ISSN 0253-9756 中国药理学报 *Acta Pharmacologica Sinica* 1992 Sep; **13** (5) : 385-390

ndrine is not a selective calcium channel blocker scular smooth muscle

Yin KWAN (关超然)[1], DENG Han-Wu (邓汉武)[2], GUAN Yong-Yuan (关永源)[3]
ch Muscle Research Program, Division of Physiology and Pharmacology, Department of dical Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada)

RACT The effects of tetrandrine (Tet) on the th properties of rat aortic ring preparations died to test the hypothesis that Tet is a Ca^{2+} nist acting on voltage-operated Ca^{2+} channels The tests were performed on contractions in- by depolarizing concentrations of KCl and by noceptor agonist, phenylephrine (Phe). ascular effects of Tet were compared to those dipine (Nif). We found that Tet behaved tively similar to, but less potent than, Nif in hibited KCl-induced contraction in a concen- -dependent fashion and its inhibitory effect ng-lasting. However, the effects on Phe- contraction of Tet was different from those of hat the extracellular Ca^{2+}-dependent contrac- s inhibited by Tet, but not by Nif. Tet (60 L^{-1}) completely inhibited the $^{45}Ca^{2+}$ uptake d by KCl and Phe in rat aortic muscle strips. he aortic muscle contractile response was in- by addition of Ca^{2+} following depletion of ular stores by Phe in the presence of smic reticulum Ca^{2+}-pump inhibitor, zonic acid, Tet (60 μmol · L^{-1}) was more ef- than Nif 1 μmol · L^{-1} in inhibiting such a re- to extracellularly added Ca^{2+}. Furthermore, t not Nif, also significantly inhibited the con- n to Phe in Ca^{2+}-free medium. Collectively, sults led us to conclude that Tet does not be- a selective VOC blocker like Nif.

WORDS tetrandrine; nifedipine; calcium l blockers; vascular smooth muscle; zonic acid

d 1992-05-18 Accepted 1992-06-09

hom correspondence and reprint request should be t. With effect from 1992 Jul 1, Professor C Y ppointed Head, Department of Physiology, Fac- Medicine, University of Hong Kong, Hong Kong.
in *Department of Pharmacology, Hu-nan Medical y, Changsha 410008, China.*
in *Department of Pharmacology, SUN Yat-Sen y of Medical Sciences, Guangzhou 510089, China.*

In view of the pivotal role of the regulation of intracellular Ca^{2+} in vascular smooth muscle in health and in diseases[1], persisting efforts have been made toward the development of new Ca^{2+} antagonists acting at the voltage-dependent *L*-type Ca^{2+} channels as therapeutic drugs or pharmacological tools via chemical synthesis[2]. However, understanding of active chemical ingredients extracted or purified from therapeutically active medicinal plants provide an alternative means of drug development. Several medicinal herb-derived chemicals have been shown to possess Ca^{2+} antagonistic properties[3-6]. Not all these previous claims have been verified or confirmed[5]. Tetrandrine, a purified vasoactive substance derived from the roots of *Stephania tetradra* S Moore, is one of the most extensively studied active ingredients extracted from the medicinal plants in China. It is chemically well defined and has become commercially available for clinical use as vasodilatory and antihypertensive drug in China, because its *in vivo* and *in vitro* pharmacological characterizations[7-10] have been best described primarily in cardiovascular tissues with special reference to its Ca^{2+} antagonistic action at the VOC sites[7]. Indeed, Tet has been shown, in direct radioligand binding studies, to interact competitively with the tritiated dihydropyridine Ca^{2+}-antagonist binding sites in the myocardial sarcolemma[11]. In myometrium[12] and in vascular smooth muscle[7], Tet was found to elicit similar action as, but much less potent than, verapamil. Since verapamil is known to

possess a number of actions other than blocking the Ca^{2+} entry process, we wish to further document the vascular effects of Tet and compare them to those of Nif, which is better defined as a Ca^{2+} antagonist at the VOC site, in order to test the hypothesis that Tet is a selective VOC Ca^{2+} antagonist in vascular smooth muscle.

MATERIALS AND METHODS

Male adult Wistar rats (300–350 g) were killed by stunning and decapitation. The thoracic aortas were promptly removed and placed in Krebs' physiological solution at pH 7.4 containing (mmol · L^{-1}) NaCl 119, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1, and glucose 11. Fat and connective tissues were removed under a dissecting microscope and cut into 4–5 mm rings. The endothelial cells were removed by rubbing against the teeth of a pair of forceps and the effectiveness of endothelial removal was confirmed functionally as the lack of relaxation to KCl-precontracted aortic rings by the addition of acetylcholine 1 μmol · L^{-1}. The aortic rings were mounted on a 3-ml organ bath connected to a force transducer (Grass FT03C) and a pen recorder. The organ baths and Krebs' solution were bubbled continuously with 95% O_2+ 5% CO_2 and warmed to 37 ℃. The solutions in the baths were changed every 20–30 min.

For contractility experiments, the aortic rings were equilibrated for 20 min before stretching the arteries to approximately 2 g and were allowed to further equilibrate for at least 90 min. Before data collection, stimulation of the arteries with KCl 60 mmol · L^{-1} was repeated every 15–20 min until a reproducible contractile response (within 10% deviation) was obtained. Such a KCl-induced response in each ring was used as a reference standard (100%) for comparison of relative magnitude of contractions. Data were not included if KCl-induced contraction at the end of the experiment (usually 5–6 h after the first response) was <75% of the initial response. For Ca^{2+}-free Krebs', Ca^{2+} was omitted and EGTA 50 μmol · L^{-1} was added. Aortic rings were allowed to be incubated in this Ca^{2+}-free solution for not more than 5 min. Such a protocol ensures total elimination of extracellular Ca^{2+} without having deleterious effects on intracellular Ca^{2+} [13].

For $^{45}Ca^{2+}$ uptake experiments, aortic rings were equilibrated at 37 ℃ for 60 min in HEPES buffer solution, then incubated in the same solution containing $^{45}CaCl_2$ 18.5 kBq · ml^{-1} for 30 min. The rings were then transferred to the $^{45}Ca^{2+}$/HEPES solution containing the agents to be tested and were incubated further for 10 min. After that, the aortic rings were placed into ice-cold HEPES solution (20 mmol · L^{-1}) containing $LaCl_3$ 75 mmol · L^{-1} and glucose 10 mmol · L^{-1} at pH 6.9 for 5 min. The tissues were blotted dry with filter paper and weighed. The $^{45}Ca^{2+}$ taken up by the tissues was extracted overnight at room temperature in 2 ml of EDTA 5 mmol · L^{-1} and was counted in a Beckman liquid scintillation counter (model LS-3801).

Tet, >98% pure, manufactured by Jing-Hua Pharmaceutical Company (Zhejiang, China), was a generous gift from the Department of Pharmacology, Tongji Medical University, Wuhan, China. All organic chemicals including Nif were purchased from Sigma. All inorganic chemicals were obtained from Fischer and Bio-Rad. Stock solution of Nif was prepared in ethanol and stored at −20 ℃ in the dark. Subsequent dilutions was made with deionized and distilled water. Stock solutions of Tet were prepared in HCl 0.1 mol · L^{-1} and added to organ baths directly in no more than 2% of the bath volume. Other drugs were dissolved in deionized and distilled water.

RESULTS

Tet inhibits responses due to Ca^{2+} entry resulting from KCl depolarization and activation of α_1-adrenoceptors with Phe Fig 1 shows the typical tracings illustrating the effect of Tet 60 μmol · L^{-1} on the contractile responses of rat aortic rings to membrane depolarization by KCl 60 mmol · L^{-1} (Fig 1a) or to α-adrenoceptor stimulation by Phe 10 μmol · L^{-1} (Fig 1b). Tet effectively inhibited the

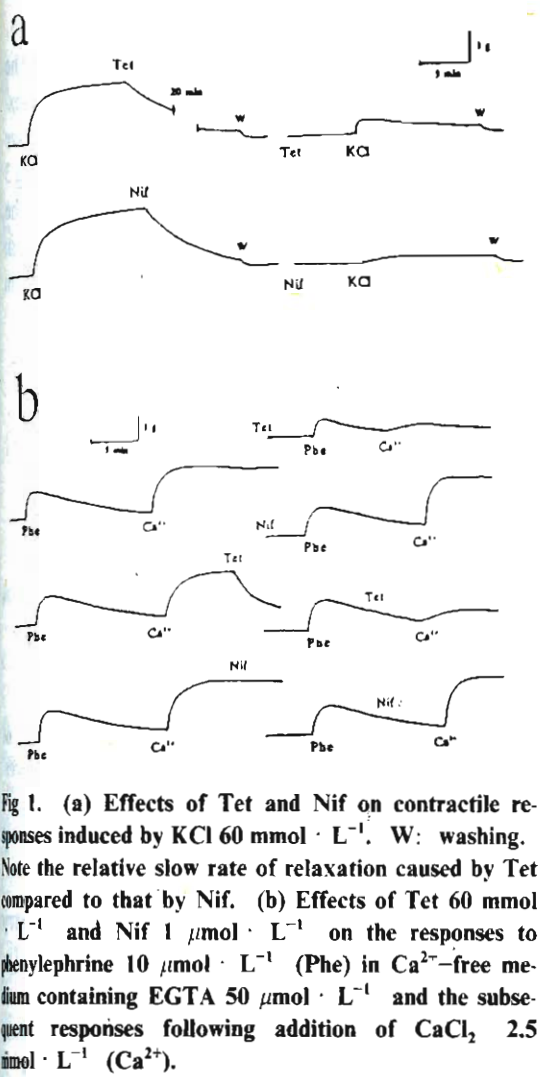

Fig 1. (a) Effects of Tet and Nif on contractile responses induced by KCl 60 mmol · L^{-1}. W: washing. Note the relative slow rate of relaxation caused by Tet compared to that by Nif. (b) Effects of Tet 60 mmol · L^{-1} and Nif 1 μmol · L^{-1} on the responses to phenylephrine 10 μmol · L^{-1} (Phe) in Ca^{2+}–free medium containing EGTA 50 μmol · L^{-1} and the subsequent responses following addition of $CaCl_2$ 2.5 mmol · L^{-1} (Ca^{2+}).

responses to KCl whether it was added prior to, or at the plateau phase of, KCl stimulation. Such an inhibitory effect of Tet was compared to that produced by Nif 1 μmol · L^{-1}. Although the inhibitory effects of these 2 substances were comparable at the above maximal concentrations (see below), the tempory development of inhibition or relaxation of the KCl–induced contraction was considerably slower for Tet than for Nif. Such a difference is unlikely due to other intracellular effects beyond the cell membrane, because the tension development of these aortic rings in the presence of Ca^{2+} selective ionophore, calcimycin (10 μmol · L^{-1}), or the skinning reagent, saponin (50 μg · ml^{-1}), was not affected by the corresponding concentrations of Nif or Tet (data not shown).

Activation of α–adrenoceptors in vascular smooth muscle is known to involve Ca^{2+} release from the internal stores and Ca^{2+} entry from the extracellular space[13]. Fig 1b shows the effect of Tet and Nif on the contractile responses of rat aortic rings to Phe (a selective α_1–adrenoceptor agonist) in Ca^{2+}–free medium and subsequent addition of $CaCl_2$ 2.5 mmol · L^{-1}. It is clear that Tet inhibited or relaxed the Phe–induced contraction, when added prior to or at the plateau phase of the tension development, respectively. Nif, on the other hand, had no effect at all on the contraction induced by Phe. Fig 1b also shows that the transient response due to the release of intracellular Ca^{2+} in Ca^{2+}–free medium was slightly inhibited by Tet but not by Nif.

Inhibitory effects of Tet is concentration–dependent The cumulative concentration–response relationship of the relaxant effects of Tet and Nif on KCl– and Phe–induced contractile responses of rat aortic rings is shown in Fig 2. Both Tet and Nif inhibited KCl–induced contraction in a concentration–dependent manner, Nif being more potent than Tet. Tet also inhibited Phe–induced contraction in a concentration–dependent manner, but Nif was totally ineffective over the entire concentration range employed. These results suggest that Tet acts by inhibiting the VOC (like Nif) as well as by blocking the Nif–insensitive, receptor–operated Ca^{2+} channels. Although the inhibitory effect of Tet on the responses induced by Phe in Ca^{2+}–free medium was much smaller than those in Ca^{2+}–containing medium, the inhibitory effect was statistically significant at >30 μmol · L^{-1} Tet (Fig 2c).

Tet inhibits $^{45}Ca^{2+}$ uptake stimulated by

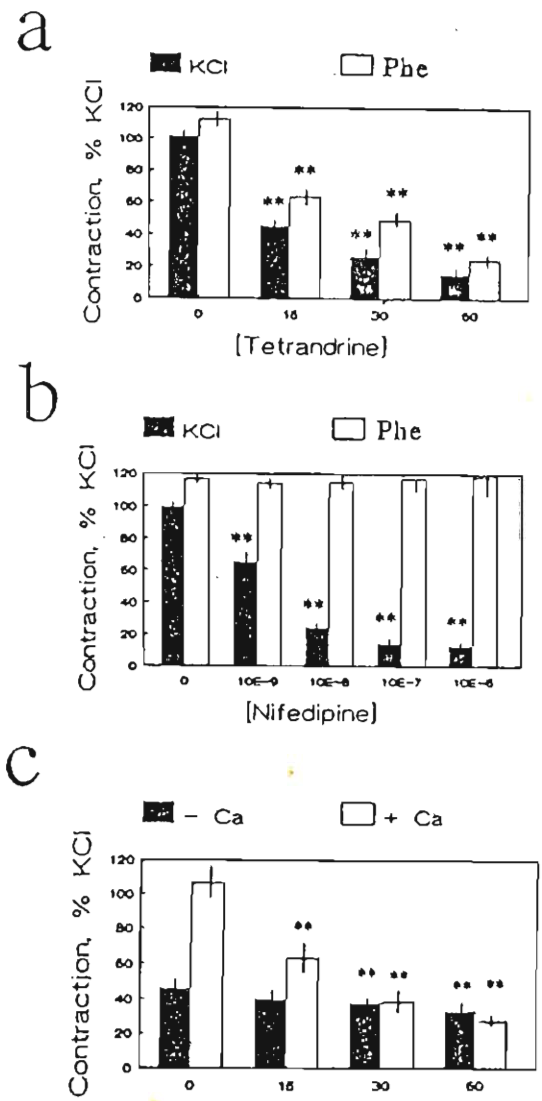

Fig 2. Effect of (a) Tet (in μmol · L^{-1}) and (b) Nif (in μmol · L^{-1}) on contractile responses to KCl 60 mmol · L^{-1} and Phe 10 μmol · L^{-1} in Krebs' solution. (c) Comparison of the effects of Tet 60 μmol · L^{-1} on peak response of transient contraction of rat aortic rings to Phe 10 μmol · L^{-1} in Ca^{2+}–free medium containing EGTA 50 μmol · L^{-1} and the subsequent sustained response to re–admission of Ca^{2+} 2.5 mmol · L^{-1}. $^{**}P<0.05$ *vs* control in the absence of Tet. $n=6$. $\bar{x}\pm s$. The response to KCl is taken as 100%. $^{**}P<0.05$ *vs* control in the absence of Tet or Nif.

KCl and Phe To further confirm the Ca^{2+} antagonistic action of Tet as suggested by the contractility studies, the effects of Tet 60 μmol · L^{-1} on the uptake of $^{45}Ca^{2+}$ by the aortic tissue was also examined under the experimental conditions similar to the above contractility studies using KCl and Phe. Fig 3 clearly shows that Tet effectively blocked the $^{45}Ca^{2+}$ uptake induced by KCl and Phe, in excellent agreement with the results in contractility studies.

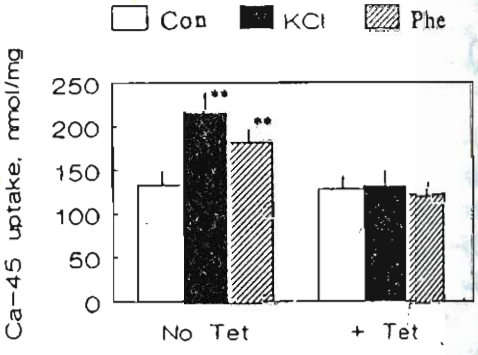

Fig 3. Effects of Tet 60 μmol · L^{-1} on uptake of $^{45}Ca^{2+}$ by rat aortic tissues in the absence and presence of KCl 60 mmol · L^{-1} or Phe 10 μmol · L^{-1}. $n=9$. $\bar{x}\pm s$. $^{**}P<0.05$ *vs* control (Con) in the absence of KCl or Phe.

Tet inhibits responses due to Ca^{2+} entry induced by cyclopiazonic acid (CPA) In creasing evidence suggests that inhibition of Ca^{2+} sequestration by internal membrane leads to slow depletion of internal Ca^{2+} stores, which then signals Ca^{2+} entry into the cytosol and we have confirmed this pathway to be operative in vascular smooth muscle using cyclopiazonic acid (CPA) as a novel pharmacological tool[14]. We have investigated the effects of Tet and Nif on the rat aortic responses due to Ca^{2+} entry via this pathway. Fig 4 shows clearly that, in the control tissue, following depletion of intracellular stores (the transient response) by maximal concentration of Phe in Ca^{2+}–free medium, re–admission of Ca^{2+} after repeated washes caused direct refilling of the intracellular stores without eliciting an

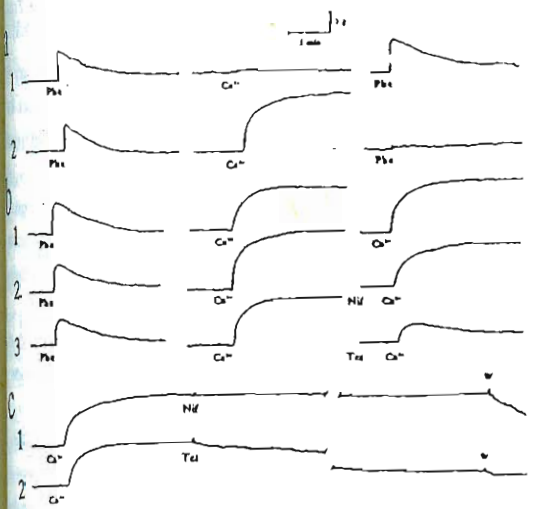

Fig 4. Effect of Tet 60 μmol · L^{-1} and Nif 1 μmol · L^{-1} on Ca^{2+}–induced response in the presence of CPA 10 μmol · L^{-1} following depletion of intracellular Ca^{2+} stores by Phe 10 μmol · L^{-1} in Ca^{2+}–free medium containing EGTA 50 μmol · L^{-1}. (a) After the transient response to Phe, vehicle (tracing 1) or CPA 10 μmol · L^{-1} (tracing 2) was added 15 min before addition of Ca^{2+} 2.5 mmol · L^{-1}. The tissues were then thoroughly washed in Ca^{2+}–free medium containing CPA and stimulated with Phe 10 μmol · L^{-1} for the second time. (b) All preparations were treated with CPA 10 μmol · L^{-1} using the same procedures as described in (a) except that vehicle (tracing 1), Nif 1 μmol · L^{-1} (tracing 2) or Tet 60 μmol · L^{-1} (tracing 3) was added following a thorough washout of the Ca^{2+}–induced contraction with Ca^{2+}–free medium containing CPA. Then, Ca^{2+} 2.5 mmol · L^{-1} was again added. Note that the inhibition of Ca^{2+}–induced contraction by Tet is more potent than that by Nif. (c) the tissues were treated the same way as in (b), showing only the part of Ca^{2+}–induced contraction. Nif (1 μmol · L^{-1}) and Tet 60 μmol · L^{-1} were added at the plateau phase of the sustained contraction induced by Ca^{2+}. Note that Nif had very little effect, but Tet, slowly relaxed tension development to nearly the resting level in 60 min.

contractile responses. The effective refilling of the internal Ca^{2+} stores without tested by the reappearance of the transient response to Phe in Ca^{2+}–free medium (tracing a1). When CPA was added prior to refilling by re–admission of Ca^{2+}, a relatively sustained contractile reponse was observed which was not accompanied by a transient contraction to the subsequent application of Phe in Ca^{2+}–free medium (tracing a2). Addition of Tet 60 μmol · L^{-1} or Nif 1 μmol · L^{-1} prior to the addition of CPA, partially inhibited the Ca^{2+}–induced response, Tet being more potent than Nif (tracing b2 *vs* b3 as compared to the control tracing b1). When Tet or Nif was added at the plateau phase of the Ca^{2+}–induced responses, Nif elicited almost no relaxant effect (tracing c1), but Tet caused a slow relaxation (tracing c2).

DISCUSSION

In this work we have demonstrated a pharmacological assessment of the vascular effects of Tet, to test the hypothesis that Tet acts selectively on VOC. Unlike the earlier studies, we have utilized several different approaches. This included [a] the use of rat aorta devoid of nerve and endothelium, [b] comparative study of Tet with Nif, which has well–defined inhibitory effects on *L*–type VOC than verapamil, [c] effects of Tet on vascular contactility with confirmation using $^{45}Ca^{2+}$ uptake technique, and [d] effect of Tet on 3 different ways of activating Ca^{2+} entry process (eg, via membrane depolarization by KCl, via activation of α_1–adrenoceptor by Phe and via inhibition of internal membrane Ca^{2+}–pump by CPA).

Indeed, we have confirmed that Tet inhibited the contractile responses by interfering with the Ca^{2+} entry via the *L*–type VOC, but its inhibitory effect was much less potent than Nif. However, the inhibitory effect of Tet on Ca^{2+} channels in rat aortic smooth muscle, unlike that of Nif, appears to be non–selective. Tet also inhibited the responses due to Ca^{2+} entry brought about by α_1–adrenoceptor activation (this reponse, presumably due to Ca^{2+} entry via the VOC,

was not affected at all by Nif). This nonselective inhibition by Tet of Ca^{2+}–entry via KCl depolarization and Phe activation was further confirmed in the $^{45}Ca^{2+}$ uptake studies. Furthermore, the contractile response induced by Ca^{2+}, in the presence of sarcoplasmic recticulum Ca^{2+}–pump inhibitor, following depletion of intracellular Ca^{2+} pools, was also effectively inhibited by Tet, but only minimally affected by Nif. More recently, additional effect of Tet on *T*–type Ca^{2+} channels in neuroblastoma cells has also been reported[15]. The above observations, together with the fact that Tet also inhibited the transient contractile responses in Ca^{2+}–free medium cast serious doubts about the specificity of its action as a Ca^{2+} antagonist. In conclusion, Tet is not a potent and selective VOC antagonist over the existing dihydropyridine Ca^{2+} antagonists. This deficiency, however, should not be taken to underestimate the usefulness of Tet as a pharmacological tool in the study of cellular Ca^{2+} signalling.

ACKNOWLEDGMENT This work was supported by a grant–in–aid and a Career Investigator Award (to CYK) from the Heart and Stroke Foundation of Ontario.

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