Effects of Ca Antagonists on the Norepinephrine Release and Contractile Responses of Isolated Canine Saphenous Veins to Transmural Nerve Stimulation

Yoshinobu TAKATA and Hitoshi KATO
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan
Accepted December 23, 1983

Abstract—Effects of verapamil, diltiazem and nicardipine on tritium overflow and contraction evoked by transmural nerve stimulation (TNS) were evaluated using canine saphenous vein strips preincubated with [3H]norepinephrine. External Ca2+ was required for both tritium overflow and contraction evoked by TNS. All the Ca antagonists tested significantly increased the spontaneous overflow of tritium in a concentration-dependent manner with no changes in basal tension. Verapamil in concentrations lower than 10^{-5} M significantly enhanced the TNS-evoked tritium overflow, but reduced it at 3 \times 10^{-5} M, while this drug at 3 \times 10^{-6} - 3 \times 10^{-5} M concentration-dependently inhibited the TNS-evoked contraction. Verapamil, 3 \times 10^{-5} M, inhibited the TNS-evoked contraction more strongly than the evoked tritium overflow. On the other hand, diltiazem and nicardipine in concentrations higher than 10^{-5} M significantly inhibited both tritium overflow and contraction evoked by TNS. There was no significant difference between inhibitions of the TNS-evoked tritium overflow and contraction by either diltiazem or nicardipine. Neither increase in the spontaneous tritium overflow nor inhibitions of the TNS-evoked tritium overflow and contraction by nicardipine appeared to be related to its phosphodiesterase inhibiting activity. These results suggest that diltiazem and nicardipine may inhibit the TNS-evoked contraction mainly by inhibiting Ca2+-dependent transmitter release from the adrenergic nerve endings, whereas verapamil may inhibit it by restricting the availability of Ca2+ at the postsynaptic sites and in the highest concentration used, by additional inhibition of transmitter release.

It is well known that Ca antagonists inhibit the transmembrane Ca2+ influx into cardiac and smooth muscle cells (1–3). Since an increase in the intracellular Ca2+ concentration is a critical event in the excitation-secretion coupling of transmitter release from electrically stimulated adrenergic fibers (4), it can be expected that Ca antagonists inhibit the release of transmitter evoked by transmural nerve stimulation (TNS). However, discrepant results have been reported regarding the effects of Ca antagonists on the release of transmitter from the adrenergic nerve endings evoked by electrical stimulation: Haeusler (5) reported that verapamil did not influence the Ca2+-dependent release of norepinephrine from isolated cat heart produced by sympathetic nerve stimulation. On the other hand, it has been reported that verapamil and other Ca antagonists decreased the Ca2+-dependent norepinephrine release from sympathetic nerves of isolated rabbit heart (6). In isolated guinea pig mesenteric artery, Suzuki et al. (7) showed that diltiazem suppressed the summation of excitatory junction potentials produced by perivascular nerve stimulation. More recently, Galzin and Langer (8) reported that verapamil, but not diltiazem, increased the electrically evoked release of [3H]norepinephrine from rabbit hypothalamic slices.

In the present study, we investigated the
effects of verapamil, diltiazem and nicardipine on both tritium overflow and contraction produced by TNS using canine saphenous vein strips preloaded with [3H]norepinephrine. In addition, in order to reestimate the dependence of the TNS-evoked transmitter overflow and contraction on external Ca\(^{2+}\) concentrations, we also investigated the effects of different Ca\(^{2+}\) concentrations in the medium on those produced by TNS.

Part of these data have been reported at the 56th General Meeting of the Japanese Pharmacological Society (Osaka, Japan).

Materials and Methods

Mongrel dogs of either sex weighing 13–32 kg were anesthetized with sodium pentobarbital, 32 mg/kg i.v. The lateral saphenous veins were excised in a length of approximately 5 cm and cleaned of adipose and connective tissues. The veins were spirally cut into strips of approximately 3 mm in width and 30 mm in length. The strips were incubated for 2 hr with Krebs-bicarbonate solution (NaCl, 118.2; KCl, 4.6; MgSO\(_4\), 1.2; CaCl\(_2\), 2.5; KH\(_2\)PO\(_4\), 1.2; NaHCO\(_3\), 24.8 and glucose, 10.0 mM) containing 3x10^{-7} M [7,8-3H]norepinephrine (specific activity of 3.2 Ci/mmol) and 5.7 x 10^{-5} M ascorbic acid. The incubation medium was maintained at 37°C and bubbled with a gas mixture of 95% O\(_2\) and 5% CO\(_2\). At the end of the incubation, the strips were rinsed in the tritium-free Krebs-bicarbonate solution and mounted vertically for superfusion as previously described by Su and Bevan (9); the venous strips were placed under an initial tension of 3 g between a pair of parallel platinum-wire electrodes of 0.5 mm diameter separated by 2 mm. The strips were superfused at a constant flow rate of 3.6 ml/min by a Perista mini-pump (Atto, SJ-1215) with Krebs-bicarbonate solution which was maintained at 37°C and bubbled with a mixture of 95% O\(_2\) and 5% CO\(_2\). The strips were allowed to equilibrate for 60 min before starting the experiments. Thereafter, TNS was repeated applied 8 times (S1-S8) every 16 min by an electric stimulator (Nihon Kohden, SEN-7103). Stimulation parameters were 0.3 msec duration, supramaximal voltage (20 V) and a frequency of 10 Hz for 15 sec. The tension developed was isometrically recorded 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 prior to the 2nd stimulation (S\(_2\)) throughout the experiment. Ten milliliters of scintillation cocktail (5.5 g DPO, 0.1 g POPOP, 667 ml toluene and 333 ml Triton X-100) were added to 1 ml samples before the determination of total radioactivity in a liquid scintillation counter (Beckman, LS 9000 or Aloka, LSC-903). Drugs tested were added to the superfusion medium for 48 min from 6 min after the 3rd stimulation (S\(_3\)) to 6 min after the 6th stimulation (S\(_6\)). The stimulation-evoked overflow of total tritium was estimated by subtracting the radioactivity obtained in the 2-min sample immediately before stimulation from the radioactivity obtained in the 2-min sample during stimulation. The effects of Ca antagonists on both stimulation-evoked tritium overflow and contraction were estimated from the ratio of the response to the 3rd stimulation (S\(_3\)) in the presence of the drug to the response to S\(_3\), and they were evaluated by comparing them with the corresponding ratio obtained from the control experiments without the drug (for details, see “Results”). Radioactivity obtained from the 2-min sample immediately before each stimulation of S\(_3\)-S\(_8\) was considered as the spontaneous overflow of total tritium and designated as Sp\(_A\)-Sp\(_B\), respectively. The effects of Ca antagonists on the spontaneous tritium overflow were estimated from the ratios Sp\(_A\)/Sp\(_B\), Sp\(_B\)/Sp\(_C\), and Sp\(_6\)/Sp\(_3\), and they were evaluated by comparing them with the corresponding ratios obtained from the control experiments without the drug.

In some experiments in which the effects of external Ca\(^{2+}\) concentration on the stimulation-evoked tritium overflow and contraction were studied, the strips were first superfused with normal Krebs solution, then with the Krebs solutions containing various concentrations of Ca\(^{2+}\), and finally with normal Krebs solution again (recovery). Changes in Ca\(^{2+}\) concentration were made in the following sequence: from 1.25 mM to 0.5 mM, Ca\(^{2+}\) deprivation and Ca\(^{2+}\) depri-
vation plus 2 mM EGTA. The superfusion period of 16 min was used for each of the Krebs solutions with various Ca²⁺ concentrations. The strips were electrically stimulated 3 times under normal Krebs solution and then were stimulated 10 min after onset of superfusion with each medium. The data are expressed as the mean and standard error of mean. Statistical analysis was performed by means of Student’s t-test.

The following drugs were used: 1-[7,8-³H]norepinephrine (New England Nuclear), verapamil hydrochloride (Eisai), diltiazem hydrochloride (Tanabe), nicardipine hydrochloride (Yamanouchi), tetrodotoxin (Sankyo), theophylline (Wako Pure Chemicals) and 3-isobutyl-1-methylxanthine (IBMX, Sigma). Three Ca antagonists (10⁻³ M), theophylline (4.5x10⁻² M) and IBMX (6.8x10⁻³ M) were originally dissolved in distilled water. Tetrodotoxin (6x10⁻⁵ M) was originally dissolved in an appropriate amount of diluted acetic acid and then was diluted with distilled water. Subsequently, all drugs used were diluted to the required concentration with Krebs solution immediately prior to the experiment.

Results

Tritium overflow and contraction evoked by electrical stimulation: Electrical stimulation at a frequency of 10 Hz for 15 sec produced a transient increase in tritium overflow and the corresponding contraction in canine saphenous veins. When the venous strips were repeatedly stimulated 8 times every 16 min, the stimulation-evoked tritium overflow declined gradually, and the ratio of the tritium overflow evoked by the 8th stimulation (S₈) to that evoked by the 3rd stimulation (S₃) was 0.84±0.05 (Table 1). On the other hand, the stimulation-evoked contraction did not decline by repetitive stimulations (Table 1).

Effects of tetrodotoxin on the stimulation-evoked tritium overflow and contraction: To confirm whether both tritium overflow and contraction evoked by electrical stimulation at a pulse duration of 0.3 msec are due to stimulation of intramural nerves, the strips were superfused with the medium containing tetrodotoxin, which blocks activation of nerves, but does not block a direct activation of the smooth muscle itself (10). As shown in Fig. 1, in the presence of 6x10⁻⁷ M tetrodotoxin, both tritium overflow and contraction evoked by three repetitive stimulations were completely abolished, indicating that these responses, under the stimulation conditions used, were due to activation of intramural nerves. Tetrodotoxin had no effects on the spontaneous tritium overflow from the veins; Sp₄/Sp₃, Sp₅/Sp₃ and Sp₆/Sp₃ in the presence of tetrodotoxin were 0.94±0.01, 0.78±0.03 and 0.72±0.01 (n=4), respectively, and these values were not significantly different from the corresponding control values of Table 2.

Effects of different Ca²⁺ concentrations in the medium on the spontaneous and TNS-evoked tritium overflows and TNS-evoked contraction: The spontaneous tritium overflow was not modified by superfusion with the Krebs solution containing 1.25 mM Ca²⁺. However, the spontaneous tritium overflows under superfusion with the medium containing 0.5 mM Ca²⁺, Ca²⁺ deprivation and Ca²⁺ deprivation with 2 mM EGTA, that is

<table>
<thead>
<tr>
<th>Table 1. Control values for tritium overflow and contraction evoked by repetitive applications of transmural nerve stimulation (TNS) in isolated canine saphenous veins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>TNS-evoked overflow</td>
</tr>
<tr>
<td>TNS-evoked contraction</td>
</tr>
</tbody>
</table>

The venous strips preloaded with [³H]norepinephrine were superfused with Krebs-bicarbonate solution, and superfusate samples were continuously collected every 2 min. The strips were transmurally stimulated 8 times (S₁–S₈) every 16 min at 10 Hz, 0.3 msec, 20 V for 15 sec. Values obtained from S₄ to S₈ are expressed as a fraction of the respective S₃ values. Values are expressed as the mean±S.E.M. from 5 preparations.
Spy/Sp3, Spy/Sp6 and Spy/Sp3, were 0.90 ±0.03, 0.91 ±0.05 and 1.06 ±0.09 (n=4), respectively; these values were significantly greater than the corresponding values obtained from the control of Table 2 (P<0.05 or P<0.01). The superfusion with Ca2+ deprivation medium with 2 mM EGTA caused a transient elevation in the spontaneous tritium overflow without any effect on basal tension (Fig. 2). This effect reached its maximum 4 min after onset of superfusion with this medium; its maximum spontaneous tritium overflow was 1.37±0.12 (n=4) times greater than the spontaneous overflow just before superfusion with this medium.

On the other hand, the TNS-evoked tritium overflow and contraction were significantly reduced to a similar extent with stepwise decreases in Ca2+ concentration in the superfusion medium and then were abolished by superfusing with Ca2+ deprivation medium with or without 2 mM EGTA (Fig. 2). Thereafter, by resuperfusion with normal Krebs solution, the TNS-evoked tritium overflow was restored, while the TNS-evoked contraction was partially restored (Fig. 2). The effects of external Ca2+ concentrations on both responses to TNS...
Table 2. Effects of verapamil, diltiazem and nicardipine on spontaneous tritium overflow from isolated canine saphenous veins

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Conc. (M)</th>
<th>In the presence of drugs</th>
<th>After removal of drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sp₄/Sp₃</td>
<td>Sp₅/Sp₃</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>0.86±0.04</td>
<td>0.77±0.03</td>
</tr>
<tr>
<td>Verapamil</td>
<td>3×10⁻⁶</td>
<td>1.17±0.09*</td>
<td>1.25±0.12**</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>1.43±0.05***</td>
<td>1.74±0.12****</td>
</tr>
<tr>
<td></td>
<td>3×10⁻⁵</td>
<td>1.72±0.08***</td>
<td>2.54±0.14****</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>3×10⁻⁶</td>
<td>0.94±0.02</td>
<td>0.95±0.03**</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>0.96±0.02</td>
<td>0.97±0.03**</td>
</tr>
<tr>
<td></td>
<td>3×10⁻⁵</td>
<td>1.12±0.04**</td>
<td>1.26±0.06***</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>3×10⁻⁶</td>
<td>0.95±0.02</td>
<td>0.99±0.04*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>0.96±0.02</td>
<td>0.99±0.02***</td>
</tr>
<tr>
<td></td>
<td>3×10⁻⁵</td>
<td>1.18±0.06**</td>
<td>1.49±0.14***</td>
</tr>
</tbody>
</table>

The venous strips preloaded with [3H]norepinephrine were superfused with Krebs-bicarbonate solution, and superfusate samples were continuously collected every 2 min. The strips were transmurally stimulated eight times every 16 min. After three pre-drug periods of stimulation, three periods of stimulation were applied in the presence of drugs, and two subsequent stimulations were applied after removal of drugs. Spontaneous tritium overflow shows radioactivity obtained for 2 min immediately prior to each stimulation, and values obtained from Sp₄ to Sp₈ are expressed as a fraction of the Sp₃ value. Values are expressed as the mean±S.E.M. from 5 experiments. Asterisks indicate significant difference from the corresponding control values: *P<0.05, **P<0.01, ***P<0.001.
are summarized in Fig. 3.

Effects of Ca antagonists on the spontaneous tritium overflow: In the control strips, the spontaneous tritium overflow from the veins slowly declined throughout the experimental period as judged by the ratios $S_{p4}/S_{p3}$–$S_{p8}/S_{p3}$ (Table 2). Verapamil, $3 \times 10^{-6}$–$3 \times 10^{-5}$ M, when added to the superfusion medium 10 min prior to the 4th stimulation, produced a significant increase in the spontaneous overflow of tritium from the veins in a concentration-dependent manner with no effect on basal tension, this effect usually reaching a plateau 30 to 40 min after the application of drug (Fig. 4). Diltiazem and nicardipine, $3 \times 10^{-6}$–$3 \times 10^{-5}$ M, also increased significantly the spontaneous overflow of tritium in a concentration-dependent manner, although both drugs again had no effects on basal tension. The order of potency in increasing the spontaneous tritium overflow was verapamil, nicardipine, and, least potent, diltiazem. The increase in the spontaneous overflow by Ca antagonists was reversed by resuperfusing with Ca antagonists-free medium, although the spontaneous tritium overflow from the strips previously superfused with the medium containing verapamil and nicardipine was maintained at a significantly higher level than that obtained for the control strips without Ca antagonists. These results are shown in Table 2.

Effects of Ca antagonists on both tritium overflow and contraction evoked by TNS: To investigate the effects of Ca antagonists on both tritium overflow and contraction evoked by TNS, the venous strips were

---

Fig. 3. Effects of external Ca$^{2+}$ concentration on both tritium overflow and contraction evoked by transmural nerve stimulation (TNS) in isolated canine saphenous veins preloaded with $[3^H]$norepinephrine. In each pair, the first column is TNS-evoked tritium overflow and the second column is TNS-evoked contraction. Values are expressed as the mean±S.E.M. from 4 preparations. Asterisks indicate significant difference from the corresponding control values: **P<0.01, ***P<0.001.

Fig. 4. Effects of verapamil on both tritium overflow and contraction evoked by transmural nerve stimulation in an isolated canine saphenous vein preloaded with [3H]norepinephrine. The strip was stimulated eight times every 16 min. The response to the first stimulation is not shown.
stimulated three times in the absence of Ca antagonists and then stimulated three times in the presence of Ca antagonists. All three Ca antagonists, as mentioned above, produced a continuous increase in the spontaneous tritium overflow until 30–40 min after their applications, which interfered with an accurate determination of the effects of these drugs on the tritium overflow evoked by the first two stimulations (S4 and S5) in the presence of drugs. Thus, the effects of Ca antagonists on the tritium overflow and contraction evoked by TNS were quantified by using the responses to the 3rd stimulation (S6) in the presence of drugs. When verapamil was added to the superfusion medium at 3×10⁻⁶ and 10⁻⁵ M, it significantly increased the TNS-evoked tritium overflow in a concentration-dependent manner, but at a concentration of 3×10⁻⁵ M, this drug conversely caused a significant decrease (S₆/S₅ = 0.55 ± 0.05 (n=5), P<0.001 when compared with the corresponding ratio of the control in Table 1. On the other hand, verapamil at 3×10⁻⁶–3×10⁻⁵ M significantly inhibited the TNS-evoked contraction in a concentration-dependent manner (S₆/S₃ for 3×10⁻⁵ M was 0.20±0.06, P<0.001). Verapamil at 3×10⁻⁵ M inhibited the TNS-evoked contraction more markedly than the evoked tritium overflow. Moreover, the inhibition of the contraction by 3×10⁻⁵ M verapamil was significantly greater than the inhibitions by diltiazem and nicardipine at 3×10⁻⁵ M (P<0.01 and P<0.001, respectively). Tracings of the effects of 3×10⁻⁶ M verapamil are shown in Fig. 4.

Diltiazem and nicardipine at 3×10⁻⁶ M had no effects on both tritium overflow and contraction evoked by TNS. Diltiazem, 10⁻⁵ and 3×10⁻⁵ M, significantly inhibited the TNS-evoked tritium overflow and the TNS-evoked contraction in a concentration-dependent manner; the ratios S₆/S₃ for 3×10⁻⁵ M were 0.46±0.07 in overflow and 0.64±0.07 in contraction. There was no significant difference between both inhibitory effects of diltiazem.

Nicardipine, 10⁻⁵ and 3×10⁻⁵ M, also caused significant inhibitions of both responses to TNS in a concentration-dependent manner; the ratios S₆/S₃ for 3×10⁻⁵ M were 0.66±0.05 in overflow and 0.77±0.09 in contraction. There was no significant differ-

![Fig. 5](image-url)
ence between both inhibitions by nicardipine. The inhibition of the evoked tritium overflow by $3 \times 10^{-5}$ M diltiazem was significantly greater than that by the same concentration of nicardipine ($P<0.05$). Diltiazem also inhibited the contraction more effectively than did nicardipine, but the inhibitions by both drugs did not reach statistical significance. Summarized data for verapamil, diltiazem and nicardipine are shown in Fig. 5.

The inhibitory effects of the three Ca antagonists on the TNS-evoked tritium overflow were rapidly reversed when the strips were stimulated 10 min after resuperfusion with normal Krebs solution; the ratios $S_7/S_3$ obtained from the strips previously superfused with $3 \times 10^{-5}$ M of verapamil, diltiazem and nicardipine were $0.78\pm0.06$, $0.73\pm0.09$ and $0.86\pm0.07$, respectively. On the other hand, the inhibitory effects of these drugs on the contraction were not completely reversed even 26 min after resuperfusion with normal Krebs solution; the ratios $S_8/S_3$ obtained from the strips with previous superfusion with $3 \times 10^{-5}$ M verapamil, diltiazem and nicardipine were $0.61\pm0.08$, $0.90\pm0.04$ and $0.79\pm0.07$, respectively.

**Effects of phosphodiesterase inhibitors on the spontaneous and TNS-evoked tritium overflows and TNS-evoked contraction:** Since nicardipine has been reported to inhibit cyclic AMP phosphodiesterase activity from canine coronary artery and beef heart (11) and since administrations of a phosphodies- terase inhibitor and dibutyryl cyclic AMP have been shown to increase the spontaneous release of norepinephrine from peripheral adrenergically innervated tissues (12, 13), we also investigated by using other phosphodiesterase inhibitors, theophylline and IBMX, whether the increase in the spontaneous tritium overflow by nicardipine is related to its phosphodiesterase inhibitory effect. Theophylline and IBMX were used in concentrations which were presumed to be as effective as $3 \times 10^{-5}$ M nicardipine in inhibiting phosphodiesterase activity from canine coronary artery (11). As shown in Table 3, neither $1.8 \times 10^{-3}$ M theophylline nor $2 \times 10^{-4}$ M IBMX caused an increase in the spontaneous tritium overflow. The simultaneous application of IBMX did not prevent the increase in the spontaneous tritium overflow produced by $3 \times 10^{-5}$ M nicardipine, although theophylline prevented it (Table 3).

On the other hand, both theophylline and IBMX enhanced the TNS-evoked tritium overflow. The values of the ratio $S_6/S_3$ for

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>5</td>
<td>0.86±0.04</td>
<td>0.77±0.03</td>
<td>0.73±0.03</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>$3 \times 10^{-5}$</td>
<td>5</td>
<td>1.18±0.06**</td>
<td>1.49±0.14**</td>
<td>1.44±0.09***</td>
</tr>
<tr>
<td>Theophylline</td>
<td>$1.8 \times 10^{-3}$</td>
<td>4</td>
<td>0.79±0.03</td>
<td>0.70±0.10</td>
<td>0.69±0.04</td>
</tr>
<tr>
<td>IBMX</td>
<td>$2 \times 10^{-4}$</td>
<td>3</td>
<td>0.93±0.04</td>
<td>0.86±0.03</td>
<td>0.76±0.04</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>$3 \times 10^{-5}$</td>
<td>+</td>
<td>0.81±0.04</td>
<td>0.80±0.05</td>
<td>0.76±0.04</td>
</tr>
<tr>
<td>Theophylline</td>
<td>$1.8 \times 10^{-3}$</td>
<td>+</td>
<td>1.54±0.29*</td>
<td>1.93±0.47*</td>
<td>1.86±0.47*</td>
</tr>
</tbody>
</table>

The venous strips preloaded with [3H]norepinephrine were superfused with Krebs-bicarbonate solution, and superfusate samples were continuously collected every 2 min. The strips were transmurally stimulated three times in the absence of drug and then three times in the presence of drug. Spontaneous tritium overflow shows radioactivity obtained for 2 min immediately prior to each stimulation, and values obtained from Sp4 to Sp6 are expressed as a fraction of the Sp3 value. Values are expressed as the mean±S.E.M. Asterisks indicate significant difference from the corresponding control values: *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

Table 3. Effects of phosphodiesterase inhibitors on spontaneous tritium overflow from isolated canine saphenous veins.
theophylline and IBMX were 1.19±0.09 (n=4) and 1.50±0.05 (n=3), respectively; these values were significantly greater than the corresponding control value given in Table 1 (P<0.05 or P<0.001). The two inhibitors markedly inhibited the TNS-evoked contraction; the values of $S_6/S_3$ for theophylline and IBMX were 0.28±0.07 and 0.12±0.07, respectively. These two inhibitors also caused a further inhibition of the contraction in the presence of nicardipine; the values of $S_6/S_3$ for theophylline and IBMX were 0.05±0.02 and 0.08±0.07, respectively, in the presence of nicardipine.

**Discussion**

In the present study, the evoked tritium overflow, but not contraction, gradually declined with repeated stimulations. Rorie et al. (14) have reported that in the canine saphenous vein, 5–20% of the electrically released norepinephrine was sequestered in the vesicles for reutilization. Furthermore, under conditions where the endogenous norepinephrine may be continuously replenished through synthesis, the newly synthesized norepinephrine was preferentially released when the sympathetic nerve was stimulated at a frequency of 30 impulses/sec (15). The relative importance of new synthesis in maintaining transmitter release may vary with the interval and the rate of stimulation as well as with the organ or species examined. Therefore, a gradual decline of the evoked tritium overflow with repeated stimulations may be caused by a decreased proportion of $[^{3}H]$norepinephrine to the total amount of norepinephrine released by stimulations. On the other hand, contractions of constant height mean that the total amount of transmitter released does not change when stimulation was repeatedly applied.

Both tritium overflow and contraction evoked by TNS were inhibited to the similar extent by lowering $Ca^{2+}$ concentration in the superfusion medium and abolished by superfusing with $Ca^{2+}$ deprivation medium, indicating that $Ca^{2+}$ is required for norepinephrine release from the adrenergic nerve endings by electrical stimulation as previously described by Boullin (16) in perfused cat colon and by Kirpekar and Misu (17) in perfused cat spleen. An increase in the spontaneous tritium overflow seen under superfusion with the medium containing 0.5 mM $Ca^{2+}$ and with $Ca^{2+}$ deprivation medium appears to be caused by mechanisms suggested by Boullin (16), who reported that removal of $Ca^{2+}$ from the medium may cause not only an increase in membrane permeability as a result of a decreased membrane stability, but also an inhibition of the transmitter uptake mechanism, both of which lead to an increase in norepinephrine efflux. When the veins were superfused with $Ca^{2+}$ deprivation medium in the presence of EGTA, a transient increase in the spontaneous overflow of tritium was also observed with no change in basal tension. Publicover and Duncan (18) reported that EGTA caused a transient rise in the frequency of miniature endplate potential in frog neuromuscular junction and that this effect was because EGTA promoted the emptying of a $Ca^{2+}$ reservoir or beneath the inner face of membrane, thus causing a rapid $Ca^{2+}$ efflux via the $Ca^{2+}$-sensitive sites that trigger exocytosis of transmitter. A similar sequence of events might also occur at the adrenergic nerve endings of canine saphenous veins.

Under the present experimental conditions, verapamil in concentrations lower than $10^{-5}$ M caused a significant increase in the TNS-evoked tritium overflow from the veins. Publicover and Duncan (19) suggested that verapamil in high concentration can either inhibit the membrane $Ca^{2+}$ pump or cause the release of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores in frog neuromuscular junction, both of which lead to an increase in $Ca^{2+}$ concentration in the motor nerve endings. If this suggestion can also be applied to the adrenergic nerve endings of canine saphenous veins, one might speculate that this drug may increase the release of transmitter evoked by TNS in spite of a possible inhibitory effect of $Ca^{2+}$ influx. More recently, Galzin and Langer (8) reported that verapamil in concentrations between 1 and 10 $\mu$M increased the electrically evoked overflow of $[^{3}H]$norepinephrine from rabbit hypothalamic slices by blocking the presynaptic $\alpha_2$-adrenoceptors. However, this drug in concentrations of $10^{-6}$–$3\times10^{-5}$ M did not increase the tritium...
overflow evoked by 40 mM K+ from canine saphenous veins, although phentolamine increased it through a possible blocking effect on the presynaptic α2-adrenoceptors (Y. Takata and H. Kato, unpublished observation). In this connection, Starke and Montel (20) reported that α2-adrenoceptor agonists and antagonists modified the KCl-evoked release of transmitter from rabbit heart when KCl concentrations up to 50 mM were used. Therefore, it is unlikely that the increase in the evoked tritium overflow by verapamil may be explained by the presynaptic α2-adrenoceptor blocking properties of this drug, although further investigations are required before a clear-cut conclusion can be drawn. It has been reported that higher concentrations of verapamil have a local anesthetic effect (21–24). Most of these reports also indicated that the local anesthetic effect of verapamil was observed at concentrations higher than 10^{-4} M (21–23) and persistent after washing out the drug (22, 24). However, the concentrations of verapamil used in the present study were lower than 10^{-4} M. Moreover, the inhibitory effects of 3×10^{-5} M verapamil on the TNS-evoked tritium overflow were rapidly reversed by removal of the drug from the superfusion medium, although the inhibitory effects of this drug on the TNS-evoked contraction were not completely reversed. Hence, it appears unlikely that the local anesthetic effect of verapamil is responsible for the inhibition of the TNS-evoked tritium overflow. Verapamil is preferably assumed to inhibit the evoked tritium overflow through suppression of Ca^{2+} influx into the nerve endings.

On the other hand, verapamil significantly inhibited the contractile responses to TNS even in concentrations in which marked increases in the evoked tritium overflow were observed. The highest concentration (3×10^{-5} M) of verapamil inhibited the contraction much more strongly than the evoked tritium overflow. Moreover, the inhibition of the contraction by 3×10^{-5} M verapamil also was much greater than that by the same concentration of diltiazem or nicardipine, although there was no significant difference between inhibitory effects of verapamil and two other Ca antagonists on the evoked tritium overflow. This remarkable inhibition of the contraction by verapamil might be related to its interaction with α-adrenoceptor as suggested by Langer and Shepperson (25) and Karliner et al. (26). Our results suggest that verapamil may inhibit the TNS-evoked contraction by restricting the availability of Ca^{2+} at the postsynaptic sites and in the highest concentration used, by additional inhibition of transmitter release from nerve endings.

Diltiazem, unlike verapamil, inhibited both tritium overflow and contraction evoked by TNS in a concentration-dependent manner (Fig. 5). This result differs from that of Galzin and Langer (8) showing that diltiazem in concentrations up to 10^{-4} M had no effect on the electrically evoked release of [3H]-norepinephrine from rabbit hypothalamic slices. This discrepancy might due to the difference between tissues or experimental conditions used. Nagao et al. (27) reported that diltiazem had a local anesthetic activity with an ED50 value of 0.28%, that is 6.2×10^{-3} M. However, it is unlikely that the inhibition of the TNS-evoked tritium overflow by diltiazem is caused by its local anesthetic activity since the concentrations of diltiazem used in the present study are lower than those required for local anesthetic activity, judging from the result of Nagao et al. (27). Langer and Shepperson (25) reported that diltiazem in concentrations up to 10^{-4} M had no significant effect on the contractile response of superfused canine saphenous vein to norepinephrine. Our results suggest that diltiazem may inhibit the TNS-evoked contraction by inhibiting transmitter release mainly by suppressing Ca^{2+} influx into the nerve endings.

On the other hand, verapamil significantly inhibited the contractile responses to TNS even in concentrations in which marked increases in the evoked tritium overflow were observed. The highest concentration (3×10^{-5} M) of verapamil inhibited the contraction much more strongly than the evoked tritium overflow. Moreover, the inhibition of the contraction by 3×10^{-5} M verapamil also was much greater than that by the same concentration of diltiazem or nicardipine, although there was no significant difference between inhibitory effects of verapamil and two other Ca antagonists on the evoked tritium overflow. This remarkable inhibition of the contraction by verapamil might be related to its interaction with α-adrenoceptor as suggested by Langer and Shepperson (25) and Karliner et al. (26). Our results suggest that verapamil may inhibit the TNS-evoked contraction by restricting the availability of Ca^{2+} at the postsynaptic sites and in the highest concentration used, by additional inhibition of transmitter release from nerve endings.

Diltiazem, unlike verapamil, inhibited both tritium overflow and contraction evoked by TNS in a concentration-dependent manner (Fig. 5). This result differs from that of Galzin and Langer (8) showing that diltiazem in concentrations up to 10^{-4} M had no effect on the electrically evoked release of [3H]-norepinephrine from rabbit hypothalamic slices. This discrepancy might due to the difference between tissues or experimental conditions used. Nagao et al. (27) reported that diltiazem had a local anesthetic activity with an ED50 value of 0.28%, that is 6.2×10^{-3} M. However, it is unlikely that the inhibition of the TNS-evoked tritium overflow by diltiazem is caused by its local anesthetic activity since the concentrations of diltiazem used in the present study are lower than those required for local anesthetic activity, judging from the result of Nagao et al. (27). Langer and Shepperson (25) reported that diltiazem in concentrations up to 10^{-4} M had no significant effect on the contractile response of superfused canine saphenous vein to norepinephrine. Our results suggest that diltiazem may inhibit the TNS-evoked contraction by inhibiting transmitter release mainly by suppressing Ca^{2+} influx into the nerve endings. Fujiwara et al. (28) suggested that diltiazem acts as a Ca antagonist at the muscle membrane and also in the nerve endings in canine basilar artery. The concentrations of nicardipine required for the inhibition of the neuromuscular transmission were much higher than that for the suppression of Ca^{2+} influx in the postsynaptic sites of guinea pig basilar artery (29). In canine saphenous vein, diltiazem inhibited the evoked tritium overflow more effectively than did nicardipine. These results indicate that diltiazem is more susceptible to the inhibition of Ca^{2+} influx in the presynaptic
Nicardipine is a Ca antagonist (29) with a cyclic AMP phosphodiesterase inhibiting activity (11). This Ca antagonist has no local anesthetic activity even in a concentration of 0.5%, that is, 9.7×10^{-3} M (30). Phosphodiesterase inhibitors have been reported to enhance the electrically evoked transmitter release from the nerve endings of adrenergically innervated tissues (13, 31, 32). When both theophylline and IBMX were used in concentrations which were assumed to be as effective as 3×10^{-5} M nicardipine in inhibiting a phosphodiesterase activity from canine coronary artery (11), the two former agents significantly enhanced the TNS-evoked tritium overflow, while nicardipine inhibited it. These two inhibitors caused much greater inhibition of the TNS-evoked contraction than did nicardipine and also caused a further inhibition in the presence of nicardipine. From these results, it appears that the inhibitory effects of nicardipine on the evoked tritium overflow and contraction may not be related to its phosphodiesterase inhibition and subsequent accumulation of cyclic AMP. Conceivably, nicardipine may inhibit the TNS-evoked contraction predominantly due to inhibiting transmitter release as a result of suppression of Ca^{2+} influx.

Nicardipine increased the spontaneous tritium overflow, while neither theophylline nor IBMX increased it. Furthermore, a simultaneous application of IBMX did not prevent the increase in the spontaneous overflow by nicardipine. These results indicate that a phosphodiesterase inhibiting action of nicardipine is not responsible for its increase in the spontaneous tritium overflow. In contrast to IBMX, the simultaneous application of theophylline prevented the increasing effect of nicardipine on the spontaneous overflow. Therefore, it appears that nicardipine increases the spontaneous overflow by a mechanism susceptible to theophylline.

Both verapamil and diltiazem also caused marked increases in the spontaneous tritium overflow. More recently, Karaki et al. (33) reported that high concentration of organic Ca antagonists increased the spontaneous [^3H]efflux from the rabbit aorta previously loaded with [^3H]norepinephrine. In the present study, we did not investigate in detail the mechanism of the increasing effects of verapamil and diltiazem on the spontaneous overflow. However, these Ca antagonists caused no changes in basal tension even when significant increases in the spontaneous tritium overflow were observed. This suggests that the increased tritium overflow does not reflect an intact [^3H]norepinephrine, but rather its [^3H]-metabolites, chiefly [^3H]3,4-dihydroxyphenylglycol and O-methylated, deaminated compounds—a mixture of [^3H]3-methoxy-4-hydroxyphenylglycol and [^3H]3-methoxy-4-hydroxymandelic acid (14, 34–36). The former metabolite is intraneuronally formed as a consequence of a continuous efflux of norepinephrine from the strage vesicles into the neuroplasm in the absence of stimulation and subsequently easily diffuses out of canine saphenous veins (34), while the latter metabolites are extraneuronally formed (35, 36). It is speculated that Ca antagonists might increase the spontaneous tritium overflow by promoting the processes mentioned above. Experiments are in progress to further investigate the mechanism by which Ca antagonists increase the spontaneous overflow.

It is concluded that diltiazem and nicardipine may inhibit the TNS-evoked contraction mainly by inhibiting transmitter release from the nerve endings, while verapamil may inhibit it by restricting the availability of Ca^{2+} at the postsynaptic sites and at the highest concentration used, by additional inhibition of transmitter release.

Acknowledgement: we thank Mr. M. Masuda and Miss A. Nakamori for excellent technical assistance.

References
2 Fleckenstein, A.: Specific pharmacology of calcium in myocardium, cardiac pacemakers and
11 Sakamoto, N., Terai, M., Takenaka, T. and Maeno, H.: Inhibition of cyclic AMP phosphodiesterase by 2-6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(N-benzyl-N-methylamino)ethyl ester 5-methyl ester hydrochloride (YC-93), a potent vasodilator. Biochem. Pharmacol. 27, 1269–1274 (1978)
16 Boullin, D.J.: The action of extravascular catecholamines on the release of the sympathetic transmitter from peripheral nerves. J. Physiol. (Lond.) 189, 95–105 (1967)


