Presynaptic Sites of Isolated Canine Saphenous Veins Are More Sensitive to Protein Kinase C than Postsynaptic Ones

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ABSTRACT—Effects of protein kinase C (PKC) activators and inhibitors on both tritium overflow and contraction evoked by 40 mM KCl were studied in canine saphenous veins preloaded with [3H]norepinephrine (NE). 12-O-Tetradecanoylphorbol 13-acetate (TPA) and phorbol 12,13-dibutyrate (PDBu) at 10^{-11} – 10^{-7}M enhanced concentration-dependently the KCl-evoked tritium overflow, which was antagonized by polymyxin B (10^{-5} M) and staurosporine (10^{-7} or 10^{-6}M). PDBu (10^{-8} and 10^{-7}M), but not TPA, potentiated the KCl-induced contraction. Only staurosporine reduced the KCl-induced contraction in the presence of PKC activators. Polymyxin B (3 \times 10^{-5} M) which failed to inhibit exogenous NE-induced contraction attenuated both responses to KCl. Staurosporine (10^{-6} M) suppressed not only both the responses to KCl but also exogenous NE-induced contraction. Phentolamine (10^{-6} M) inhibited almost completely the KCl-induced contraction and augmented remarkably the evoked tritium overflow. PDBu (10^{-8} and 10^{-7}M) still potentiated both responses to KCl in the phentolamine-treated veins. An additional treatment with nifedipine (10^{-6} M) inhibited markedly the potentiation of the KCl-induced contraction by PDBu in the presence of phentolamine without affecting the evoked overflow. These results suggest that PKC may modulate KCl-evoked NE release from the adrenergic nerve endings of canine saphenous veins and that PKC is more sensitive to presynaptic than postsynaptic sites.

Although it is well-known that a rise in the intraneuronal concentration of Ca^{2+} triggers exocytotic norepinephrine (NE) release from adrenergic nerve endings, the intraneuronal processes linking an increased Ca^{2+} concentration and NE release are not established. Protein kinase C (PKC), which is a Ca^{2+}- and phospholipid-enzyme, is distributed in presynaptic nerve endings (1) and various other tissues (2), and it is activated by both tumor-promoting phorbol esters (3) and the endogenous PKC activator 1,2-diacylglycerol (4). Activation and inhibition of PKC have been reported to facilitate and to suppress depolarization-evoked release of neurotransmitters, respectively (5–10). These findings suggest that PKC may play a role in the regulation of neurotransmitter release. Phorbol esters have been also utilized to evaluate the role of PKC in neurotransmitter-induced contraction of vascular smooth muscle (11–13). There are, however, few reports that have simultaneously investigated the involvement of PKC in vascular adrenergic neurotransmission...
and associated contraction of smooth muscle. Recently, we have reported that PKC activators enhance electrically evoked NE release from adrenergic nerve endings of the canine saphenous veins, but have no apparent effect on the corresponding contraction (14).

In the tissues with rich adrenergic innervation, KCl causes exocytotic release of NE through depolarization of adrenergic nerve endings (15). Thus, KCl as well as electrical nerve stimulation has been extensively employed for the studies on the effects of various agents on neurotransmitter release from central (16, 17) and peripheral (15, 18) adrenergic nerve endings. In the present study, to further investigate the role of PKC in presynaptic and postsynaptic sites, we studied simultaneously the effects of PKC activators and inhibitors on both NE release and contraction in response to KCl in the canine isolated saphenous veins preloaded with [3H]NE.

MATERIALS AND METHODS

Isotope experiments

Mongrel dogs of either sex, weighing 8–17 kg, were anesthetized with sodium pentobarbital, 32 mg/kg, i.v. Helical strips (3 x 30 mm) of the canine saphenous veins were incubated at 37°C for 2 hr in Krebs-bicarbonate (Krebs) solution containing 1.34x10^{-7} M [7-3H]NE (10 μCi in 5 ml of incubation medium) and 5.7x10^{-5} M ascorbic acid. This concentration of [3H]NE ensures adequate labelling of the tissues (19). The Krebs solution had the following composition: 118.2 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM KH2PO4, 24.8 mM NaHCO3 and 10.0 mM dextrose. After the incubation, the strips were mounted for superfusion according to our previous report (20) and superfused at a rate of 3.6 ml/min with Krebs solution. The 6-min application of 40 mM KCl was repeated either 3 or 7 times as described below. The 40 mM KCl solution was prepared by substituting NaCl with equimolar KCl. 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 every 2 min from 4 min before the second application of 40 mM KCl throughout the experiment. One ml of each sample was mixed with 6 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 (Aloka, LSC-903). The tritium overflow evoked by 40 mM KCl was calculated by subtracting the radioactivity present in the 2-min sample just before KCl application from that in the 6-min of KCl application plus subsequent 2-min sample, and the sum of the differences was taken as the KCl-evoked tritium overflow. Thus, we have measured total evoked tritium overflow, and avoided a chromatographic separation of the metabolites, because it more closely reflects neurotransmitter release than does the overflow of [3H]NE (21).

Experimental protocol

The first series of experiments was carried out to study the effect of PKC activators, 12-O-tetradecanoylphorbol 13-acetate (TPA) and phorbol 12,13-dibutyrate (PDBu), on the 40 mM KCl-evoked tritium overflow and contraction in the presence or absence of PKC inhibitors, polymyxin B and staurosporine. The stimulation with 40 mM KCl was repeated 7 times (S1–S7). Either PKC activators (10^{-11}–10^{-7} M) or their vehicle, dimethyl sulfoxide (DMSO), at stepwise increasing concentrations was superfused for 22 min each from 10 min before the onset of each stimulation of S3 to S7. The effect of PKC activators on the depolarization-evoked tritium overflow and contraction was expressed as the ratios of the responses to each stimulation (S3–S7) during the application of PKC activators to the re-
sponse to S2, that is, S3/S2 - S7/S2, and they were evaluated by comparing them with the corresponding ratios obtained from the vehicle-superfused veins. PKC inhibitors were superfused from 10 min before S3 throughout the experiment. The effect of PKC inhibitors in combination with PKC activators was also expressed as the ratios S3/S2 - S7/S2. 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 each stimulation of S2 - S7 was considered as the spontaneous tritium overflow and designated as Sp2 - Sp7, respectively. The effect of drugs on the spontaneous tritium overflow was estimated from the ratios, Sp3/Sp2 - Sp7/Sp2, and they were compared with the corresponding ratios in the absence of the drugs.

In the first series of experiments, higher concentrations of PDBu potentiated both tritium overflow and contraction in response to 40 mM KCl. Therefore, the second series of experiments was performed to examine whether the PDBu-potentiated contraction is due to increased NE release or increased Ca^{2+} influx as a result of depolarization of the venous smooth muscle cells, because these two mechanisms are responsible for the contraction elicited by KCl in the canine saphenous vein (18) and because PDBu activates the voltage-dependent Ca^{2+} channels in the smooth muscle of the canine saphenous vein (22). The stimulation with 40 mM KCl and the superfusion with PDBu (10^{-11} - 10^{-7} M) were identical to those described for the first series of experiments. Phentolamine and nifedipine at 10^{-6} M were superfused from 10 min before S3 throughout the experiment in order to block the components in response to released NE and depolarization of the smooth muscle cells, respectively, of the 40 mM KCl-induced contraction.

The third series of experiments was performed to investigate the effect of PKC inhibitors on the 40 mM KCl-evoked tritium overflow and contraction. The veins were stimulated 3 times (S1 - S3) with 40 mM KCl. PKC inhibitors were superfused 10 min before S3. The effects of the inhibitors on the responses to 40 mM KCl and on the spontaneous tritium overflow were expressed as the ratios S3/S2 and Sp3/Sp2, respectively, and they were compared with the corresponding ratios in the vehicle-treated veins.

The fourth series of experiments was carried out to investigate whether inhibition of K^+ channels leads to an enhancement of the KCl-evoked tritium overflow, because PDBu inhibits K^+ channels following PKC activation (23). In these experiments, the 40 mM KCl treatment was repeated 3 times (S1 - S3) and 10^{-3} M tetraethylammonium (TEA), a K^+ channel blocker, was superfused 10 min before S3. The effect of TEA was expressed and evaluated, as in the third series of experiments.

**Non-isotope experiments**

To further examine whether the inhibition of the KCl-induced contraction by higher concentration of PKC inhibitors is only due to a decrease in the evoked tritium overflow, the effect of PKC inhibitors on exogenous NE was investigated. In these experiments, the 6-min application of 40 mM KCl was repeated twice, and NE was then injected 3 times at 30 min intervals in a volume of 0.1 ml into the superfusion stream (NE1 - NE3). The amount (6 - 20 nmol) of NE used was sufficient to cause a contraction similar to that elicited by the second application of 40 mM KCl. PKC inhibitors were superfused 20 min before NE3. The effect of PKC inhibitors on the contraction induced by exogenous NE was expressed as the ratio NE3/NE2, which was evaluated by comparing it with the corresponding ratio in the vehicle-treated veins.

**Statistical analysis**

All data are expressed as the mean ± S.E.M. Statistical analyses were performed using an unpaired Student's t-test for two-sample comparison and one-way analysis of variance followed by Dunnett test or the wholly significant difference method for multiple comparisons. In each case, P values less than 0.05
were considered significant.

Drugs

The following drugs were used: 1-[7-\textsuperscript{3}H(N)]norepinephrine (NE) (specific activity, 14.9 Ci/mmol; New England Nuclear), 12-O-tetradecanoylphorbol 13-acetate (Sigma), phorbol 12,13-dibutyrate (Sigma), phorbol (Sigma), polymyxin B sulfate (Sigma), stauros- porine (Boehringer Mannheim), phenolamine mesylate (Ciba-Geigy), nifedipine (Bayer), tetraethylammonium (TEA) chloride (Sigma), and NE bitartrate (Sigma). PKC activators (10\textsuperscript{-2} M) and staurosporine (10\textsuperscript{-3} M) were dissolved in DMSO to prepare stock solutions. Nifedipine (3 \times 10\textsuperscript{-3} M) was freshly dissolved in ethanol. Handling of nifedipine was carried out under light from a sodium vapor lamp. Polymyxin B and TEA were dissolved in distilled water. All drugs used were diluted with Krebs solution just before use.

RESULTS

Effects of PKC activators on 40 mM KCl-evoked tritium overflow and contraction

Figure 1 shows the effects of stepwise increasing concentrations of PKC activators on both tritium overflow and contraction induced by 40 mM KCl. TPA and PDBu (10\textsuperscript{-11}–10\textsuperscript{-7} M) were...
PKC activating phorbol esters, enhanced significantly the KCl-evoked tritium overflow in a concentration-dependent manner, compared with the vehicle (DMSO)-superfused veins. The augmentation of the KCl-evoked tritium overflow by PDBu (10^{-8} and 10^{-7} M) was significantly (P < 0.05) greater than that by the same concentration of TPA. TPA at all concentrations used had no significant effects on the KCl-induced contraction, whereas PDBu at 10^{-8} and 10^{-7} M potentiated significantly the contraction. Phorbol (10^{-11}–10^{-7} M), which is inactive on PKC (24), affected neither the tritium overflow nor the contraction induced by KCl.

TPA, PDBu and phorbol at all concentrations employed failed to alter the spontaneous tritium overflow (the ratio Sp7/Sp2 for DMSO was 0.77 ± 0.06; the ratios Sp7/Sp2 for 10^{-7} M of TPA, PDBu and phorbol were 0.72 ± 0.02, 0.73 ± 0.02 and 0.76 ± 0.02, respectively. PDBu at 10^{-8} M or more elevated the resting tension. The elevation in the resting tension by PDBu corresponded to 15.5 ± 1.9% for 10^{-8} M and 62.0 ± 5.2% for 10^{-7} M of the contraction (3.56 ± 0.53 g, n = 6) induced by S2. TPA caused no marked rise in the resting tension.

Effects of PKC activators on 40 mM KCl-evoked tritium overflow and contraction in the presence of PKC inhibitors

As shown in Fig. 2, the facilitatory effects of TPA and PDBu on the KCl-evoked tritium overflow were significantly reduced by polymyxin B (10^{-5} M) and staurosporine (10^{-7} or 10^{-6} M). There was no difference between the inhibitory actions of the two inhibitors. The KCl-induced contraction in the presence of TPA and PDBu was suppressed by staurosporine but not by polymyxin B (Fig. 2). The inhibitory action of staurosporine was
concentration- and superfusion time-dependent under stepwise increasing concentrations of TPA or PDBu (the ratios \(S_3/S_2\) and \(S_7/S_2\) for \(10^{-7}\) staurosporine were 0.96 ± 0.02 and 0.39 ± 0.05 in the TPA-treated veins, respectively; the corresponding ratios for \(10^{-6}\) M staurosporine were 0.82 ± 0.05 and 0.14 ± 0.08 in the PDBu-treated veins, respectively). Neither Polymyxin B nor staurosporine altered the spontaneous tritium overflow. The PDBu-induced increase in the resting tension was completely abolished by concurrent superfusion with \(10^{-6}\) M staurosporine.

Effects of phentolamine or phentolamine plus nifedipine on the action of PDBu on 40 mM KCl-evoked tritium overflow and contraction

Phentolamine (\(10^{-6}\) M) alone facilitated significantly the KCl-evoked tritium overflow, whereas it almost completely inhibited the contraction (Fig. 3). The inhibitory effect of phentolamine on the contraction progressively increased with time: the ratios \(S_3/S_2\) and \(S_7/S_2\) were 0.13 ± 0.03 and 0.02 ± 0.01, respectively. On the other hand, the tritium overflow enhancing effect of phentolamine was almost constant regardless of the superfusion time. In the phentolamine-treated veins, PDBu at

![Graph showing effects of phentolamine and nifedipine on KCl-evoked tritium overflow and contraction.](image)

**Fig. 3.** Effect of phentolamine (Phent) or phentolamine plus nifedipine (Nife) on the action of phorbol 12,13-dibutyrate (PDBu) on 40 mM KCl-evoked tritium overflow and contraction in veins preloaded with \([\text{H}]\)norepinephrine. PDBu was superfused as shown in Fig. 1. Phentolamine and nifedipine were superfused from 10 min before the onset of \(S_3\) throughout the experiment. Values are expressed as the mean ± S.E.M. from 5 to 6 experiments. *\(P < 0.05\), **\(P < 0.01\).
10^{-11} - 10^{-7} M caused a significant and concentration-dependent enhancement of the KCl-evoked tritium overflow; and at 10^{-8} and 10^{-7} M, it potentiated significantly the KCl-induced contraction (Fig. 3). Nifedipine (10^{-6} M) failed to affect the facilitatory effect of PDBu (10^{-11} - 10^{-7} M) on the KCl-evoked tritium overflow in the presence of phentolamine, but this Ca entry blocker inhibited markedly the KCl-induced contraction observed in a combined superfusion with PDBu (10^{-8} and 10^{-7} M) and phentolamine (Fig. 3). Neither phentolamine nor nifedipine had a significant effect on the spontaneous tritium overflow from the PDBu-treated veins. Although phentolamine did not affect the rise in the resting tension by PDBu (10^{-8} and 10^{-7} M), nifedipine inhibited significantly (P < 0.01) the elevating action of PDBu on the resting tension in the presence of phentolamine: the elevation of the resting tension by 10^{-8} M and 10^{-7} M PDBu in combination with nifedipine and phentolamine corresponded to 2.7 ± 2.0% and 16.8 ± 8.1%, respectively, of the contraction (3.07 ± 0.29 g, n = 5) induced by S2.

**Effects of PKC inhibitors on 40 mM KCl-evoked tritium overflow and contraction**

Table 1. Effects of polymyxin B and staurosporine on spontaneous and KCl-evoked tritium overflows and KCI-induced contraction in veins preloaded with [3H]norepinephrine

| Drugs                  | Conc (M) | Spontaneous \[^3^H\] overflow | KCl-evoked \[^3^H\] overflow | contraction |
|------------------------|----------|-------------------------------|-----------------------------|-------------|
|                        |          | \(S_p/S_p\)                   | \(S_3/S_2\)                 | \(S_3/S_2\) |
| Control (None)         |          | 0.90 ± 0.02                   | 0.99 ± 0.03                 | 0.99 ± 0.03 |
| Polymyxin B            | 10^{-5}  | 0.96 ± 0.02                   | 0.88 ± 0.04                 | 0.92 ± 0.02 |
|                        | 3 \times 10^{-5} | 0.92 ± 0.03 | 0.57 ± 0.08** | 0.71 ± 0.02** |
| Control (0.1% DMSO)    |          | 0.97 ± 0.04                   | 0.93 ± 0.02                 | 0.97 ± 0.03 |
| Staurosporine          | 10^{-7}  | 0.96 ± 0.03                   | 0.85 ± 0.04                 | 0.03 ± 0.04 |
|                        | 10^{-6}  | 0.96 ± 0.04                   | 0.75 ± 0.05*                | 0.63 ± 0.11* |

The veins were stimulated 3 times (S1–S3) with 40 mM KCl. Each protein kinase C inhibitor was superfused 10 min before S1. The radioactivities in the 2-min sample just before S2 and in the four 2-min samples after onset of S2 were 4560 ± 324 and 21281 ± 1483 dpm (n = 31), respectively. The contraction was 3.29 ± 0.27 g. Values are expressed as the mean ± S.E.M. from 5 to 6 experiments. Asterisks indicate significant difference from the respective control, *P < 0.05, **P < 0.01.

Polymyxin B at 10^{-5} M had no effects on the tritium overflow and contraction in response to 40 mM KCl, but at 3 \times 10^{-5} M, it caused a significant inhibition of both responses to 40 mM KCl (Table 1). Staurosporine at 10^{-6} M but not at 10^{-7} M inhibited significantly the KCl-evoked tritium overflow and contraction (Table 1). The two PKC inhibitors did not alter the spontaneous tritium overflow and the resting tension.

**Effects of TEA on 40 mM KCl-evoked tritium overflow and contraction**

TEA (10^{-3} M) had no significant effects on the 40 mM KCl-evoked tritium overflow and contraction (the ratios S3/S2 in overflow were 0.99 ± 0.03 (n = 5) for the control and 1.04 ± 0.04 (n = 5) for TEA; the ratios S3/S2 in contraction were 0.99 ± 0.03 for the control and 1.01 ± 0.02 for TEA). This indicates that blockade of K\(^+\) channels is not responsible for the 40 mM KCl-evoked tritium overflow mechanisms.

**Effects of PKC inhibitors on exogenous NE-induced contraction**

As shown in Table 2, there was no significant difference between tensions developed by the 2nd 40 mM KCl and the 2nd NE before
superfusion with polymyxin B or staurosporine. Polymyxin B (3 × 10⁻⁵ M) did not reduce the contractile response to exogenous NE, while staurosporine (10⁻⁷ and 10⁻⁶ M) caused a significant and concentration-dependent inhibition of the contraction induced by exogenous NE (Table 2).

**DISCUSSION**

The involvement of PKC in stimulation-secretion coupling processes has been demonstrated by using tumor-promoting phorbol esters that mimic the action of the endogenous PKC activator 1,2-diacylglycerol (see the Introduction). In the present study, PKC activating phorbol esters, TPA and PDBu, produced a significant and concentration-dependent facilitation of the 40 mM KCl-evoked tritium overflow in the canine saphenous veins preloaded with [³H]NE. Phorbol, which lacks the ability to activate PKC (24), failed to enhance the KCl-evoked tritium overflow. PKC inhibitors, polymyxin B (25) and staurosporine (26), by themselves, inhibited the evoked tritium overflow and counteracted the enhancing action of PKC activators on the evoked tritium overflow. These results consistently suggest that PKC may be involved in exocytotic NE release from the canine saphenous veins.

Although TPA has been reported to be more potent or equivalent to PDBu in activating PKC in vitro (24, 27), PDBu (10⁻⁸ and 10⁻⁷ M) potentiated the KCl-evoked tritium overflow more notably than did TPA, as previously reported in NE release from the rabbit hippocampus (6) and the mouse atria (28). Allgaier et al. (6) speculated that the difference in the ability of TPA and PDBu to potentiate NE release may be due to the higher lipid solubility of TPA, because lipophilic drugs need more time to equilibrate in the extracellular space compared with more hydrophilic analogues (29). On the other hand, Musgrave et al. (28) suggested that sympathetic nerves have multiple forms of PKC which are differentially activated by TPA or PDBu, because the enhancing effect of TPA on NE release was identical when the pretreatment time was prolonged from 15 to 75 min and because PDBu facilitated significantly NE release even in the presence of the maximally effective concentration of TPA. These two views also might account for the results that PDBu (10⁻⁸ and 10⁻⁷ M), but not TPA, produced the rise in the resting tension and the

| Drugs                | Conc (M) | KCl₂-induced contraction (g) | Exogenous NE-induced contraction |
|----------------------|----------|-------------------------------|---------------------------------|
| Control (None)       | -        | 3.37 ± 0.31                   | 3.16 ± 0.25                    | 1.00 ± 0.01 |
| Polymyxin B          | 3 × 10⁻⁵ | 3.01 ± 0.16                   | 2.97 ± 0.26                    | 1.00 ± 0.02 |
| Control (0.1% DMSO)  | -        | 3.18 ± 0.38                   | 3.04 ± 0.36                    | 1.00 ± 0.02 |
| Staurosporine        | 10⁻⁷     | 3.07 ± 0.29                   | 3.00 ± 0.30                    | 0.87 ± 0.02* |
|                      | 10⁻⁹     | 3.00 ± 0.31                   | 2.98 ± 0.28                    | 0.71 ± 0.05** |

The 6-min application of 40 mM KCl was repeated twice (KCl₁ and KCl₂) and norepinephrine (6–20 nmol) was then injected 3 times at 30 min intervals into the superfusion stream (NE₁ – NE₂). Each protein kinase C inhibitor was superfused 20 min before NE₂. The amounts of norepinephrine injected were 11.2 ± 2.3 nmol in the control (none), 12.0 ± 2.0 nmol in 3 × 10⁻⁵ M polymyxin B, 10 nmol in the control (0.1% DMSO), 12.0 ± 1.2 nmol in 10⁻⁵ M staurosporine and 12.2 ± 2.4 nmol in 10⁻⁶ M staurosporine. Values are expressed as the mean ± S.E.M. from 5 experiments. Asterisks indicate significant difference from the respective control. *P < 0.05, **P < 0.01.
enhancement of the KCl-induced contraction.

The accurate mechanism underlying the enhancing effect of PKC activators on NE release remains unclear. PDBu also enhanced significantly the KCl-evoked tritium overflow in the presence of phentolamine, indicating that the enhancement caused by PDBu is not due to blockade of presynaptic α2-adrenoceptors. Furthermore, it has been reported that inhibition of neuronal NE reuptake is not responsible for the phorbol ester-potentiated NE release (30, 31). PKC activation causes blockade of K⁺ currents (23) or facilitation of Na⁺ (32) and Ca²⁺ (33) currents, each of which results in an enhancement of NE release. The present study with TEA, a K⁺ channel blocker, reveals that blockade of K⁺ channels is not responsible for the 40 mM KCl-evoked tritium overflow mechanisms. We have shown that the 40 mM KCl-evoked tritium overflow from the canine saphenous veins is not modified by the drugs affecting Na⁺ channel function (20). Therefore, it seems that neither blockade of K⁺ channels nor facilitation of Na⁺ channels is the mechanism of the enhancing action of PKC activators on the 40 mM KCl-evoked tritium overflow. Ca²⁺ and PKC act synergistically on stimulus-secretion coupling processes (34), which is due to an increase in the sensitivity to Ca²⁺ of exocytotic NE release processes following the enzyme activation (35). PKC activation enhances Ca²⁺ currents and ⁴⁵Ca²⁺ accumulation in Aplysia neurons, cultured sympathetic neurons of the chick embryo and rat adrenal chromaffin cells (27, 33, 36). Since phorbol esters facilitate Ca²⁺-dependent but not Ca²⁺-independent NE release (6, 30), it is most likely that PKC activation may result in either sensitization to Ca²⁺ of exocytotic processes or increased influx of Ca²⁺ into the nerve endings to potentiate NE release.

PKC activators and inhibitors had no effect on the spontaneous tritium overflow in agreement with the results in other tissues (10, 30, 31, 35, 37), suggesting that PKC may be responsible for modulating rather than for mediating NE release from the canine saphe-
nous veins.

TPA (10⁻¹¹ – 10⁻⁷ M) and PDBu (10⁻¹¹ – 10⁻⁹ M) failed to potentiate the 40 mM KCl-induced contraction in spite of a significant enhancement of the evoked tritium overflow. Recently, we reported that PDBu (10⁻⁷ M) did not increase the contractile response of canine saphenous vein to exogenous NE (14). These results suggest that presynaptic sites may be more sensitive to PKC activators than postsynaptic ones. When the KCl concentration in the superfusion medium was increased from 40 to 50 mM, the evoked tritium overflow and contraction increased by about 2.7- and 1.4-fold over the 40 mM KCl responses, respectively (Y. Takata et al., unpublished data). Therefore, the enhancement of the 40 mM KCl-evoked overflow by PKC activators seems to be insufficient for a significant potentiation of the contraction. This view allowed us to speculate that the enhancing effects of PDBu (10⁻⁸ and 10⁻⁷ M) on the contraction produced by 40 mM KCl are not due to increased tritium overflow. Recent biological studies indicate that brain PKC can be separated into three distinct fractions, designated types I, II and III, by chromatography on a hydroxyapatite column (38, 39). Brandt et al. (40) suggested that PKC-I is located postsynaptically, while PKC-II and PKC-III are located presynaptically. The distinct distributions of the PKC family might be responsible for the higher sensitivity of presynaptic sites to PKC activators; however, whether multiple forms of PKC exist in pre- and postsynaptic sites of the canine saphenous vein remains unknown.

It has been reported that the KCl-induced contraction of the canine saphenous vein is caused not only by release of endogenous NE but also by depolarization of the smooth muscle cells (18). The present results with phenolamine suggest that under the experimental conditions used, a large part of the contraction elicited by 40 mM KCl is attributable to NE released endogenously. Although the contraction which was still observed in the presence of phenolamine is thought to occur via de-
polarization of the smooth muscle cells, the possibility that the reversal of the inhibitory effect of phentolamine on postsynaptic \( \alpha \)-adrenoceptors by increased release of NE may contribute to the residual contraction cannot be excluded, because nifedipine did not reduce significantly the KCl-induced contraction observed under a combined superfusion with PDBu (up to \( 10^{-9} \) M) and phentolamine. Even in the phentolamine-treated veins, PDBu (\( 10^{-8} \) and \( 10^{-7} \) M) potentiated significantly the KCl-induced contraction and an additional superfusion with nifedipine suppressed markedly the increased contraction with no significant inhibition of the evoked tritium overflow. Therefore, the potentiation of the KCI-induced contraction by PDBu in the presence of phentolamine, presumably as well as that in the absence of phentolamine, seems to be mainly due to an activation of the mechanism of contraction through depolarization of the venous smooth muscle. This notion is supported by the findings (22) that PDBu activates the voltage-dependent Ca\(^{2+}\) channels in these smooth muscle cells and that a combined treatment with PDBu and 20 mM KCl results in a marked contraction.

Polymyxin B at \( 10^{-5} \) M had no effects on the KCl-induced contraction regardless of the presence of PKC activators, while at \( 3 \times 10^{-5} \) M, it inhibited significantly the KCl-evoked tritium overflow and contraction. The inhibitory effect of polymyxin B on the contraction is exclusively due to a decrease in the evoked tritium overflow, because polymyxin B at \( 3 \times 10^{-5} \) M did not reduce the contraction elicited by exogenous NE. The contraction induced by exogenous NE was significantly reduced by the 20-min pretreatment with staurosporine (\( 10^{-7} \) M), but the contraction induced by 40 mM KCl was not inhibited by the 10-min pretreatment, although the magnitude of the contractile response to exogenous NE was similar to that induced by 40 mM KCl. The differences in the staurosporine effects are most likely due to staurosporine requiring a longer time to induce a significant inhibition. This view is supported by the present result that the inhibition of the KCl-induced contraction by staurosporine was time-dependent despite the superfusion with stepwise increasing concentrations of TPA or PDBu. The inhibitory effect of staurosporine (\( 10^{-7} \) M) on the KCl-induced contraction in the presence of TPA is not attributable to decreased release of NE, because polymyxin B (\( 10^{-5} \) M), which inhibited the evoked tritium overflow to a similar extent, failed to suppress the KCl-induced contraction. Staurosporine at \( 10^{-6} \) M reduced significantly the KCl-evoked tritium overflow and contraction regardless of the presence of PDBu. Together with the result that staurosporine inhibited the exogenous NE-induced contraction, it appears that staurosporine (\( 10^{-6} \) M) decreases the KCl-induced contraction through its action at pre- and postsynaptic sites. Staurosporine has been reported to also possess a direct inhibitory action on myosin light chain kinase (41). PDBu at \( 10^{-8} \) and \( 10^{-7} \) M elevated the resting tension without any change in the spontaneous tritium overflow, which was not modified by phentolamine. The elevation of the resting tension produced by PDBu was inhibited by nifedipine and was abolished by staurosporine. These results suggest that PDBu may activate the voltage-dependent Ca\(^{2+}\) channels, presumably via PKC stimulation. A direct inhibitory effect on myosin light chain kinase may be also responsible for the abolition of increased resting tension.

In conclusion, the present study indicates that PKC modulates depolarization-evoked NE release from adrenergic nerve endings of the canine saphenous veins and presynaptic sites are more sensitive to PKC than postsynaptic ones.

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