Naunyn-Schmiedeberg's Arch Pharmacol (1998) 357: 441–445

ORIGINAL ARTICLE

Ulf Bickmeyer · Frank Weinsberg · Erika Müller
Herbert Wiegand

Blockade of voltage-operated calcium channels, increase in spontaneous catecholamine release and elevation of intracellular calcium levels in bovine chromaffin cells by the plant alkaloid tetrandrine

Received: 13 October 1997 / Accepted: 14 January 1998

Abstract Experiments were performed in bovine chromaffin cells in short term primary culture. Tetrandrine is a plant alkaloid from the chinese medical herb Stefania tetrandra. The aim of the present study was to investigate the mechanisms by which tetrandrine interacts with calcium signalling and to provide a quantitative description of effects. Tetrandrine blocked voltage-operated calcium channel currents concentration-dependently as shown in whole cell patch-clamp recordings. The blockade of calcium channels reduced the potassium-stimulated catecholamine release. Besides, the drug increased the spontaneous (not stimulated) release of catecholamines in the presence of extracellular calcium. Measurements of intracellular calcium levels [Ca]i showed a calcium release from intracellular stores by tetrandrine. This tetrandrine-induced [Ca]i elevation was higher in calcium containing as compared to calcium free solution. Tetrandrine effects partially overlap with those of thapsigargin, but tetrandrine has additional targets, since it increased [Ca]i in cells pretreated with thapsigargin. We conclude that tetrandrine blocks voltage-operated calcium channels and increases [Ca]i by blocking endoplasmic and other calcium pumps.

Keywords Tetrandrine · Calcium channel currents · Catecholamine · Intracellular calcium · Thapsigargin

Introduction

Tetrandrine is a bis-benzyl-isoquinoline alkaloid, which can be isolated from the roots of the chinese medical herb Stefania tetrandra. It has long been used for the treatment of hypertension, inflammation and, more recently, silicosis (Bingci et al. 1982). The isolated active compound tetrandrine has biological activities in vascular and lymphatic tissue as well as in lung tissue (Miles et al. 1993), smooth muscle (Kwan and Wang 1993) and nervous tissue from mammals and insects (King et al. 1988; Bickmeyer et al. 1994; Wiegand et al. 1996). It has been described not only to affect the binding properties of other compounds to calcium channels (King et al. 1988), but also to block calcium channels in different tissues: L-type channels, T-type channels, N-type channels (Felix et al. 1992; Liu et al. 1992; Rossier et al. 1993; Bickmeyer and Wiegand 1993) and P-type channels (Weinsberg et al. 1994; Weinsberg 1995). Several investigations suggest, that tetrandrine additionally interacts with the intracellular calcium homeostasis of such different cells like lymphocytes and neuroblastoma cells (Miles et al. 1993; Bickmeyer et al. 1996). The aim of the present study is to investigate the mechanisms by which tetrandrine interacts with calcium signalling in bovine chromaffin cells, which are well suited for investigations with different methods like patch-clamp, determination of catecholamine release and calcium fluorimetry.

U. Bickmeyer · F. Weinsberg · E. Müller · H. Wiegand (✉)
Medizinisches Institut für Umwelthygiene,
Abteilung Neurotoxikologie, Auf'm Hennekamp 50,
D-40225 Düsseldorf, Germany

Materials and methods

Culture method. Primary cultures of bovine chromaffin cells were prepared (Marxen et al. 1989) and kept in culture medium containing: Dulbecco's modified eagle medium (DMEM) 10 g/l, glucose 33 mM, $NaHCO_3$ 36 mM, glutamine 7 mM, 10% fetal calf serum, 100 U/ml penicillin/streptomycine, fluordesoxyuridine 10 µM, uridine 10 µM and cytosinarabinoside 10 µmM. Cells were cultured at a densitiy of 500.000/ml.

Electrophysiology. The recordings were done with the EPC-7 patch clamp amplifier (List electronics) and analyzed with the computer programm CED Electrophysiology package V5.5 (Cambridge Electronic Design). All recordings were carried out between Day 1 and Day 3 of culture. The bath solution contained: tetraethylammonium-chloride (TEA) 135 mM, N-(2hydroxyethyl)piperazine-N-2-ethanesulfonicacid (HEPES) 10 mM, $MgCl_2$ 1.2 mM, $BaCl_2$ 10 mM, tetrodotoxin (TTX) 2 µM (pH was adjusted to 7.2 with TEA-OH). Barium currents through calcium channels were recorded with firepolished patch pipettes with 2–7M(resistance. Pipette solution: CsCl 135 mM, HEPES 10 mM, ethyleneglycol-bis(2-aminoethylether)-N,N,N'-tetraaceticacid (EGTA) 10 mM, $MgCl_2$ 2 mM, Na-ATP 4 mM (pH was adjusted to 7.2 with TEA-OH). Calcium channel currents were evoked from a holding potential of –70 to 0 mV for 100 ms

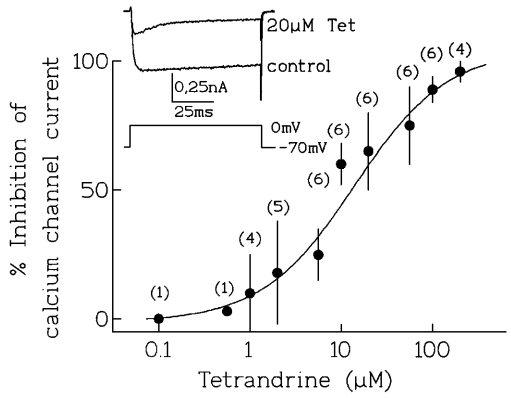

Fig. 1 Inhibition of calcium channel currents of chromaffin cells by various concentrations of tetrandrine, measured in the whole cell configuration of the patch clamp technique. The number of cells treated with equal concentrations is indicated above the error bars. The IC_{50} was 10.4 μM. Cells were clamped to –70 mV and depolarized to 0 mV for 100 ms as indicated in the *inset* which shows a current trace during treatment with 20 μM tetrandrine

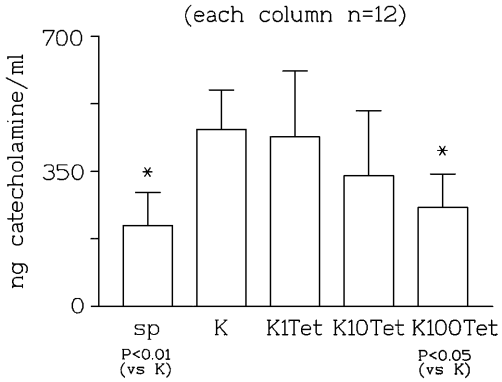

Fig. 2 Amount of catecholamines (ng/ml) released in the absence or presence of the depolarizing stimulus 50 mM K^+ (5 min). (K) represents the stimulated control, (sp) represents the unstimulated control (5 min). K1Tet, K10Tet and K100Tet represents the amount of catecholamines released by K^+ in the presence of 1 μM, 10 μM and 100 μM tetrandrine, respectively. * $P<0.05$ for comparison with K

every 30 s. For concentration effect curves, current amplitudes were determined before and 10 min after tetrandrine application. Current amplitudes were measured 90 ms after the onset of stimuli lasting for 100 ms.

Analysis of catecholamine release. Cells were washed twice using as experimental buffer HEPES buffered saline (HBS, containing in mM: NaCl 150, KCL 5.4, $CaCl_2$ 1.8, HEPES 20, $MgSO_4$ 0.8, d-glucose 20). For chemical depolarisation of the cells, 50 mM K^+ was applied for 5 min and the catecholamine content of the buffer was determined using high-performance-liquid-chromatography (HPLC). Effects of tetrandrine on spontaneous catecholamine release were measured by detecting the release during a 15-min incubation of cells with different tetrandrine concentrations. The samples were filtered through a 0.2 μm pore filter, dihydrobenzoylamine (DHBA, 340 ng/ml) was added as standard and L(+)ascorbic acid (24 mM final concentration) was used as antioxidant. The samples were frozen at –20C° and the catecholamine content was measured within one week by HPLC and electrochemical detection (Biometra). Because of the low amount of dopamine released in control experiments (<3% of catecholamines), only epinephrine and norepinephrine were included in the HPLC analysis.

Fluorometric measurements of [*Ca*]*i.* For video imaging experiments, cells were cultured on glass dishes covered with collagen in 60 mm petridishes.

The medium was removed from the petridishes and replaced with HBS, pH 7.3 containing 1 μM fura-2/acetoxymethylester. After incubation for 45 min at 37°C, the fura-2 containing HBS was removed and cells were washed for 30 min in fura-2 -free HBS in an open perfusion microincubator (Bachofer) mounted on an inverted microscope (Zeiss Axiovert). Fluorescence from chromaffin cells was monitored simultaneously using digital imaging fluorescence microscopy (PTI) of 2–7 cells/coverslip. Fluorescence images were obtained through an oil immersion objective (40↔) and visualized with a video camera (SIM-ICCD-04). Ratios were obtained by division of two background-corrected images, one obtained at the excitation wavelength of 340 nm, the other at 380 nm. For determination of R-min and R-max values we used HBS with 10 μM ionomycin and 10 μM gramicidin in the presence of 10 mM $CaCl_2$ (R-max) and HBS without calcium supplemented with 1 mM EGTA (R-min) in a set of calibrating experiments. Fluorescence ratios were converted in calcium concentrations by the formula given by Grynkievics et al. (1985).

Tetrandrine was a gift from the Institute of Health, Bejing, Peoples Republic of China. It was dissolved in acidified distilled water to stock solutions of 2 mM or 4 mM. The addition of tetrandrine to the HBS did not significantly alter the pH of the solution nor did it interact with fura-2 at a concentration of 100 μM. Thapsigargin was from Sigma.

Statistics. The results are presented as mean ±SD. For statistical testing we used ordinary Anova and a nonparametric posttest (Dunn's), if not indicated otherwise.

Results

Tetrandrine blocked calcium channel currents in bovine chromaffin cells in a concentration-dependent manner. Currents were measured in the whole cell configuration and were elicited by voltage steps from a holding potential of –70 mV to 0 mV. Different concentrations of tetrandrine were applied after control currents became stable. After 10 min, the blockade induced by tetrandrine reached a steady-state level. The IC_{50} was 10.4±1.6 μM (Fig. 1).

A blockade of voltage-operated calcium channels should result in a reduction of the depolarisation-induced catecholamine release, measured by catecholamine determination in the buffer solution. We chemically depolarised the cells with 50 mM K^+ (5 min) and compared the catecholamine release of tetrandrine-treated cells (1 μM, 10 μM, 100 μM) with that of untreated controls. Tetrandrine reduced the secretion of catecholamines significantly, an effect which is probably related to the blockade of voltage-operated calcium channels. 100 μM tetrandrine reduced the K^+-evoked release significantly (Fig. 2).

To assess effects of tetrandrine on the spontaneous catecholamine release cells were incubated for 15 min with different tetrandrine concentrations. Tetrandrine itself elevated the amount of spontaneously released catecholamines. 100 μM tetrandrine increased the amount of released catecholamines significantly (Fig. 3). 1 μM (data not shown) and 10 μM tetrandrine showed no significant effect.

In calcium-free buffer we found no increase of spontaneously released catecholamines up to a concentration of 200 μM tetrandrine (data not shown).

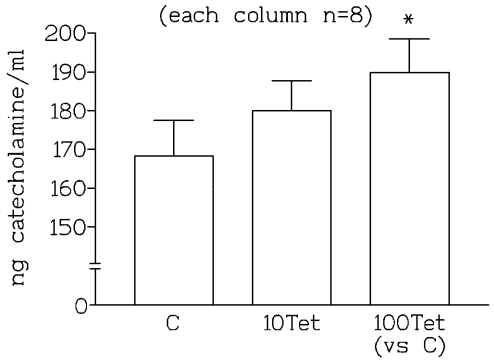

Fig. 3 Amount of catecholamines (ng/ml) spontaneously released under control (C) conditions and in the presence of 10 µM and 100 µM tetrandrine (15 min). * $P<0.05$ for comparison with C

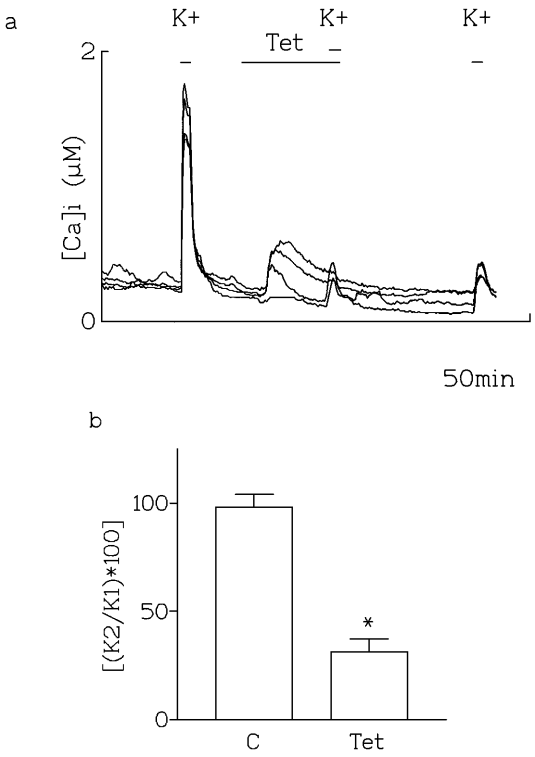

Fig. 4 **a** Depolarization-evoked (K^+) increase of [Ca]i in 4 cells in the absence and presence of 100 µM tetrandrine (Tet). Tetrandrine blocks depolarization induced calcium entry and this effect is hardly reversible. Note the tetrandrine-induced increase of [Ca]i. **b** Increase of [Ca]i measured during subsequent stimulation with 50 mM K^+ in controls and tetrandrine treated cells. Data are calculated by dividing the values during a second K^+-depolarization (K2) through the values during the first K^+-depolarization (K1) in controls (C) and in tetrandrine treated cells. * $P<0.0001$; Students *t*-test

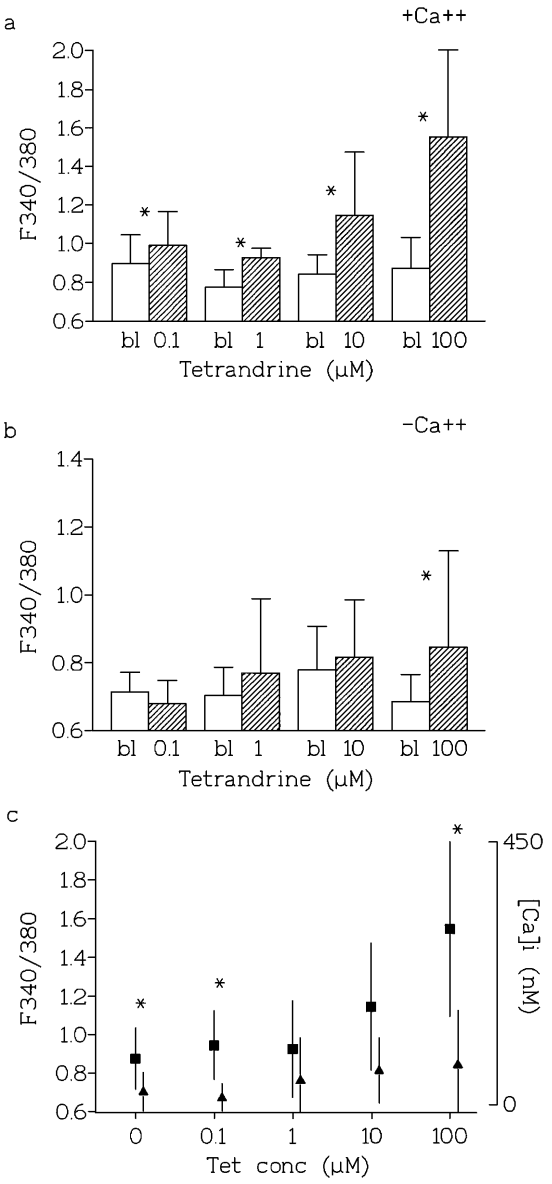

Fig. 5 **a** Elevation of fluorescence ratio induced by various concentrations of tetrandrine in calcium-containing buffer. bl represents baselines in cells before treatment. * $P<0.05$, Anova for repeated measures, Wilcoxon signed rank test. *Each column* represents data from at least 14 cells. **b** Elevation of fluorescence ratio induced by various concentrations of tetrandrine in calcium-free buffer (2–3 min). bl represents the baseline of cells before treatment. * $P<0.05$, Anova for repeated measures, Wilcoxon signed rank test. *Each column* represents data from at least 9 cells. **c** Elevation of [Ca]i induced by various concentrations of tetrandrine in the presense (■) and in the absense of extracellular calcium (▲). *Each point* represents the result from at least 9 cells. * $P<0.05$ between both conditions at the same concentration

To investigate whether tetrandrine induces elevation of [Ca]i, we performed fluorometric measurements using fura-2 as calcium indicator and a video imaging system on the single cell level.

The first experiment was carried out to measure [Ca]i during chemical depolarisation with 50 mM K^+ similar to the catecholamine release experiments. 50 mM K^+ induced a strong elevation of [Ca]i which was blocked by 100 µM tetrandrine (Fig. 4). This was expected from our electrophysiological investigations and catecholamine release experiments. Similar to electrophysiological experiments (Bickmeyer and Wiegand 1993; Weinsberg et al. 1994), the blockade was hardly reversible. There was a large tetrandrine-induced [Ca]i elevation (Fig. 4). We performed fur-

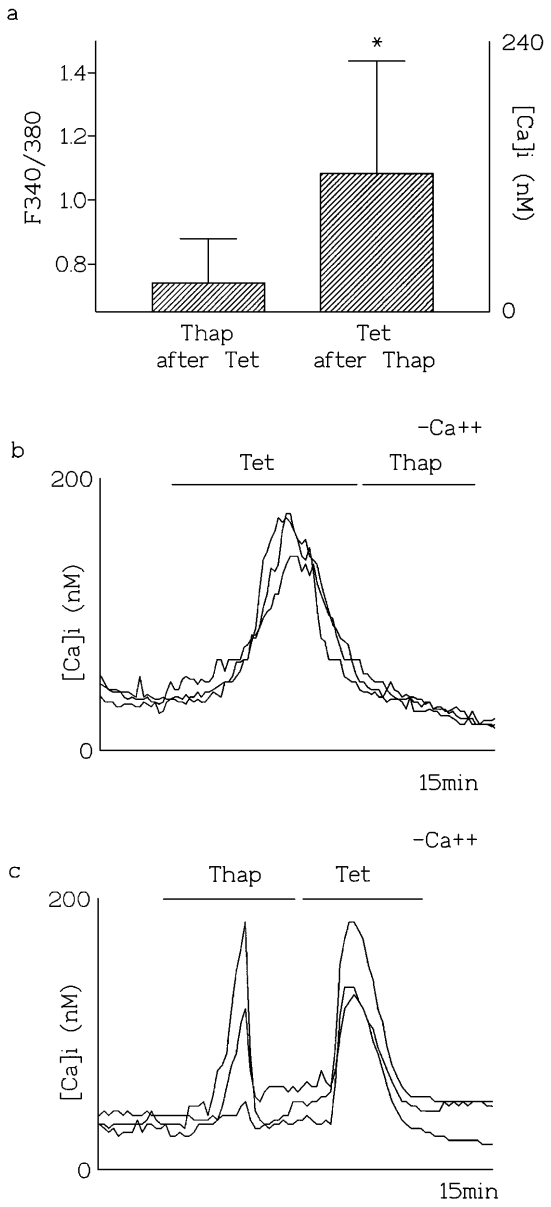

Fig. 6 **a** [Ca]i levels during treatment with 5 μM thapsigargin after pretreatment with 100 μM tetrandrine (Thap after Tet; n = 22) and 100 μM tetrandrine after pretreatment with 5 μM thapsigargin (Tet after Thap; n = 15) in calcium free solution. * P<0.05 (nonparametric test; Mann-Whitney). **b** Original traces of [Ca]i of 3 cells, treated with 100 μM tetrandrine (Tet) and successively with 5 μM thapsigargin (Thap). **c** Original traces of 3 cells treated with 5 μM thapsigargin (Thap) and successively with 100 μM tetrandrine (Tet)

ther experiments to investigate the concentration-response relationship of this tetrandrine effect. Tetrandrine was used in the concentration range between 0.1 μM and 100 μM and we found a concentration-dependent elevation of [Ca]i in response to tetrandrine in calcium-containing buffer (Fig. 5a). In calcium-containing buffer there was a significant [Ca]i elevation at all tested tetrandrine concentrations (Fig. 5a; Anova repeated measure, Wilcoxon signed rank test; P<0.05).

We performed similar experiments as described above to obtain a possible concentration-response relationship in calcium-free buffer (supplemented with 1 mM EGTA) and also found a significant elevation of [Ca]i by tetrandrine at a concentration of 100 μM (Fig. 5b).

Comparing the [Ca]i responses to tetrandrine obtained in calcium-free and calcium-containing buffer (Fig. 5c), we conclude that there is a major contribution of extracellular calcium to the tetrandrine-induced elevation of [Ca]i. Nevertheless, these data demonstrate the ability of tetrandrine to release calcium from intracellular stores.

Because of the relatively long latency (1–2 min) of [Ca]i elevations by tetrandrine, we favoured the idea of an inhibition of calcium-pumps as suggested by Leung et al. (1994) for HL-60 cells. Therefore we investigated the interaction of tetrandrine with thapsigargin- sensitive calcium pumps (Thastrup et al 1990; Robinson et al. 1992). We used calcium-free buffer and applied tetrandrine at a concentration of 100 μM, and after 5–8 min we removed tetrandrine and added thapsigargin at a concentration of 5 μM. Under this conditions we found no response to thapsigargin (Fig. 6a,b). Tetrandrine seemed to deplete thapsigargin sensitive calcium stores. We then added thapsigargin first and after 5–8 min we applied tetrandrine and found a response to tetrandrine (Fig. 6a,c). The observation of a tetrandrine-effect after pretreatment with thapsigargin showed that tetrandrine has additional, thapsigargin-insensitive targets.

Discussion

Tetrandrine is a natural organic substance which blocked voltage-operated calcium channels of different types and in different tissues (King et al. 1988, Liu et al. 1992, Wang and Lemos 1995, Bickmeyer et al. 1996). Additionally it elevated intracellular calcium levels in lymphocytes (Miles et al. 1993) and was suggested to act similarily to thapsigargin in HL60 cells by blocking endoplasmic reticulum calcium ATPases (Leung et al. 1994).

Our results from bovine chromaffin cells showed a concentration-dependent block of voltage-operated calcium channel currents by tetrandrine, a concentration-dependent reduction of K^+-stimulated catecholamine release and a concentration-dependent increase of the spontaneous catecholamine release. We also showed a concentration-dependent elevation of [Ca]i levels in the absence and presence of extracellular calcium, demonstrating the ability of tetrandrine to release calcium from intracellular stores. A depletion of intracellular stores led to a store-operated calcium influx in chromaffin cells, which weakly triggered a release of catecholamines (Powis et al. 1996). This pathway might be one reason for the increase in spontaneous catecholamine release by tetrandrine in our experiments. Takemura et al. (1995) reported a block of store-operated calcium entry in specific cell types, and in other cells like PC12 cells tetrandrine only partially blocked this pathway. In chromaffin cells tetrandrine did not block store-operated calcium entry (unpublished observations).

Tetrandrine inhibited effects of subsequently applied thapsigargin, but increased [Ca]i after pretreatment with thapsigargin. Thapsigargin is believed to block selectively calcium ATPases of the endoplasmic reticulum (Thastrup et al. 1990). Tetrandrine obviously had additional targets which, we suppose, may include plasmalemmal calcium pumps. Removal of extracellular calcium decreased [Ca]i levels in chromaffin cells, suggesting that under resting conditions the [Ca]i level is partially maintained by calcium influx. Therefore an inhibition of plasmalemmal calcium pumps would increase [Ca]i levels more efficiently than thapsigargin and would be more effective in calcium-containing than in calcium-free solution.

We conclude that tetrandrine blocks voltage-operated calcium channels and increases [Ca]i by a blockade of endoplasmic and other calcium pumps.

Acknowledgements We thank Karl-Heinz Redelings for the determination of catecholamines using HPLC and two anonymus referees for constructive criticism.

References

Bickmeyer U, Wiegand H (1993) Tetrandrine effects on calcium currents in cultured neurones of fetal mice. NeuroReport 4:938–940

Bickmeyer U, Rössler W, Wiegand H (1994) Calcium channel currents in cultured pars intercerebralis neurosecretory cells of adult locusta migratoria. J Exp Biol 197:393–398

Bickmeyer U, Hare MF, Atchison WD (1996) Tetrandrine blocks voltage-dependent calcium entry and inhibits the bradykinin-induced elevation of intracellular calcium in NG108-15 cells. NeuroToxicology 17:335–342

Bingci L, Changqi Z, Yurui L (1982) Studies on the contents of glycosaminoglycans from lungs of silicotic rats and tetrandrine treated silicotic rats. Ecotoxicol Environ Safety 7:323–329

Felix JP, King VF, Shevell JL, Garcia ML, Kaczorowski GJ, Bick IRC, Slaughter RS (1992) Bis(benzylisoquinoline) analogs of tetrandrine block L-type calcium channels: evidence for interaction at the diltiazem-binding site. Biochemistry 31:11793–11800

Grynkievicz G, Poenie M, Tsien RY (1985) A new generation of calcium indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450

King VK, Garcia ML, Himmel D, Reuben JP, Lam YT, Pan JX, Han GQ, Kaczorowski GJ (1988) Interaction of tetrandrine with slowly inactivating calcium channels. J Biol Chem 263:2238–2244

Kwan C, Wang Z (1993) Tetrandrine: a vasodilator of medicinal herb origin with a novel contractile effect on dog saphenous vein. Eur J Pharmacol 238:431–434

Leung YM, Kwan CY, Loh TT (1994) Dual effects of tetrandrine on cytosolic calcium in human leukaemic HL-60 cells: intracellular calcium release and calcium entry blockade. Br J Pharmacol 113:767–774

Liu QY, Karpinsky E, Pang PKT (1992) Tetrandrine inhibits both T and L calcium channel currents in ventricular cells. J Cardiovasc Pharmacol 20:513–519

Marxen P, Fuhrmann U, Bigalke H (1989) Gangliosides mediate inhibitory effects of tetanus and botulinum A neurotoxins on exocytosis in chromaffin cells. Toxicon 27:849–859

Miles PR, Bowman L, Ma JHK, Ma JYC (1993) Alterations in alveolar type II cell metabolism induced by tetrandrine and other alkaloids. Toxicol Appl Pharmacol 119:142–149

Powis DA, Clark CL, O'Brien KJ (1996) Depleted internal store-activated Ca^{++} entry can trigger neurotransmitter release in bovine chromaffin cells. Neurosci Lett 204:165–168

Robinson IM, Cheek TR, Burgoyne RD (1992) Ca^{2+} influx induced by the Ca^{2+}-ATPase inhibitors 2,5-di-(t-butyl)-1,4-benzohydroquinone and thapsigargin in bovine adrenal chromaffin cells. Biochem J 288:457–463

Rossier MF, Python CP, Capponi AM, Schlegel W, Kwan CY, Vallaton MB (1993) Blocking T-type calcium channels with tetrandrine inhibits steroidgenesis in bovine adrenal glomerulosa cells. Endocrinology 132:1035–1042

Takemura H, Kwan CY, Ohshika H (1995) Calcium antagonistic actions of tetrandrine depend on cell types. Res Commun Mol Pathol Pharmacol 90:59–68

Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca stores by specific inhibition of the endoplasmatic reticulum Ca-ATPase. Proc Natl Acad Sci USA 87:2466–2470

Wang G, Lemos JR (1995) Tetrandrine: a new ligand to block voltage-dependent Ca^{2+} and Ca^{2+}-activated K+ channels. Life Sci 56:295–306

Weinsberg, F (1995) Wirkungen des Pflanzenalkaloids Tetrandrine auf Ionenströme und die Katecholaminsekretion chromaffiner Zellen aus dem Nebennierenmark von Rindern. Thesis, Universität of Düsseldorf

Weinsberg F, Bickmeyer U, Wiegand H (1994) Effects of tetrandrine on calcium channel currents of bovine chromaffin cells. Neuropharmacol 33:885–890

Wiegand H, McIntosh LJ, Gotzsch U, Krämer K (1996) Effects of tetrandrine on spontaneous and evoked release of acetylcholine at the mouse neuromuscular junction. J Pharmacol Exp Ther 279:891–901