetradecanoylphorbol 13-acetate (TPA), phorbol 12,13-dibutyrate (PDBu) or mezerein caused a significant and concentration-dependent enhancement of the tritium overflow evoked by TNS, while the activators failed to affect the corresponding contraction except with the highest concentration of PDBu when the contraction was significantly reduced. Phorbol, which is inactive on PKC, had no effects on the tritium overflow and contraction induced by TNS.

3 PKC inhibitors, polymyxin B (1 and 10 μ M) and the isoquinolinesulphonamide, H-7 (1 μ M), inhibited significantly the phorbol ester-potentiated tritium overflow evoked by TNS with no effects on the contraction. H-7 and the related inhibitor H-8 at 10 μ M reduced significantly both responses to TNS in the presence of TPA, while they suppressed only the TNS-induced contraction in the absence of TPA.

4 None of the PKC activators or inhibitors affected the spontaneous tritium overflow.

5 PDBu (0.01 and 0.1 μ M) elevated resting tension of the veins more effectively than TPA and mezerein.

6 These results suggest that PKC may modulate electrically stimulated noradrenaline release from adrenergic nerve endings of the canine saphenous veins and the PKC activators may act more selectively on presynaptic than postsynaptic sites, but have no apparent effect on postjunctional noradrenergic mechanisms.

Keywords: Protein kinase C activator; noradrenaline release; contraction; canine saphenous vein

Introduction

Depolarization of adrenergic nerve endings by electrical nerve stimulation leads to an elevation of intraneuronal Ca^{2+} levels, which triggers exocytotic release of noradrenaline (NA) (Blaustein, 1979). The intraneuronal responses between an increased Ca^{2+} concentration and NA release are not established. Recently, Ca^{2+} -phospholipid-dependent protein kinase (protein kinase C, PKC) has been shown to play a role in various biological responses including neurotransmitter release and neurotransmitter-induced contraction of vascular smooth muscle (Nishizuka, 1986). PKC is activated by 1,2-diacylglycerol, one of the products of phosphatidylinositol 4,5-bisphosphate hydrolysis in response to extracellular signals (Takai *et al.*, 1979), and is distributed in sympathetic nerves (Malhotra *et al.*, 1988) and various other tissues (Kuo *et al.*, 1980). Since tumour-promoting phorbol esters mimic the action of the endogenous PKC activator (Nishizuka, 1986), 1,2-diacylglycerol, the esters, 12-O-tetradecanoylphorbol 13-acetate (TPA) and phorbol 12,13-dibutyrate (PDBu) have been widely used as tools to study the involvement of PKC in the release of adrenergic neurotransmitters. Most of the investigations, however, have been performed with central neuronal tissues (Allgaier & Hertting, 1986; Allgaier *et al.*, 1987; Versteeg & Florijn, 1987; Versteeg & Ulenkate, 1987; Wang & Friedman, 1987; Hertting & Allgaier, 1988; Huang *et al.*, 1989), and few previous workers have examined simultaneously the implication of PKC in vascular adrenergic neurotransmission and subsequent neurotransmitter-induced contraction of smooth muscle. In the present study we investigated simultaneously the effect of PKC activators or inhibitors on both tritium overflow and following contraction

evoked by transmural nerve stimulation (TNS) in canine isolated saphenous veins prelabelled with [³H]-NA.

Methods

Isotope experiments

Mongrel dogs of either sex, weighing 12–26 kg, were anaesthetized with sodium pentobarbitone (32 mg kg⁻¹, i.v.). Helical strips (3 × 30 mm) of the canine saphenous veins were incubated at 37°C for 2 h in Krebs-bicarbonate (Krebs) solution containing 134 nM (–)-[7-³H]-NA (10 μ Ci in 5 ml of incubation medium) and 57 μ M ascorbic acid, and mounted for superfusion according to our previous paper (Takata & Kato, 1984). The Krebs solution had the following composition (in mM): NaCl 118.2, KCl 4.6, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 24.8 and dextrose 10.0. The strips were then superfused at a rate of 3.6 ml min⁻¹ with the Krebs solution maintained at 37°C and bubbled with 95% O₂ and 5% CO₂. After a 60 min equilibration period, the veins were electrically stimulated (10 Hz, 0.3 ms, supramaximum voltage of 20 V, 150 pulses) at 16 min intervals through a pair of parallel platinum-wire electrodes with an electric stimulator (Nihon Kohden, SEN-7103). The TNS-induced contraction corresponds to a nearly half-maximal response, judging from the stimulus-contraction relationship. The TNS was repeated 8 times (S₁–S₈), if not indicated otherwise. Developed tension was recorded isometrically on an ink-writing oscillograph (Nihon Kohden, WI-640G) through a force-displacement transducer (Nihon Kohden, TB-611T). The superfusate samples were continuously collected at 2 min intervals from 4 min before S₂ throughout the experiment. One ml of each sample was mixed

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with 10 ml of scintillation mixture (5.5 g DPO, 0.1 g POPOP, 667 ml toluene and 333 ml Triton X-100) and then counted in a liquid scintillation counter (Beckman, LS-9000 or Aloka, LSC-903). The TNS-evoked overflow of total tritium was estimated by subtracting the radioactivity obtained in the 2 min sample just before TNS from that in the 2 min sample during TNS. Thus, we have measured total evoked tritium overflow, and avoided lengthy chromatographic separation of the metabolites, because it more closely reflects neurotransmitter output than does the overflow of [^3H]-NA (Rorie *et al.*, 1980). Either PKC activators (0.01 nM–1 μM) or their vehicle, dimethyl sulphoxide (DMSO), at stepwise increasing concentrations was superfused 10 min before the onset of each stimulation of S_3 to S_8 . The effects of PKC activators on the stimulation-evoked tritium overflow and contraction were expressed as the ratios of the responses to the stimulations (S_3 – S_8) during the superfusion with PKC activators to the response to S_2 , that is S_3/S_2 – S_8/S_2 , and they were evaluated by comparing them with the corresponding ratios obtained from the DMSO-superfused veins. PKC inhibitors were superfused from 10 min before S_3 throughout the experiment. The effects of PKC inhibitors in combination with PKC activators were also expressed as the ratios, S_3/S_2 – S_8/S_2 . These ratios were compared with the corresponding ratios obtained in the veins superfused with PKC activators alone. Radioactivity obtained from the 2 min sample just before stimulation of S_2 – S_8 was considered as the spontaneous tritium overflow and designated as Sp_2 – Sp_8 , respectively. The effects of drugs on the spontaneous tritium overflow were estimated from the ratios, Sp_3/Sp_2 – Sp_8/Sp_2 , and they were compared with the corresponding ratios in the absence of the drugs.

In some experiments the effect of 0.1 μM PDBu on the TNS-evoked tritium overflow was also investigated in the presence of tetraethylammonium (TEA), a K^+ channel blocker, because PKC activation causes blockade of K^+ currents (Baraban *et al.*, 1985) and because the blockade of K^+ currents results in augmentation of the stimulation-evoked NA release from canine saphenous veins (Kato & Takata, 1987). In this case the TNS was repeated 4 times (S_1 – S_4). TEA (1 mM) was superfused from 10 min before S_3 throughout the experiment, and either PDBu (0.1 μM) or DMSO (0.001%) was further added to the superfusion medium from 10 min before S_4 .

Non-isotope experiments

In these experiments, the TNS was applied twice every 16 min under the stimulation conditions described above. Thereafter, NA (10 nmol) was injected 3 times at 30 min intervals in a volume of 0.1 ml into the superfusion stream (NA_1 – NA_3). The amount of NA used was sufficient to cause a contraction similar to that produced by the second TNS. Either PDBu (0.1 μM) or DMSO (0.001%) was superfused 10 min before NA_3 . The effect of PDBu on the contraction induced by exogenous NA was expressed as the ratio NA_3/NA_2 and it was evaluated by comparing with the corresponding ratio in the DMSO-treated group.

Statistical analysis

All data are expressed as the mean $\pm$ s.e.mean. Statistical analyses were performed with an unpaired Student's two-tailed *t* test for two-sample comparison and one-way analysis of variance followed by Fisher's test of least significant difference or Dunnett test for multiple comparisons. In each case, *P* values less than 0.05 were considered significant.

Drugs

The following drugs were used: (–)-[7- ^3H (N)]-NA (specific activity, 14.9 Ci mmol $^{-1}$, New England Nuclear), TPA (Sigma), PDBu (Sigma), mezerein (Sigma), phorbol (Sigma), polymyxin B sulphate (Sigma), 1-(5-isoquinolinesulphonyl)-2-

methyl-piperazine dihydrochloride (H-7) (Seikagaku Kogyo), N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8) (Seikagaku Kogyo), tetrodotoxin (Sigma), TEA chloride (Sigma), NA bitartrate (Sigma). PKC activators (10 mM) were dissolved in DMSO to prepare stock solution. Stock solutions (10 mM) of PKC inhibitors were prepared in distilled water. All drugs used were diluted with Krebs solution just before use.

Results

Effects of protein kinase C activators on TNS-evoked tritium overflow and contraction

In preliminary experiments we confirmed that tetrodotoxin (0.6 μM) abolished the TNS-evoked tritium overflow and the corresponding contraction during superfusion with PKC activators in the veins prelabelled with [^3H]-NA.

Figure 1 shows the effects of increasing concentrations of PKC activators on both tritium overflow and contraction induced by TNS. TPA and PDBu (0.01 nM–1 μM), PKC activating phorbol esters, caused a significant augmentation of the TNS-evoked tritium overflow in a concentration-dependent manner, compared with the DMSO-superfused veins. The

Figure 1 Effects of protein kinase C (PKC) activators on tritium overflow and contraction evoked by transmurial nerve stimulation (TNS) in veins prelabelled with [^3H]-noradrenaline. Each PKC activator at increasing concentrations was superfused 10 min before each stimulation of S_3 to S_8 . The radioactivities in the 2 min sample just before S_2 and the 2 min sample during S_2 were 4920 ± 265 and 13617 ± 1023 d.p.m. ($n = 25$), respectively. The corresponding contraction was 3.03 ± 0.20 g. Values are expressed as the mean from 5 experiments with s.e.mean shown by vertical bars. Symbols are as follows: DMSO ($\circ$); 12-O-tetradecanoylphorbol 13-acetate ($\bullet$); phorbol 12,13-dibutyrate (Δ); mezerein ($\blacktriangle$); phorbol ($\square$). Asterisks indicate significant difference from the veins treated with dimethylsulphoxide: ** $P < 0.01$.

enhancement of the evoked tritium overflow caused by higher concentrations of PDBu (0.1 and $1\mu\text{M}$) was significantly ($P < 0.01$) greater than that by TPA. The TNS-induced contraction remained unchanged in the presence of TPA and PDBu except with the highest concentration of PDBu ($1\mu\text{M}$) when the contraction produced by TNS was significantly ($P < 0.01$) inhibited. Mezerein (0.01 nM– $1\mu\text{M}$), a non-phorbol PKC activator (Miyake *et al.*, 1984), also enhanced significantly the TNS-evoked tritium overflow with no effects on the associated contraction. The facilitatory effect of mezerein was not significantly different from that of PDBu. Phorbol (0.01 nM– $1\mu\text{M}$), which is inactive on PKC (Castagna *et al.*, 1982), failed to affect either tritium overflow or the contraction caused by TNS.

The three PKC activators and phorbol had no significant effects on spontaneous tritium overflow (the ratio Sp_8/Sp_2 for DMSO was 0.68 ± 0.05 ; the ratios Sp_8/Sp_2 for each $1\mu\text{M}$ of TPA, PDBu, mezerein and phorbol were 0.78 ± 0.04 , 0.72 ± 0.03 , 0.79 ± 0.05 and 0.79 ± 0.04 , respectively). PDBu at 1 nM or more produced a concentration-dependent elevation of resting tension, the degree of the elevation being equivalent to $19.2 \pm 3.4\%$ for 1 nM, $22.2 \pm 2.5\%$ for $0.01\mu\text{M}$, $50.7 \pm 8.7\%$ for $0.1\mu\text{M}$ and $89.6 \pm 10.6\%$ for $1\mu\text{M}$ of the contraction ($3.08 \pm 0.22\text{ g}$, $n = 5$) induced by S_2 . There was a slight increase in the resting tension in the presence of the other PKC activators corresponding to $15.6 \pm 4.2\%$ ($n = 5$) for $1\mu\text{M}$ TPA and $17.8 \pm 4.7\%$ ($n = 5$) for $1\mu\text{M}$ mezerein over that induced by S_2 . Phorbol failed to elevate resting tension.

Effects of protein kinase C inhibitors on the action of PKC activators on TNS-evoked tritium overflow and contraction

Polymyxin B (1 and $10\mu\text{M}$), a PKC inhibitor (Kuo *et al.*, 1983), produced a significant inhibition of the facilitatory effects of TPA on the TNS-evoked tritium overflow with no alteration of the contraction in comparison with the TPA-treated veins (Figure 2a). H-7 (1 and $10\mu\text{M}$), an isoquinolinesulphonamide PKC inhibitor (Hidaka *et al.*, 1984), also significantly inhibited the enhancement of the evoked

tritium overflow by TPA, which was not significantly different from the inhibitory action of polymyxin B (Figure 2b). The corresponding contraction was inhibited only by $10\mu\text{M}$ H-7 (Figure 2b). In the presence of TPA, H-8 ($1\mu\text{M}$), another isoquinolinesulphonamide PKC inhibitor (Hidaka *et al.*, 1984), had no effects on the TNS-evoked tritium overflow and contraction, but at $10\mu\text{M}$ it inhibited significantly both responses to TNS (Figure 2c).

The facilitation of the TNS-evoked tritium overflow by PDBu was significantly reduced by superfusion with $1\mu\text{M}$ of each polymyxin B and H-7 but without effects on the contraction induced by TNS (Figure 3). There was no significant difference between the inhibitory actions of the two inhibitors.

The three PKC inhibitors used, by themselves, did not affect the TNS-evoked tritium overflow, whereas H-7 and H-8 ($10\mu\text{M}$), but not polymyxin B, significantly inhibited the associated contraction (Figure 4). These inhibitors caused no change in spontaneous tritium overflow in the presence and absence of TPA and PDBu compared with the DMSO-treated veins.

Effects of phorbol 12,13-dibutyrate on TNS-evoked tritium overflow and contraction in the presence of tetraethylammonium

As shown in Table 1, TEA (1 mM) enhanced significantly both responses to TNS; the ratios S_4/S_2 were 2.16 ± 0.15 in overflow and 1.24 ± 0.03 in contraction. Since TEA (1 mM) does not increase the contraction induced by exogenous NA (Takata & Kato, 1988), the enhancing effect of TEA on the TNS-evoked contraction is due to an increased neurotransmitter release. PDBu ($0.1\mu\text{M}$) in combination with TEA produced a further significant facilitation of the TNS-evoked tritium overflow, while it caused no additional increase in the contraction.

Effects of phorbol 12,13-dibutyrate on the contraction induced by exogenous noradrenaline

To confirm further a more selective action of PKC activators on presynaptic than postsynaptic sites, the effects of PDBu on

Figure 2 Effects of polymyxin B (a), H-7 (b) and H-8 (c) on tritium overflow and contraction evoked by transmurial nerve stimulation (TNS) in the presence of 12-O-tetradecanoylphorbol 13-acetate (TPA) in veins prelabelled with [^3H]-noradrenaline. TPA at increasing concentrations was superfused 10 min before each stimulation of S_3 to S_8 . Each protein kinase C inhibitor ($1\mu\text{M}$ (●), $10\mu\text{M}$ (△), no inhibitor (○)) was superfused from 10 min before S_3 throughout the experiment. Values are expressed as the mean from 5 to 6 experiments with s.e.mean shown by vertical bars. Asterisks indicate significant difference from the veins treated with TPA alone (no inhibitor): * $P < 0.05$; ** $P < 0.01$.

Figure 3 Effects of poloxymyxin B and H-7 on tritium overflow and contraction evoked by transmurial nerve stimulation (TNS) in the presence of phorbol 12,13-dibutyrate (PDBu) in veins prelabelled with [³H]-noradrenaline. PDBu at increasing concentrations was superfused 10 min before each stimulation of S₃ to S₈. Each protein kinase C inhibitor (1 μM poloxymyxin B (●), 1 μM H-7 (Δ), no inhibitor (○)) was superfused from 10 min before S₃ throughout the experiment. Values are expressed as the mean from 5 experiments with s.e.mean shown by vertical bars. Asterisks indicate significant difference from the veins treated with PDBu alone (no inhibitor): **P* < 0.05; ***P* < 0.01.

the contraction induced by exogenous NA (10 nmol) were investigated. In the vehicle group, the increases in tension to the second TNS and the second application of NA were 3.12 ± 0.43 and 3.40 ± 0.49 g (*n* = 5), respectively. The corresponding values in another group of veins before superfusion

Table 1 Effects of phorbol 12,13-dibutyrate (PDBu, 0.1 μM) on transmurial nerve stimulation (TNS)-evoked tritium overflow and contraction in the presence of tetraethylammonium (TEA, 1 mM) in veins prelabelled with [³H]-noradrenaline

Drugs	TNS-evoked	
	³ H overflow (S ₄ /S ₂)	Contraction (S ₄ /S ₂)
Control	0.99 ± 0.02	1.03 ± 0.03
TEA + DMSO	2.16 ± 0.15**	1.24 ± 0.03**
TEA + PDBu	3.21 ± 0.25***††	1.33 ± 0.06**

TEA was superfused from 10 min before S₃ throughout the experiment. PDBu was added to the superfusion medium for 16 min from 10 min before S₄. Values are expressed as the mean ± s.e.mean from 5 experiments. Significantly different from the control veins (***P* < 0.01) or from the veins treated with TEA plus 0.001% dimethylsulphoxide (DMSO) (††*P* < 0.01).

with 0.1 μM PDBu were 2.95 ± 0.49 and 3.22 ± 0.61 g (*n* = 5), respectively. No significant difference between the contractions caused by the TNS and NA was observed in either group. PDBu (0.1 μM) produced no significant potentiation of the contraction induced by 10 nmol NA (the ratios NA₃/NA₂ for the vehicle and PDBu groups were 1.07 ± 0.04 (*n* = 5) and 1.10 ± 0.02 (*n* = 5), respectively).

Discussion

In the present study TPA and PDBu caused a significant and concentration-dependent enhancement of tritium overflow induced by TNS in canine saphenous veins prelabelled with [³H]-NA, while phorbol, which is insensitive to PKC (Castagna *et al.*, 1982), failed to enhance it. Mezerein, a non-phorbol PKC activator (Miyake *et al.*, 1984), also facilitated the TNS-evoked tritium overflow in a concentration-dependent manner. Together with the evidence that PKC is distributed in sympathetic nerves (Malhotra *et al.*, 1988), the results of the present study suggest that PKC may be involved in exocytotic NA release from the canine saphenous veins. Our present findings are qualitatively similar to previous

Figure 4 Effects of poloxymyxin B (a), H-7 (b) and H-8 (c) on tritium overflow and contraction evoked by transmurial nerve stimulation (TNS) in veins prelabelled with [³H]-noradrenaline. Each protein kinase C inhibitor (1 μM (●), 10 μM (Δ), no inhibitor (○)) was superfused from 10 min before S₃ throughout the experiment. Values are expressed as the mean from 5 experiments with s.e.mean shown by vertical bars. Asterisks indicate significant difference from the control veins (no inhibitor): ***P* < 0.01.

reports on neurotransmitter release from both central (see Introduction) and peripheral adrenergic nerve endings (Wakade *et al.*, 1985; Shuntoh & Tanaka, 1986; Ishac & De Luca, 1988; Musgrave & Majewski, 1989). Polymyxin B and H-7, PKC inhibitors, counteracted significantly the PKC activator-induced facilitation of the tritium overflow evoked by TNS. H-7, but not polymyxin B (Mazzei *et al.*, 1982), also inhibits adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase at about the same concentration range (Hidaka *et al.*, 1984). Since the agents, which elevate cyclic AMP content, enhance NA release evoked by depolarization from the adrenergic nerve endings of canine saphenous veins (Takata & Kato, 1984; 1985), we investigated whether H-7 reduced the enhancing effects of PKC activators on the evoked tritium overflow by inhibiting cyclic AMP-dependent protein kinase, but not PKC. For this purpose, we used H-8, which is a more potent inhibitor of cyclic AMP-dependent protein kinase than PKC and is less potent than H-7 in inhibiting PKC (Hidaka *et al.*, 1984). Because H-8 did not inhibit the TPA-induced enhancement of the evoked tritium overflow more strongly than did H-7, these results suggest that the antagonistic action of H-7 may be due to inhibition of PKC and that the enhancing effect of PKC activators on the TNS-evoked NA release from the canine saphenous veins is attributable to activation of PKC. In spite of these suggestions, neither polymyxin B nor H-7, by themselves, inhibited the tritium overflow evoked by TNS. Since the two PKC inhibitors at 10 μM or more were previously shown not to inhibit stimulation evoked neurotransmitter release (Allgaier & Hertting, 1986; Wang & Friedman, 1987; Shuntoh *et al.*, 1988) although higher doses ($> 30 \mu\text{M}$) were effective, it is possible that the concentration of PKC inhibitors used to reduce the evoked tritium overflow in the present study may be too low.

PDBu at higher concentrations enhanced the tritium overflow evoked by TNS more markedly than did TPA, although TPA is more potent or equivalent to PDBu in activating PKC *in vitro* (Castagna *et al.*, 1982; Malhotra *et al.*, 1988). The reason for such potency differences is unknown but a greater potency of PDBu was also reported in NA releases from the rabbit hippocampus (Allgaier *et al.*, 1987) and from the mouse atria (Musgrave *et al.*, 1989). The former authors believe that a major reason for the reduced potency of TPA is that this highly lipophilic drug requires more time to equilibrate in the extracellular space compared with more hydrophilic analogues (Dunwiddie & Fredholm, 1985). On the other hand, Musgrave *et al.* (1989) showed that a smaller effect of TPA is not simply due to TPA requiring a longer time to produce its effect, because the potentiation caused by TPA was identical when the pretreatment period was prolonged from 15 to 75 min, and because PDBu produced a further enhancement of NA release even in the presence of the maximally effective concentration of TPA. From these results, they suggested that sympathetic nerves of the mouse atria possess multiple forms of PKC which are differentially activated by TPA and PDBu.

The precise mechanism by which PKC activators potentiate NA release remains unclear. The studies with rat salivary gland (Wakade *et al.*, 1985) and mouse atria (Musgrave & Majewski, 1989) indicate that the facilitatory action of PKC activators on electrically stimulated NA release is not due to blockade of presynaptic α_2 -adrenoceptors. Furthermore, it has been reported that inhibition of neural NA reuptake is not responsible for the phorbol ester-induced potentiation of the evoked NA release (Shuntoh *et al.*, 1988). PKC activation causes blockade of K^+ currents (Baraban *et al.*, 1985) or facilitation of Na^+ (Dicker & Rozengurt, 1981) and Ca^{2+} currents (Wakade *et al.*, 1986), each of which leads to facilitation of the evoked NA release. PDBu caused a further significant enhancement of the TNS-evoked tritium overflow even in the presence of TEA. If PKC activators potentiate the depolarization-evoked NA release via facilitation of Na^+ currents following the enzyme activation, then it would be antici-

pated that the activators enhance the tritium overflow evoked by TNS, but not by elevated potassium ions, because the NA release induced by 40 mM KCl is not modified by drugs affecting Na^+ channel function in canine saphenous veins (Takata & Kato, 1985). However, the 40 mM KCl-evoked tritium overflow from canine saphenous veins preloaded with [^3H]-NA was also enhanced by PKC activators (unpublished data). Therefore, it appears that neither blockade of K^+ currents nor facilitation of Na^+ currents accounts for the PKC activator-potentiated tritium overflow. There is evidence for a synergistic action of PKC and Ca^{2+} in the stimulation-secretion coupling process (Nishizuka, 1984). The synergism occurs as a result of the increased sensitivity to Ca^{2+} of NA release processes triggered by Ca^{2+} following PKC activation (Shuntoh & Tanaka, 1986). Phorbol ester also causes a marked enhancement of electrically stimulated $^{45}\text{Ca}^{2+}$ accumulation in cultured sympathetic neurones of the chick embryo facilitating NA release (Malhotra *et al.*, 1988). This, together with the result that phorbol esters enhance only the Ca^{2+} -dependent NA release (Wakade *et al.*, 1985; Allgaier *et al.*, 1987), make it most likely that PKC activation and the resultant phosphorylation of neuronal proteins responsible for release processes lead to either enhanced influx of Ca^{2+} into the nerve endings or sensitization to Ca^{2+} of exocytotic processes facilitating NA release. The neuronal proteins which are substrates for PKC during NA release processes in the canine saphenous veins are still unknown.

Neither PKC activators nor inhibitors caused significant effects on spontaneous tritium overflow, in agreement with the findings in other tissues (Wakade *et al.*, 1985; Shuntoh & Tanaka, 1986; Ishac & De Luca, 1988; Shuntoh *et al.*, 1988; Musgrave & Majewski, 1989), suggesting that PKC activation may be responsible for modulating rather than for mediating neurotransmitter release.

PKC activators facilitated the TNS-evoked tritium overflow without producing a significant increase in subsequent contraction. Furthermore, PDBu failed to enhance the contraction produced by exogenous NA. Thus, it seems that PKC activators are more selective in their presynaptic than postsynaptic effects in the canine saphenous veins in contrast to the results with the perfused rat kidney (Sehic & Malik, 1989). Since the contraction produced by TNS was significantly increased in the presence of 1 mM TEA, the finding that PKC activators did not enhance the TNS-induced contraction in spite of an increase in the evoked tritium overflow cannot be explained by the view that no further increase in contraction occurred after PKC activators because the contraction was already maximal. The present study indicates that 1 mM TEA augmented electrically evoked tritium overflow 2.16 fold, whereas it enhanced the contraction only 1.24 fold. Therefore, the enhancement of the TNS-evoked overflow by PKC activators appears to be insufficient for significant potentiation of the contraction. Moreover, it is unlikely that under the conditions employed PKC plays a major role in the contractile response of the canine saphenous veins to NA released. A significant inhibition of the TNS-evoked contraction by the highest concentration (1 μM) of PDBu is likely to be due to a marked elevation of resting tension.

H-7 or H-8 (10 μM) inhibited significantly the TNS-evoked contraction regardless of the presence of PKC activators. The inhibitory effects of the two inhibitors are not due to a decrease in the evoked tritium overflow, since polymyxin B, which inhibited the evoked tritium overflow to a similar extent, did not reduce the corresponding contraction. Both H-7 and H-8 inhibit not only PKC but also Ca^{2+} -calmodulin-dependent protein kinase, the K_i values of H-7 and H-8 for Ca^{2+} -calmodulin-dependent kinase being 97 and 68 μM , respectively (Hidaka *et al.*, 1984). On the other hand, polymyxin B is over 100 fold more potent as an inhibitor of PKC than of Ca^{2+} -calmodulin-dependent kinase (K_i value; 6.5 μM vs 950 μM) (Wrenn & Wooten, 1984). It appears, therefore, that the inhibitory effects of H-7 and H-8 on the TNS-induced contraction may be due to the inhibition of Ca^{2+} -calmodulin-

dependent kinase rather than PKC. This view is also supported by the result that the PKC activators did not increase the contraction produced by TNS.

Higher concentrations of PDBu elevated resting tension much more effectively than TPA and mezerein. Phorbol did not raise resting tension. These findings suggest that the elevation of resting tension by the activators is probably due to PKC activation. Chiu *et al.* (1988) have reported that PDBu may activate the voltage-dependent Ca^{2+} channels through PKC activation to contract the canine saphenous veins. The lesser potency of TPA in raising resting tone might be in part explained by its highly lipophilic property as mentioned

above. The elevation of resting tension induced by TPA and PDBu was not significantly suppressed by polymyxin B and H-7, in agreement with the result that H-7 at $10\text{ }\mu\text{M}$ caused no inhibition of the TPA-induced contraction in the aorta of normotensive Wistar Kyoto rats (Shibata *et al.*, 1990). On the other hand, Khalil & van Breemen (1988) found that H-7 at $20\text{ }\mu\text{M}$ reversibly inhibited the TPA-induced contraction in the rabbit aorta. Differences in animal species and experimental conditions might account for this discrepancy.

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