Existence of two components in the tonic contraction of rat aorta mediated by α_1 -adrenoceptor activation

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1 The mechanisms involved in the contraction of rat aorta induced by the activation of α_1 -adrenoceptors were studied. Phenylephrine induced a phasic contraction in the aorta incubated in Ca^{2+} -free medium containing 0.5 mM EGTA. Subsequent addition of Ca^{2+} induced a tonic contraction, which exhibited a stepwise development, an initial phase lasting 3 to 6 min (tonic-I) followed by a superimposing second phase (tonic-II).

2 2-Nitro-4-carboxyphenyl-N,N-diphenylcarbamate, which has been reported to inhibit phosphatidylinositol turnover, and H-7, a protein kinase C inhibitor, inhibited the tonic-I phase more effectively than the tonic-II phase. On the other hand, the tonic-II phase was more sensitive to nifedipine and cromakalim.

3 The rate of $^{45}\text{Ca}^{2+}$ influx during the tonic-I phase in phenylephrine-treated muscles was not different from that in untreated muscles, while that during the tonic-II phase was significantly greater. Nifedipine inhibited the increased $^{45}\text{Ca}^{2+}$ influx during the tonic-II phase, whereas H-7 did not affect the uptake during either phase.

4 These results suggest that the tonic contraction of rat aorta following α_1 -adrenoceptor activation involves two different mechanisms: one is directly related to consequences of the polyphosphoinositide cascade, probably to protein kinase C, and the other dependent on Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels.

Introduction

The contraction of vascular smooth muscle induced by α_1 -adrenoceptor activation has been believed to be caused by either Ca^{2+} release from the intracellular stores or Ca^{2+} influx through Ca^{2+} channels. Experimentally, α_1 -adrenoceptor-mediated contraction can be separated into phasic and tonic components: the phasic component seems to depend on intracellularly stored Ca^{2+} while the tonic component depends on extracellular Ca^{2+} . The activation of the α_1 -adrenoceptor is linked to the hydrolysis of polyphosphoinositide into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Villalobos-Molina *et al.*, 1982; Hashimoto *et al.*, 1986; Rapoport, 1987). IP_3 can cause a Ca^{2+} release from sarcoplasmic reticulum and a contraction in vascular smooth muscle (Suematsu *et al.*, 1984; Somlyo *et al.*, 1985; Walker *et al.*, 1987). Therefore IP_3 could act as a second messenger responsible for α_1 -adrenoceptor-mediated Ca^{2+} release and the phasic component (Hashimoto *et al.*, 1986; Rapoport, 1987). On the other hand, it is still unclear whether the tonic component depends entirely on Ca^{2+} influx, and how the component is related to polyphosphoinositide hydrolysis.

The sustained contraction of rat aorta following α_1 -adrenoceptor activation is partially inhibited by Ca^{2+} channel blockers (Godfraind *et al.*, 1982; Beckeringh *et al.*, 1984; Chiu *et al.*, 1986; Bogner & Enero, 1988). The existence of a component in the contraction, which is resistant to Ca^{2+} channel blockers, provides two possibilities: (1) that this component does not require the enhanced Ca^{2+} entry, or (2) that Ca^{2+} entry through 'receptor-operated Ca^{2+} channels' which are insensitive to organic Ca^{2+} channel blockers (Bolton, 1979; Cauvin *et al.*, 1983) contributes to this contraction.

We here report that the tonic contraction due to α_1 -adrenoceptor activation of rat aorta is composed of two components, one clearly related to the cascade of PI hydrolysis and the other due to Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels. Preliminary results of this study have been given elsewhere (Nishimura & Ito, 1989).

Methods

Tension experiments

Rectangular preparations, 1–2 mm wide and 5–6 mm long, were made by cutting open the thoracic aorta isolated from male rats older than 13 weeks of age (300–400 g). The endothelial layer was removed by gently rubbing the intimal surface with a cotton bud. The preparations were suspended in a Magnus bath containing 5 ml physiological saline solution (PSS) aerated with 95% O_2 and 5% CO_2 at 37°C and were equilibrated under a resting tension of 1 g for 1 h before the experiment. The isometric tension was measured by an isometric transducer (Nihon Kohden SB-1T) and recorded on a pen-writing recorder (Rikadenki R-14).

$^{45}\text{Ca}^{2+}$ influx experiments

The rate of $^{45}\text{Ca}^{2+}$ influx into rat aortic smooth muscle was measured by a cold lanthanum method (Karaki & Weiss, 1979) simultaneously with a tension recording. The thoracic aorta denuded of endothelium was cut into helical strips (4–5 mm in width, 25–30 mm in length and 8–12 mg wet weight) and suspended in a Magnus bath containing 10 ml PSS gassed with 95% O_2 and 5% CO_2 at 37°C. After the equilibration under a resting tension of 1 g for 1 h the isometric tension was recorded as mentioned above. The muscle was exposed to Ca^{2+} -free PSS for 15 min, then challenged by phenylephrine 1×10^{-6} M, and further 10 min later Ca^{2+} 2.5 mM was reintroduced. At an appropriate time after reintroduction of Ca^{2+} , the bath solution was changed to PSS containing $^{45}\text{CaCl}_2$ ($1 \mu\text{Ci ml}^{-1}$, cold Ca^{2+} 2.5 mM) and phenylephrine (1×10^{-6} M) within 5 s. The change of solution did not affect the level of tension. As control, the same protocol was performed but without phenylephrine. After the incubation with $^{45}\text{Ca}^{2+}$ for 2 min, each preparation was quickly transferred to La^{3+} solution aerated with 100% O_2 at 0.5°C to displace extracellular $^{45}\text{Ca}^{2+}$ with La^{3+} . After 30 min washing in cold La^{3+} solution, the preparation was blotted between filter papers (Whatman No. 2) for 10 s under a weight of 3.0 g cm^{-2} , weighed and placed in a scintillation vial. The

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muscle was solubilized by 0.5 ml Soluene-350 (Packard) overnight at room temperature. After the addition of 2 ml of scintillation cocktail (ACS-II) the radioactivity was counted in a liquid scintillation counter (Aloka LSC 903).

Solutions and drugs

PSS contained (mM): NaCl 136.8, MgCl₂ 0.2, CaCl₂ 2.5, KCl 5.4, NaHCO₃ 11.9 and glucose 5.5 (pH 7.2–7.4). for the Ca²⁺-free PSS, CaCl₂ was omitted from PSS and 0.5 mM ethylene glycol bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) was added. Free Ca²⁺ concentration in the medium-containing EGTA was calculated with the programme of Fabiato & Fabiato (1979). La³⁺ solution for measurement of ⁴⁵Ca²⁺ influx had the following composition (mM): LaCl₃ 73.8, glucose 5.5 and Tris-HCl 24, and pH was adjusted to 6.8–6.9 to avoid the precipitation of lanthanum (H. Karaki, personal communication).

The drugs used were phenylephrine (Wako Pure Chemicals), 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC, Sigma), cromakalim (Beecham), nifedipine (Sigma), verapamil (Eisai) and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7, Seikagaku Kogyo).

Statistics

All values are expressed as a mean $\pm$ s.e.mean (n = number of preparations). To test the effects of drugs on phenylephrine-induced contraction, at first the control response to phenylephrine was obtained, and then the response in the presence of test drug was obtained. The statistical significance was tested for paired data. In the ⁴⁵Ca²⁺ influx study the statistical significance was performed by non-paired t test. For both studies the significance was considered at the level of $P < 0.05$.

Results

Patterns of phenylephrine-induced contraction

In the presence of external Ca²⁺ (2.5 mM), phenylephrine 1×10^{-6} M, induced a contraction in which a phasic component and a tonic component fused but they could be distinguished by the rate of rise. The tonic contraction looked monophasic (Figure 1a). When phenylephrine and 2.5 mM Ca²⁺ were applied simultaneously after preincubation with Ca²⁺-free PSS (with 0.5 mM EGTA) for more than 15 min, the contraction developed in a stepwise manner. After the rapid upstroke of phasic contraction, the first tonic phase (tonic-I) developed, which lasted 30 s to 4 min, and then the second tonic phase (tonic-II) was superimposed on it (Figure 1b). If the preincubation period with Ca²⁺-free PSS was less than 15 min, the tonic contraction did not exhibit a biphasic development.

The biphasic tonic contraction was also observed with another protocol (Figure 1c). When phenylephrine was

applied during the 15 min exposure to Ca²⁺-free PSS containing 0.5 mM EGTA, the phasic component was distinctly observed. After the phasic contraction subsided, a small sustained contraction remained as long as the muscle was soaked in Ca²⁺-free PSS. Subsequent readmission of 2.5 mM Ca²⁺ at 10 min after phenylephrine induced a stepwise development of tension. The initial phase of the tonic contraction (tonic-I) had an amplitude of 45–65% of maximum contraction, and 2–6 min later the second phase of the tonic contraction (tonic-II) superimposed on it. Often a rapid rising phase was distinguished at the beginning of tonic-I. With this protocol the separation of tonic contraction into two phases was observed even when the muscle was preincubated in Ca²⁺-free PSS for only 1 min before addition of phenylephrine, although in this case tonic-II developed earlier (within 2 min after readmission of Ca²⁺).

Sensitivity to Ca²⁺ channel blockers and cromakalim

To investigate whether the Ca²⁺ influx through the nifedipine-sensitive Ca²⁺ channel contributes to the tonic contraction, the effect of nifedipine on phenylephrine-induced tonic contraction was examined. As shown in Figure 2a, nifedipine (1×10^{-7} M) applied during the tonic-II phase decreased the tension close to the level of the tonic-I phase. On the other hand, when nifedipine (1×10^{-7} M) was applied before the addition of Ca²⁺, the tonic-II phase did not appear on the tonic-I phase (Figure 2b). Figure 3a shows the effect of pretreatment with nifedipine, 1×10^{-7} M, on the time course of the tonic contraction. The inhibition by nifedipine was much greater on the later phase than on the early phase. Table 1 shows the dose-effect of nifedipine on phenylephrine-contraction. Since usually at 2 min after the addition of Ca²⁺ the tension stayed in the tonic-I phase and at 10 min the tension reached the steady state, the tension at 2 min may be considered as the tonic-I phase and that at 10 min indicates the sum of the tonic-I and tonic-II phases. Therefore it can be said that nifedipine at above 1×10^{-7} M selectively blocked the tonic-II phase. Another Ca²⁺ channel blocker, verapamil (1×10^{-6} M) also abolished the tonic-II phase while leaving the tonic-I phase unchanged.

The possibility was considered that the incubation of muscles in Ca²⁺-free PSS containing EGTA for 25 min until the readmission of Ca²⁺ made the membrane abnormally leaky to Ca²⁺ and the Ca²⁺ channel insensitive to Ca²⁺ channel blockers (Ahn *et al.*, 1984; Guan *et al.*, 1988). We checked these points in two experiments. When Ca²⁺ (2.5 mM) was applied to muscles pre-incubated in Ca²⁺-free PSS containing 0.5 mM EGTA for 30 min with no phenylephrine, the tension did not develop. This suggests that the membrane permeability to Ca²⁺ did not change much by treatment with EGTA. In other experiments, after 15 min exposure to Ca²⁺-free PSS containing 0.5 mM EGTA, KCl (20 mM) was added. Subsequent addition of Ca²⁺ (2.5 mM) (10 min later) induced a contraction, which was the same as that induced by 20 mM

Figure 1 Typical examples of phenylephrine (PE)-induced contraction of rat aorta with different protocols. (a) A contraction induced by 10^{-6} M phenylephrine in normal (2.5 mM Ca²⁺) PSS. (b) Two-step development of tonic contraction when phenylephrine and 2.5 mM Ca²⁺ were applied simultaneously to the muscle preincubated in Ca²⁺-free PSS containing 0.5 mM EGTA for 15 min. (c) Separation of phenylephrine-induced contraction into phasic, tonic-I and tonic-II components. In this example, after the muscle was preincubated in Ca²⁺-free PSS (with 0.5 mM EGTA) for 15 min, phenylephrine (1×10^{-6} M) was applied and 10 min later Ca²⁺ (2.5 mM) was added. This protocol was mainly used for the later experiments.

Figure 2 Effects of nifedipine on two components of tonic contraction due to phenylephrine in rat aorta. Phenylephrine (PE)-contraction was elicited by the protocol shown in Figure 1c. Nifedipine (10^{-7} M) was applied during the tonic-II phase (a) or 5 min before the addition of Ca^{2+} (b).

KCl in normal PSS and was abolished by pretreatment with nifedipine (1×10^{-7} M). Therefore it is unlikely that the procedure of incubation in Ca^{2+} -free, EGTA-containing PSS for up to 25 min made the permeability to Ca^{2+} abnormally high or made Ca^{2+} channels resistant to nifedipine.

Nifedipine and verapamil are known to block selectively voltage-dependent Ca^{2+} channels. The greater sensitivity of the tonic-II phase to these substances suggests that the change in membrane potential might be involved in this component. Although here we did not measure the membrane potential of the rat aorta, we tested the effect of cromakalim, which relaxes vascular smooth muscles by a hyperpolarizing action

Figure 3 Effects of nifedipine, NCDC and H-7 on the time course of tonic contraction induced by phenylephrine in rat aorta. The protocol shown in Figure 1c was used. (a) Nifedipine 1×10^{-7} M ($n = 8$), (b) NCDC 3×10^{-5} M ($n = 12$), (c) H-7 1×10^{-5} M ($n = 10$). Nifedipine was applied 5 min before the readmission of Ca^{2+} (5 min after phenylephrine) while NCDC and H-7 were applied 15 min before the addition of phenylephrine (1×10^{-6} M). At 0 min Ca^{2+} (2.5 mM) was added. In each graph: (○) control and (●) presence of each drug. Each point represents the mean and vertical lines the s.e.mean. The arrow indicates average time of initiation of the tonic-II phase. With nifedipine, the tonic-II phase did not appear. Statistical significance tested for paired samples, which is not shown in figures, was observed for every point except 0 min for nifedipine and NCDC.

Table 1 Effects of nifedipine, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7) on the phenylephrine-induced contraction

	Developed tension (%)				Initiation of tonic-II (min)		n
	2 min		10 min		Control	Treated	
	Control	Treated	Control	Treated			
<i>Nifedipine</i>							
3 × 10 ⁻⁸ M	56 ± 3	52 ± 6	100 ± 0	84 ± 4*	3.0 ± 0.3	2.7 ± 0.3	8
1 × 10 ⁻⁷ M	57 ± 2	46 ± 2*	100 ± 0	67 ± 7†	2.7 ± 0.2	—	8
3 × 10 ⁻⁷ M	64 ± 2	49 ± 2*	100 ± 0	63 ± 6†	2.5 ± 0.1	—	6
<i>NCDC</i>							
1 × 10 ⁻⁵ M	52 ± 3	39 ± 2†	100 ± 0	92 ± 0*	4.4 ± 0.4	6.0 ± 0.4*	8
3 × 10 ⁻⁵ M	51 ± 2	30 ± 1†	100 ± 0	79 ± 3†	4.7 ± 0.3	5.9 ± 0.3†	12
1 × 10 ⁻⁴ M	45 ± 1	20 ± 2†	100 ± 0	38 ± 4†	3.6 ± 0.3	—	8
<i>H-7</i>							
5 × 10 ⁻⁶ M	57 ± 3	20 ± 3†	100 ± 0	73 ± 4*	2.8 ± 0.2	4.8 ± 0.2†	7
1 × 10 ⁻⁵ M	66 ± 3	23 ± 2†	100 ± 0	74 ± 2*	2.7 ± 0.2	3.8 ± 0.1*	10
2 × 10 ⁻⁵ M	66 ± 3	7 ± 1†	100 ± 0	44 ± 2†	2.7 ± 0.2	4.8 ± 0.1†	6

The contraction was elicited with the protocol shown in Figure 1c. After the observation of control response each drug was applied in Ca^{2+} -free PSS 15 min before the addition of 1×10^{-6} M phenylephrine. The tension is expressed as the relative value of the tension development at 10 min after addition of 2.5 mM Ca^{2+} in control responses. With 1×10^{-7} and 3×10^{-7} M nifedipine, and 1×10^{-4} M NCDC the tonic-II did not appear. The data are mean ± s.e.mean. * $P < 0.05$, † $P < 0.01$ (paired t test).

Figure 4 Effect of pretreatment with cromakalim on the development of the phenylephrine (PE)-induced tonic contraction in rat aorta. The protocol shown in Figure 1c was repeated twice for the same muscle. Upper trace, control response in the absence of cromakalim. Lower trace, cromakalim (3×10^{-7} M) was applied 10 min before the addition of phenylephrine. Fifteen min after the addition of Ca^{2+} , nifedipine (1×10^{-7} M) was applied.

(Hamilton *et al.*, 1986; Weir & Weston, 1986; Standen *et al.*, 1989). As shown in Figure 4, cromakalim (3×10^{-7} M) suppressed the tonic-II phase with a small depression of the tonic-I phase. Subsequent addition of nifedipine in the presence of cromakalim did not cause a further inhibition.

Sensitivity to various substances related to phosphoinositide cascade

To determine the dependence of each component of tonic contraction on polyphosphoinositide hydrolysis, the effects of substances which might affect phosphatidylinositol cascade were tested. We used NCDC (Walenga *et al.*, 1980; Nakaki *et al.*, 1985), which has been reported to inhibit phospholipase C, an enzyme that catalyzes phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol. NCDC was applied 15 min before phenylephrine. Figure 3b shows the effect of NCDC (3×10^{-5} M) on the development of the tonic contraction, and Table 1 summarizes the dose-related effects. Unlike nifedipine, NCDC (1×10^{-5} – 1×10^{-4} M) decreased both the early and later phases of the contraction to a similar extent. This means that the decrease of the later phase could result from the inhibition of the tonic-I phase. With 1×10^{-5} and 3×10^{-5} M NCDC, the muscle exhibited a two step development of tonic contraction while the initiation of the tonic-II phase was delayed. With 1×10^{-4} M NCDC, the tonic contraction became monophasic in 6 of the 8 preparations. On the other hand, NCDC at 1×10^{-5} M did not affect the contraction induced by 60 mM KCl in normal PSS, and at 3×10^{-5} M or 1×10^{-4} M it decreased the contraction by $4 \pm 1\%$ ($n = 4$) or $19 \pm 2\%$ ($n = 4$), respectively.

Polyphosphoinositide hydrolysis following α_1 -adrenoceptor activation produces diacylglycerol, which activates protein kinase C (C-kinase) (Nishizuka, 1984). Therefore C-kinase can be involved in the phenylephrine-induced tonic contraction. Hidaka *et al.* (1984) reported that H-7 inhibited C-kinase in various tissues, and Khalil & Van Breeman (1988) used this substance to inhibit the enzyme in vascular smooth muscle activated by a phorbol ester or phenylephrine. Here the effect of H-7 on phenylephrine-contraction was tested with the protocol of Figure 1c to see whether C-kinase played a role in the phenylephrine-tonic contraction. Figure 5 shows examples of phenylephrine-contraction in the presence of 1×10^{-5} and 2×10^{-5} M H-7. H-7 decreased the phasic component due to

Figure 5 Effect of H-7 on the phenylephrine-induced tonic contraction. The protocol shown in Figure 1c was adapted: (a) the control contraction due to phenylephrine (PE) applied in Ca^{2+} -free PSS and the subsequent addition of Ca^{2+} at 10 min; (b) and (c) H-7 (1×10^{-5} , 2×10^{-5} M) was added 15 min before the application of phenylephrine. In (c), nifedipine 1×10^{-7} M was applied when the tension reached the plateau in the presence of H-7 2×10^{-5} M.

phenylephrine and abolished the residual tonic response in Ca^{2+} -free PSS, consistent with the data on rabbit aorta obtained by Khalil & Van Breeman (1988). In addition, H-7 decreased the amplitude of the tonic-I phase and delayed the onset of the tonic-II phase. The data shown in Figure 3c and Table 1 suggest that the inhibition of the contraction by H-7 mainly resulted from the inhibition of the tonic-I phase rather than the tonic-II phase. On the other hand, the inhibition of the 60 mM KCl-induced contraction in normal PSS by H-7 was $2 \pm 1\%$, $6 \pm 1\%$ and $18 \pm 2\%$ at 5×10^{-6} M, 1×10^{-5} M and 2×10^{-5} M ($n = 4$ for each concentration), respectively.

The effects of nifedipine and H-7 on phenylephrine-induced contraction were additive. The addition of nifedipine (1×10^{-7} M) at the plateau of the tonic-II phase pretreated with H-7 (2×10^{-5} M) decreased the tension close to the original level (Figure 5). When a contraction was elicited by phenylephrine (1×10^{-6} M) in normal PSS, the pretreatment with nifedipine (1×10^{-7} M) inhibited the contraction by $41 \pm 3\%$ ($n = 9$) while H-7 inhibited it by $49 \pm 4\%$ ($n = 7$). The presence of both compounds almost completely inhibited the phenylephrine-contraction (inhibition; $96 \pm 2\%$, $n = 6$). Thus it seems that phenylephrine-contraction involves nifedipine-sensitive and H-7-sensitive components.

$^{45}\text{Ca}^{2+}$ influx

To determine the relationship between both phases of phenylephrine-induced tonic contraction and the Ca^{2+} influx from the extracellular fluid we examined the rate of $^{45}\text{Ca}^{2+}$ influx. To correlate the $^{45}\text{Ca}^{2+}$ influx to contraction, the change of tension was recorded for every preparation. The rate of $^{45}\text{Ca}^{2+}$ influx was measured only for the strip which

Figure 6 The rate of $^{45}\text{Ca}^{2+}$ influx during the tonic-I and tonic-II components of phenylephrine-induced contraction. The same protocol as in Figure 1c was adapted. At 0 min 2.5 mM Ca^{2+} was added to control muscles (○) or phenylephrine-pretreated muscles (●). At the desired time PSS was changed to $^{45}\text{Ca}^{2+}$ -containing PSS for 2 min and then the muscles were transferred to cold La^{3+} solution. (▲) The $^{45}\text{Ca}^{2+}$ influx in the presence of phenylephrine and H-7 $1 \times 10^{-5}\text{ M}$; (■) the $^{45}\text{Ca}^{2+}$ influx in the presence of phenylephrine and nifedipine $1 \times 10^{-7}\text{ M}$. * Significantly different from control ($P < 0.05$). Each point represents a mean of 8 preparations with s.e.mean shown by vertical lines. * Significantly different from the influx in phenylephrine-treated group.

showed two step development of tonic contraction with the regular protocol (Figure 1c). Here we found that the tonic-I phase lasted 2–3 min in this experimental system and the tonic-II phase reached the steady state within 3 min from the initiation of the phase. After the tension response to phenylephrine was observed without $^{45}\text{Ca}^{2+}$, the same protocol was repeated for $^{45}\text{Ca}^{2+}$ influx measurement. After 0.5, 2, 4, 7 and 10 min application of 2.5 mM Ca^{2+} to control and phenylephrine-treated muscles the medium was changed to $^{45}\text{Ca}^{2+}$ -containing medium and 2 min later the muscle was quickly transferred to La^{3+} solution to remove extracellular $^{45}\text{Ca}^{2+}$ (Figure 6). The rate of $^{45}\text{Ca}^{2+}$ influx was greatest at 0.5 min in both control- and phenylephrine-treated muscles. This suggests that the Ca^{2+} entry is more rapid when Ca^{2+} is entering the cells for replenishment of Ca^{2+} stores than when the cellular Ca^{2+} distribution is close to equilibrium. There was no difference in the rate of $^{45}\text{Ca}^{2+}$ influx between control and phenylephrine-treated groups at 0.5 and 2 min, although tension developed in phenylephrine-treated muscles but not in control muscles. On the other hand, the rate of the influx was significantly greater in the phenylephrine-treated group than in the control at 4, 7 and 10 min, which corresponded to the tonic-II phase.

The rate of $^{45}\text{Ca}^{2+}$ influx at 2 min of application of Ca^{2+} was the same as that at 10 min in the phenylephrine-treated group. The effects of nifedipine ($1 \times 10^{-7}\text{ M}$) and H-7 ($1 \times 10^{-5}\text{ M}$) on the rate of $^{45}\text{Ca}^{2+}$ influx at 2 and 10 min were examined. Nifedipine did not affect the influx at 2 min but significantly decreased it at 10 min. H-7 did not affect the influx during either phase (Figure 6).

Discussion

By preincubation of the rat aorta in Ca^{2+} -free PSS containing 0.5 mM EGTA , the α_1 -adrenoceptor-mediated contraction was divided into at least three components, a phasic component and two components of tonic contraction. In addition, a small residual contraction persisting in the absence of extracellular Ca^{2+} may be another component. The phasic contraction may be due to Ca^{2+} release from sarcoplasmic reticulum and IP_3 may be a second messenger responsible for this (Hashimoto *et al.*, 1986; Rapoport, 1987). Each phase of tonic

contraction following the addition of Ca^{2+} showed pharmacologically different responsiveness to various substances, i.e., the tonic-I phase was more sensitive to NCDC and H-7 than the tonic-II phase, whereas the tonic-II phase was more sensitive to nifedipine, verapamil and cromakalim than the other. This suggests that the two phases depend on different mechanisms.

The contraction induced by phenylephrine in normal PSS was partially sensitive to either H-7 or nifedipine, and it was almost completely abolished when both agents were present. Therefore the tonic contraction due to α_1 -receptor activation in normal medium consists of two different phases which appear separately by use of the present protocol. Although at present we do not have sufficient data to explain this separation, the protocol used in this study could be useful in investigating the mechanisms involved in the α_1 -receptor-mediated contraction.

Before interpreting the data we must consider the specificity of substances used in this study which might affect polyphosphoinositide hydrolysis and C-kinase. Whether NCDC has an action other than an inhibition of phospholipase C is not clear. In this study, NCDC at $1 \times 10^{-4}\text{ M}$ considerably decreased the contraction induced by high KCl. Therefore, some non-specific action may be at least partly involved in the inhibition of phenylephrine-contraction by NCDC when the highest concentration was used. H-7 does not selectively inhibit C-kinase (K_i $6.0\text{ }\mu\text{M}$); it also inhibits cyclic AMP-dependent protein kinase (A-kinase, K_i $3.0\text{ }\mu\text{M}$), guanosine 3':5'-cyclic monophosphate (cyclic GMP)-dependent protein kinase (G-kinase, K_i $5.8\text{ }\mu\text{M}$) and myosin light chain kinase at a high concentration (K_i $97\text{ }\mu\text{M}$; Hidaka *et al.*, 1984). However, because A-kinase and G-kinase are responsible for the relaxation of vascular smooth muscle rather than its contraction (Rüegg & Paul, 1982; Ignarro & Kadowitz, 1985), it is difficult to suppose that the inhibition of A-kinase or G-kinase by H-7 causes the depression of contraction. At the highest concentration ($2 \times 10^{-5}\text{ M}$) H-7 decreased the KCl-induced contraction by 17.6%. This could be ascribed to the inhibition of myosin light chain kinase and this effect might be partly involved in the inhibition of all phases of phenylephrine-contraction at that concentration. Since the inhibition by H-7 of KCl-contraction was much less at lower concentrations, the major cause of inhibition by this agent of tonic-I could be ascribed to inhibition on C-kinase.

Overall, we can say that at the submaximal concentrations used in this study, NCDC or H-7 inhibited the tonic-I phase mainly through an effect on phospholipase C or C-kinase, respectively. Although these effects may have caused the inhibition of the tonic-II phase when the highest concentrations were used, these effects must be less or indirect as compared to the effects on the tonic-I phase.

On the other hand, although the tonic-II phase was inhibited by Ca^{2+} channel blockers the tonic-I phase was not. The resistance of the tonic-I phase to nifedipine was not due to alterations of Ca^{2+} channels following treatment with EGTA. In some studies α_1 -adrenoceptor-mediated contraction of rat aorta was partially resistant to Ca^{2+} channel blockers (Beckerlingh *et al.*, 1984; Chiu *et al.*, 1986; Bogner & Enero, 1988). The tonic-I and tonic-II phases may correspond to the Ca^{2+} channel blocker-resistant and -sensitive parts of the contraction, respectively.

We measured the Ca^{2+} uptake by the short (2 min) application of $^{45}\text{Ca}^{2+}$ at the desired time during the tonic contraction. This procedure would minimize the $^{45}\text{Ca}^{2+}$ efflux, hence the increased $^{45}\text{Ca}^{2+}$ uptake may represent the unidirectional Ca^{2+} influx (Van Breeman *et al.*, 1985). The rate of $^{45}\text{Ca}^{2+}$ influx during the tonic-II phase was significantly greater in phenylephrine-treated muscles than in the control ones. The additional Ca^{2+} influx during this period may contribute to the formation of the tonic-II phase which is sensitive to nifedipine. On the contrary, the rate of $^{45}\text{Ca}^{2+}$ influx during the tonic-I phase in phenylephrine-treated muscles was not different from that in the controls, but a contraction appeared

only in phenylephrine-treated muscles. Furthermore, the inhibition of the tonic-I phase by H-7 did not accompany the inhibition of $^{45}\text{Ca}^{2+}$ influx. These findings indicate that not only the Ca^{2+} influx but also other mechanisms are involved in the initiation of the tonic-I phase and this mechanism is likely to be associated with C-kinase.

Among the many reports that C-kinase activation induces contraction of vascular smooth muscles, some workers showed that phorbol esters, well-known C-kinase activators, induced a contraction even when a muscle was incubated in Ca^{2+} -free medium (Gleason & Flaim, 1986; Chiu *et al.*, 1987; Sawamura *et al.*, 1987) or when intracellular Ca^{2+} did not increase (Jiang & Morgan, 1987; Litten *et al.*, 1987). Besides, Itoh *et al.* (1986, 1988) reported that a phorbol ester could induce a contraction of skinned vascular smooth muscles by enhancing the Ca^{2+} sensitivity of the contractile protein. Therefore, the activation of C-kinase can cause contraction before the Ca^{2+} channels open.

H-7 suppressed the small residual contraction in Ca^{2+} -free PSS. This small contraction might not be due to Ca^{2+} release (Ito *et al.*, 1986) but result from C-kinase activation (Khalil & Van Breemen, 1988). Since C-kinase activity depends on the concentration of Ca^{2+} (Nishizuka, 1984), the readmission of Ca^{2+} to a muscle pre-exposed to Ca^{2+} -free PSS would enhance C-kinase if diacylglycerol is present, and consequent-

ly potentiate the residual contraction to the level of the tonic-I phase. H-7 also decreased the phasic contraction at all concentrations. This inhibition could result from the inhibition of the residual contraction which underlay the phasic contraction.

As well as nifedipine, cromakalim inhibited the tonic-II phase. In the presence of cromakalim the addition of nifedipine did not cause a further inhibition, indicating that the nifedipine-sensitive component equals the cromakalim-sensitive one. Although we did not measure the membrane potential, the data suggest that a depolarization occurred during the tonic-II phase and that it was responsible for the opening of the voltage-dependent Ca^{2+} channel. A question is whether some event(s) in phosphatidylinositol cascade is associated with the change in membrane potential and/or the Ca^{2+} channel opening. Phorbol esters were reported to increase Ca^{2+} influx at concentrations higher than the threshold to induce contraction (Gleason & Flaim, 1986; Litten *et al.*, 1987). Therefore, the relation of C-kinase to nifedipine-sensitive Ca^{2+} channels may be important. Further studies are needed to clarify the role of the phosphatidylinositol cascade in Ca^{2+} channel opening.

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