ELSEVIER

Biochemical Pharmacology 64 (2002) 677–687

Biochemical Pharmacology

Biphasic effects of oxethazaine, a topical anesthetic, on the intracellular Ca^{2+} concentration of PC12 cells

Yasusuke Masuda[a,*], Tamami Oguma[a], Akira Kimura[b]

[a]*Division of Toxicology, Niigata College of Pharmacy, 5-13-2, Kamishin'ei-cho, Niigata 950-2081, Japan*
[b]*Department of Medical Technology, Kitasato Junior College of Health and Hygienic Sciences, Niigata 949-7241, Japan*

Received 29 June 2001; accepted 13 February 2002

Abstract

There have been few reports on the mechanism(s) of action of oxethazaine (OXZ) despite its potent local anesthetic action. Generally, local anesthetics (LAs) not only inhibit Na^+ channels but also affect various membrane functions. In the present study, using PC12 cells as a nerve cell model, the effects of OXZ on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were examined in relation to cytotoxicity and dopamine release. $[Ca^{2+}]_i$ was determined by the quin2 method. In resting cells, $(6-10) \times 10^{-5}$ M OXZ produced lactate dehydrogenase leakage, which was Ca^{2+}-dependent, inhibited by metal Ca^{2+} channel blockers, and preceded by a marked increase in $[Ca^{2+}]_i$. Some other LAs showed no cytotoxicity at these concentrations. In K^+-depolarized cells, however, lower concentrations of OXZ (10^{-6}–10^{-7} M), that had no effect on resting $[Ca^{2+}]_i$, inhibited both the dopamine release and the increase of $[Ca^{2+}]_i$ in parallel. The inhibitory potency against the $[Ca^{2+}]_i$ increase was in the order of nifedipine > OXZ ~ verapamil > diltiazem, and OXZ acted additively on the Ca^{2+} channel blockers. OXZ showed the least effect on K^+-depolarization as determined by bisoxonol uptake. OXZ also inhibited the increase in $[Ca^{2+}]_i$ induced by S(−)-BAY K 8644, a Ca^{2+} channel agonist. These observations suggested that low concentrations of OXZ interact with L-type Ca^{2+} channels. The biphasic effects of OXZ on Ca^{2+} movement may be due to a unique chemical structure, and may participate in and complicate the understanding of the potent pharmacological and toxicological actions of OXZ.

Keywords: Oxethazaine; PC12 cells; Intracellular Ca^{2+}; Dopamine release; L-type Ca^{2+} channels

1. Introduction

OXZ is a potent topical anesthetic. Its potency as an LA markedly exceeds those of cocaine, procaine, lidocaine, and dibucaine [1]. Since OXZ is highly effective topically even at the acid pH of the gastric environment and has a large margin of safety in intragastric administration [1,2], it has long been prescribed clinically for the control of pain due to a variety of gastrointestinal disorders such as esophagitis, chronic gastritis, and peptic ulcers [3]. OXZ inhibits gastric acid secretion [4,5], which is also favorable for clinical application. In acute toxicity studies [2], however, toxicity following intravenous and intrapulmonary administration was high, accompanying depression of myocardial contractility and impaired conduction. Despite its long clinical use and potent local anesthetic action, there have been few reports on the mechanism(s) of action of OXZ.

It is generally thought that all LAs, in addition to inhibition of Na^+ channels, non-specifically affect various membrane functions at concentrations that produce anesthesia [6]. We previously reported that high concentrations of OXZ cause the contraction of portal vein branches in the perfused liver [7,8] and also markedly enhance hemolysis of red blood cells *in vitro* [9], both of which are Ca^{2+}-dependent. These Ca^{2+}-dependent actions of OXZ are not shared by other LAs [7,9], and do not appear to coincide with the local anesthetic action of OXZ.

PC12 cells, originally established from a rat pheochromocytoma [10], are widely used for various types of neurological studies. These cells can differentiate to neuron-like cells in the presence of nerve growth factor and also retain various functional characteristics of excitable

* Corresponding author. Tel.: +81-25-269-3170, ext. 205; fax: +81-25-268-1230.
E-mail address: masuda@niigata-pharm.ac.jp (Y. Masuda).

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DA, dopamine; DTPA, diethylenetriaminepentaacetic acid; KRH, Krebs–Ringer–HEPES buffer; LA(s), local anesthetic(s); LDH, lactate dehydrogenase; OXZ, oxethazaine.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01249-2

nerve cells [10–13]. For example, undifferentiated PC12 cells release DA when depolarized by high K^+, and the release requires the influx of Ca^{2+} through voltage-dependent L-type Ca^{2+} channels, which are inhibited by dihydropyridine-type Ca^{2+} channel blockers [13]. The cells also have cholinergic and other receptors, the stimulation of which sometimes causes Ca^{2+} influx [13].

In the present study, we further examined the action of OXZ on Ca^{2+} movement, using undifferentiated PC12 cells as a nerve cell model.

2. Materials and methods

2.1. Materials

The following chemicals were obtained commercially: OXZ, bupivacaine, nifedipine, and S(−)-BAY K 8644 (Sigma Chemical Co.); quin2, quin2 AM, EGTA, and DTPA (Dojindo Lab.); bisoxonol (Molecular Probes); and verapamil, diltiazem, dibucaine, lidocaine, procaine, and tetracaine (Wako Pure Chemical Ind., Ltd.). OXZ was dissolved in an equimolar HCl solution at a concentration of 10 mM. PC12 cells (American Type Culture Collection; batch, F-12704), RPMI 1640 medium, horse serum, and fetal bovine serum were purchased from the Dainippon Pharmaceutical Co.

2.2. Cell culture and preparation of cell suspension

PC12 cells were cultured in collagen-coated plastic flasks (25 cm^2) in RPMI 1640 medium fortified with heat-inactivated horse serum (1/10 vol.) and fetal bovine serum (1/20), at 37° in a humidified atmosphere of 5% CO_2 in air [14]. The cells were subcultured once a week with a split number of 1:6, and confluent cell sheets of 6- to 7-day cultures up to 20 passages were used. At the time of the experiments, the cells were detached by pipetting and then gently passed several times through three micropipette tips connected in a train, which enables dispersion of the cells with a cell viability of 97–98% (trypan blue exclusion test). The suspension was centrifuged at 800 *g* for 1 min at room temperature, and the pelleted cells were washed once with the buffer to be used for the following experiments.

2.3. LDH leakage

PC12 cells (10^6/mL, in 2-mL microcentrifuge tubes) were suspended in KRH (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 5 mM $NaHCO_3$, 6 mM D-glucose, buffered with 25 mM HEPES and NaOH to pH 7.4) or in EGTA–KRH (without added Ca^{2+}, but containing 2.4 mM $MgSO_4$ and 1 mM EGTA). The cell suspension was preincubated at 37° for 3 min, at which time drugs were added, and further incubated for up to 30 min with shaking. The cells were pelleted by centrifugation at 5000 *g* for 15 s at 4°, and the supernatant was used for the LDH assay [15]. LDH leakage was expressed as a percentage of the initial total cell activity measured after solubilization of cells with 0.1% Triton X-100.

2.4. Measurement of DA release

The method described by Isom and Borowitz [14] was applied. The cells were suspended in KRH, incubated in the presence of drugs, and centrifuged as in the case of LDH leakage. To 0.4 mL of the supernatant was added 50 μL each of 0.1 M perchloric acid and 5% EDTA Na_2, and 10 μL of 100 nM 3,4-dihydroxybenzylamine hydrochloride as an internal standard, and the mixture was kept at −80°. The frozen samples were dissolved, centrifuged at 10,000 *g* for 15 min at 4°, and subjected to HPLC. An HPLC system (EICOM) consisting of a degasser (DG-300), pump system (EP-300), column (EICOMPAK CA-5 ODS, 2.1 mm × 150 mm), and ECD detector (ECD-300, APP:450 mV) was used with a mobile phase containing 0.1 M sodium–phosphate buffer (pH 6.0) (80%; v/v), methanol (20%), sodium 1-octane sulfonate (500 mg/L), and EDTA Na_2 (50 mg/L) at a flow rate of 0.23 mL/min.

2.5. Determination of cytosolic Ca^{2+} by quin2

Cytosolic Ca^{2+} concentration was determined by the methods described by Tsien and Pozzan [16] and Thomas [17]. The cells obtained from a confluent culture flask were suspended in 4.4 mL of loading medium (RPMI 1640 containing 1% fetal bovine serum, 5 mM $NaHCO_3$, and 20 mM HEPES, adjusted to pH 7.4 with NaOH at 37°). The suspension (approximately 30×10^6 cells) was divided into 1:3 portions and prewarmed for 3 min at 37°. The 3.3-mL portion was loaded with 4 μL of 10 mM quin2/AM solution in dimethyl sulfoxide and incubated for 30 min in the dark, while the other portion was used as the unloaded control. Under these conditions, intracellular quin2 content ranged between 600 and 700 pmol/10^6 cells, and the cell viability was well preserved. The cell suspension was divided to give a cell number of about 2×10^6/2-mL sample tube and kept in a 15° water bath in the dark. The divided cells were washed twice and finally suspended in 2 mL of KRH in a cuvette. Fluorescence was measured at 37° using a single-beam Hitachi fluorescence spectrophotometer (650–10M, Hitachi Ltd.) with a micro-stirrer unit, at excitation and emission wavelengths of 339 and 492 nm, respectively. Initially, 50 μM $MnCl_2$ and then 200 μM DTPA were added to correct the fluorescence signal due to extracellular quin2. Drugs were then added. Finally, 100 μM digitonin and then 4 mM EGTA–30 mM Tris, pH 8.3, were added to obtain F_{max} and F_{min} values, respectively. $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K_d(F_2 - F_{min})/(F_{max} - F_1)$, where F_1 is the

fluorescence intensity at appropriate times, F_2 is the corrected value of F_1 for extracellular quin2 content, and the $K_d = 115$ nM.

2.6. Measurement of membrane potential

Bisoxonol, a fluorescent indicator, was used [18]. To the PC12 cell suspension (10^6 cells/mL, in KHB) in a fluorometer-cuvette kept at 37° was added 100 nM bisoxonol, and after equilibration, the cells were depolarized by adding KCl solution (final concentration 50 mM). The changes in fluorescence intensity after each addition were recorded at excitation and emission wavelengths of 540 and 580 nm, respectively.

2.7. Statistics

Data are presented as means ± SEM unless otherwise noted. Unpaired data were analyzed by the *t*-test after one-way ANOVA by the *F*-test, and the paired data were analyzed by the *F*-test after two-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxic profiles of OXZ in resting PC12 cells

First, the effects of OXZ on cell viability were examined by measuring LDH leakage. In regular KRH medium (1.2 mM Ca^{2+} included), OXZ up to 3×10^{-5} M caused no or very slight LDH leakage during the 30-min incubation period, whereas at 6×10^{-5} to 10^{-4} M considerable LDH leakage occurred (Fig. 1A). In the absence of Ca^{2+} (in EGTA–KRH), the LDH leakage caused by 6×10^{-5} to 10^{-4} M OXZ was quite small (Fig. 1B), although still higher concentrations (3×10^{-4} to 6×10^{-4} M) caused LDH leakage. The Ca^{2+}-dependent cytotoxicity was evident at much lower Ca^{2+} concentrations than are present physiologically (Fig. 1C). Furthermore, the Ca^{2+}-dependent LDH leakage caused by OXZ in KRH was markedly prevented by the non-selective metal Ca^{2+} channel blockers Co^{2+}, Ni^{2+}, and Mn^{2+} [19], whereas no protection was observed in EGTA–KRH (Table 1).

Other LAs such as procaine, lidocaine, tetracaine, and bupivacaine did not cause LDH leakage irrespective of the presence or absence of Ca^{2+} up to 1 mM, although dibucaine induced some LDH leakage (Table 2).

Table 1
Effects of CO^{2+}, Ni^{2+} and Mn^{2+} on cell death induced by OXZ in the presence and absence of Ca^{2+}

	LDH leakage (%)	
	KRH (OXZ: 10^{-4} M)	EGTA–KRH (OXZ: 3×10^{-4} M)
Control		
0 min	4.4 ± 0.4	9.4 ± 1.4
30 min	5.5 ± 0.8	10.0 ± 0.5
OXZ only	91.4 ± 3.4	61.8 ± 0.8
OXZ + $COCl_2$ (10^{-3} M)	10.1 ± 1.1[a]	79.5 ± 0.8[a]
OXZ + $NiCl_2$ (10^{-4} M)	10.6 ± 0.7[a]	63.8 ± 0.6
OXZ + $MnCl_2$ (5×10^{-3} M)	5.9 ± 1.8[a]	64.5 ± 1.6

OXZ was added to the PC12 cell suspension (10^6 mL) preincubated with metals for 3 min at 37°. Cell death was induced by 10^{-4} and 3×10^{-4} M OXZ in KRH and EGTA–KRH, respectively. After 30 min, LDH leakage into the medium was determined and expressed (mean ± SEM, N = 14) as a percentage of the total LDH activity (1138 ± 90 nmol NADH oxidized/min/1 mL of the cell suspension).

[a] Significantly different from the value with OXZ alone $P < 0.01$.

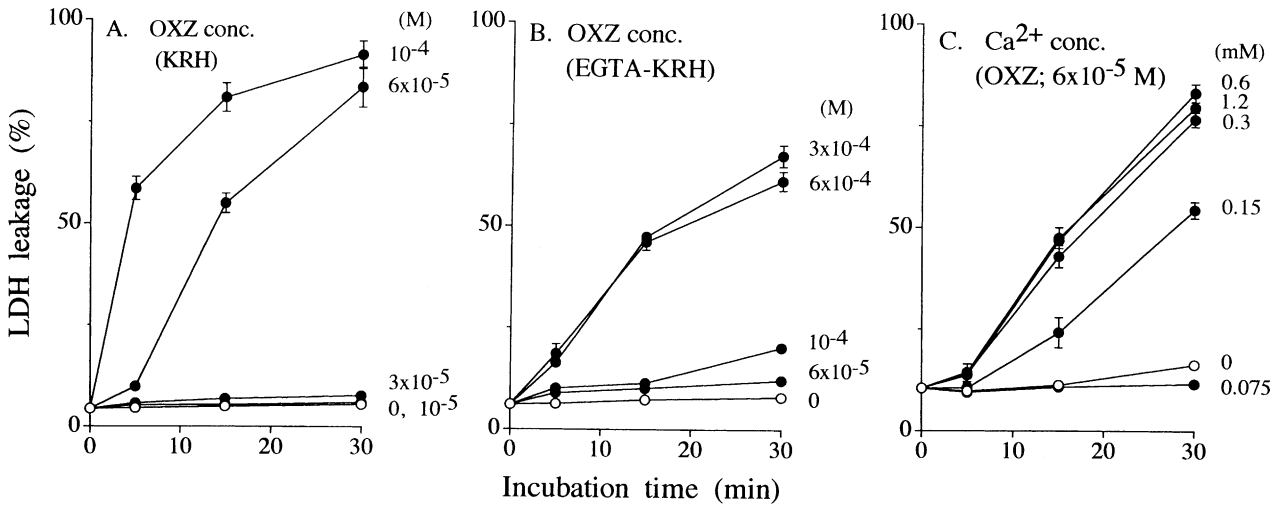

Fig. 1. Cytotoxic effects of OXZ on PC12 cells in the presence and absence of extracellular Ca^{2+}. PC12 cells (10^6 cells/mL) were incubated at 37° with various concentrations of OXZ in the presence of 1.2 mM Ca^{2+} (KRH) (A) or in its absence (EGTA–KRH) (B), or with varied Ca^{2+} concentrations at 6×10^{-5} M OXZ (C). In (C), 1 mM EGTA was included at 0 mM Ca^{2+}. At the time points indicated, LDH leakage into the medium was measured and expressed as a percentage of the initial total activity. Each point represents the mean ± SEM of 4 experiments. The total activity throughout the experiments was 1069 ± 64 (N = 12) nmol NADH oxidized/min/1 mL of cell suspension (10^6 cells).

Table 2
Effects of local anesthetics on the viability of PC12 cells in the presence and absence of Ca^{2+}

	Concentration (M)	LDH leakage (%)	
		KRH	EGTA–KRH
Control			
0 min		5.0 ± 0.3	9.4 ± 1.4
30 min		6.2 ± 0.1	10.0 ± 0.5
Procaine	10^{-4}	6.2 ± 0.5	9.5 ± 0.5
	10^{-3}	5.9 ± 0.5	9.8 ± 1.0
Lidocaine	10^{-4}	5.9 ± 0.6	9.8 ± 1.2
	10^{-3}	4.7 ± 0.4	8.3 ± 0.7
Dibucaine	10^{-4}	5.8 ± 0.3	10.2 ± 0.7
	10^{-3}	53.5 ± 1.3[a]	26.6 ± 2.6[a]
Tetracaine	10^{-4}	5.2 ± 0.8	10.5 ± 0.5
	10^{-3}	5.7 ± 0.6	9.9 ± 1.4
Bupivacaine	10^{-4}	5.5 ± 0.5	8.9 ± 1.8
	10^{-3}	5.2 ± 0.3	9.2 ± 1.4

PC12 cells (10^6 mL) were incubated with local anesthetics in KRH or EGTA–KRH for 30 min at 37°. LDH leakage into the media was determined and expressed (mean ± SEM N = 4) as a percentage of the total LDH activity (1110 ± 97 nmol NADH oxidized/min/1 mL of the cell suspension).
[a] Significantly higher than the control value $P < 0.01$.

3.2. $[Ca^{2+}]_i$ increase by cytotoxic concentrations of OXZ

As shown in Fig. 2A, in KRH, the cumulative addition of 10^{-6}–10^{-4} M OXZ to quin2-loaded cells increased fluorescence intensity in a concentration-dependent manner with no effect at 10^{-6} M, whereas only slight changes were observed in EGTA–KRH or with quin2-unloaded cells. These observations indicated that the increase in fluorescence intensity was due largely to an increase in $[Ca^{2+}]_i$. Fig. 2B shows the time course and concentration-dependency of the $[Ca^{2+}]_i$ increase following the addition of various concentrations of OXZ in KRH. OXZ at 10^{-6} M did not change $[Ca^{2+}]_i$ at least up to 10 min. OXZ at 3×10^{-6} to 3×10^{-5} M increased $[Ca^{2+}]_i$ from the basal level of about 100 nM to 150–350 nM within a few minutes. Higher concentrations (6×10^{-5} to 10^{-4} M) produced a rapid, sustained, and much greater increase of $[Ca^{2+}]_i$, far exceeding 1 μM. This marked elevation of $[Ca^{2+}]_i$ preceded the LDH leakage by OXZ.

The following experiments were performed at OXZ concentrations of 10^{-6} M or less.

3.3. Effects of OXZ on DA release in K^+-depolarized PC12 cells

In preliminary experiments, depolarization of PC12 cells by the addition of 50 mM KCl (final 56 mM) induced the release of DA into the medium, which was almost linear for at least up to 10 min; the addition of 80 mM KCl enhanced the release only slightly, 20 mM KCl was too low

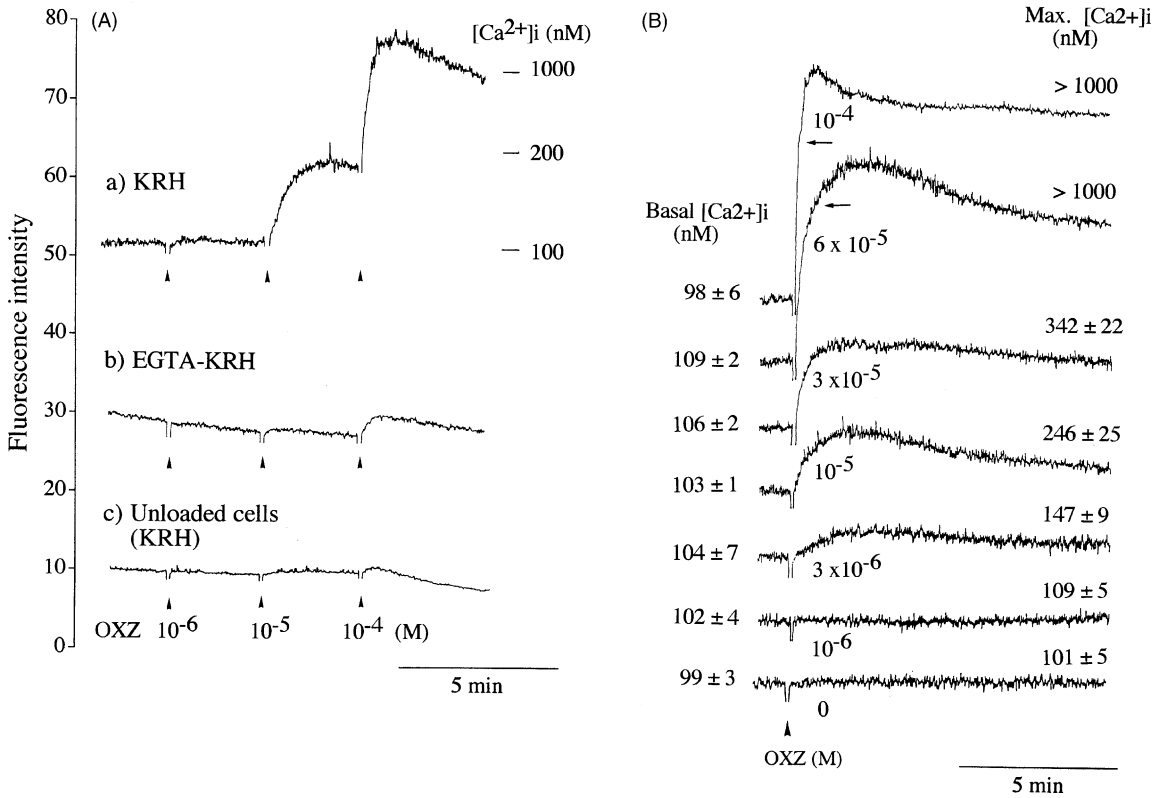

Fig. 2. Effects of OXZ on $[Ca^{2+}]_i$ in PC12 cells. (A) Changes in fluorescence intensity were followed after cumulative addition of OXZ to the quin2-loaded cell suspension (10^6 cells/mL) in the presence of 1.2 mM Ca^{2+} (KRH) (a), in the absence of Ca^{2+} (EGTA–KRH) (b), and to the quin2-unloaded cells in KRH (c). Typical recordings from 3 experiments with similar results are shown. (B) To the quin2-loaded cell suspensions in KRH were added various concentrations of OXZ, and the fluorescence intensity was recorded for 10 min. Representative recordings are shown with their basal and maximum $[Ca^{2+}]_i$, means ± SEM (N = 4). No significant differences were seen in basal $[Ca^{2+}]_i$ values. OXZ concentrations above 10^{-5} M produced significant increases in $[Ca^{2+}]_i$ ($P < 0.01$).

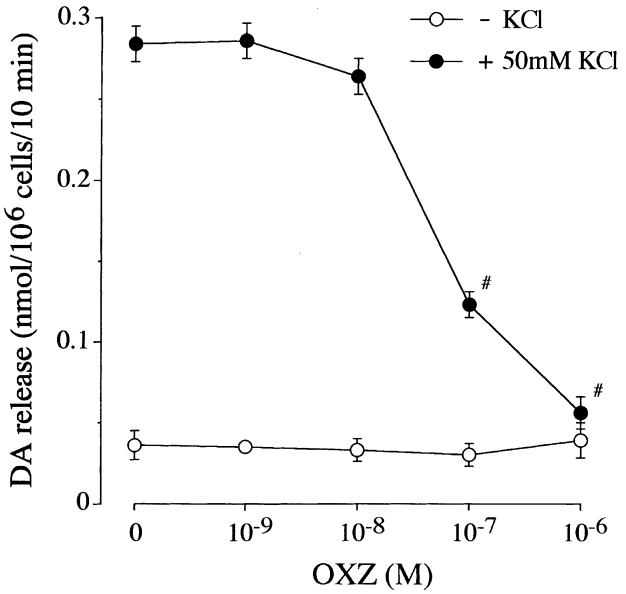

Fig. 3. Effects of low concentrations of OXZ on DA release in PC12 cells depolarized by high K^+. PC12 cells (10^6 cells/mL) in KRH were preincubated for 5 min with various concentrations of OXZ, and then 50 mM KCl was added. After a further 10-min incubation, DA released into the medium was determined as described in "Section 2". Open and closed symbols indicate DA release without and with added KCl (50 mM), respectively. Zero time values have been subtracted. Each point represents the mean ± SEM of 5 experiments, each performed in duplicate. OXZ showed no significant effects on the basal DA release. Key: (#) significantly lower than the control value at 0 μM OXZ ($P < 0.01$).

to stimulate the release, and no release occurred with 50 mM choline chloride or in the presence of 1.3 mM EGTA, in agreement with previous reports [11,20]. As shown in Fig. 3, the release of DA from PC12 cells by 50 mM K^+ was inhibited concentration-dependently by the presence of 10^{-8} to 10^{-6} M OXZ, with near complete inhibition at 10^{-6} M, whereas the resting DA release was unaffected.

3.4. Effects of OXZ on the $[Ca^{2+}]_i$ increase induced by high K^+

Since DA release by K^+-depolarization is well known to be coupled with a rapid and sustained increase of $[Ca^{2+}]_i$ [21], the effects of OXZ on the $[Ca^{2+}]_i$ increase were examined. Addition of 50 mM KCl to the quin2-loaded cell suspension in KRH medium caused an abrupt increase in fluorescence intensity (Fig. 4A, left panel), whereas preincubation of the loaded cells with 10^{-6} M OXZ for 5 min markedly inhibited the increase in fluorescence caused by the addition of KCl (Fig. 4A, right panel). Since KCl treatment of the cells in EGTA–KRH and the unloaded cells produced minimal effects, the changes in fluorescence intensity both in the absence and presence of OXZ were not artifacts but may have reflected changes in $[Ca^{2+}]_i$. As shown in Fig. 4B, preincubation of the quin2-loaded cells with 10^{-8} to 10^{-6} M OXZ inhibited the $[Ca^{2+}]_i$ increase by K^+-depolarization in a concentration-dependent manner, which was nearly parallel with the inhibition of DA release (Fig. 3).

3.5. Effects of OXZ on the membrane potential change caused by high K^+

This was tested indirectly using a potential-sensitive, negatively-charged fluorescent probe, bisoxonol, which enters depolarized cells to increase fluorescence intensity [18]. As shown in Fig. 5 (top traces), in control cells, the addition of bisoxonol produced an abrupt increase in fluorescence intensity, which faded slightly to reach near equilibrium by 10 min. At this time, depolarization by 50 mM KCl further increased the fluorescence. In the cells preincubated with 10^{-6} M OXZ for 5 min, the fading of bisoxonol fluorescence was enhanced slightly and the response to KCl decreased by about 17% as compared with that of the control cells (much less with 10^{-7} M OXZ) (Fig. 5, box). The value could be smaller when considering the quenching of bisoxonol fluorescence by OXZ even in the absence of the cells (Fig. 5, bottom traces). It is unlikely that such changes are responsible for the near complete inhibition of the $[Ca^{2+}]_i$ increase and DA release by OXZ.

3.6. Comparison with Ca^{2+} channel blockers

OXZ also effectively decreased the elevated $[Ca^{2+}]_i$ levels when applied to the depolarized PC12 cells. In this way, the potency of OXZ was compared qualitatively with those of some Ca^{2+} channel blockers (Fig. 6A); the order of potency was nifedipine > OXZ ~ verapamil > diltiazem. OXZ acted additively to the action of each Ca^{2+} channel blocker when OXZ was applied after the blocker (Fig. 6B, top traces), although the additive action was blurred when the order of addition of OXZ and the blockers was reversed, even at higher concentrations of the blockers, especially verapamil and diltiazem (Fig. 6B, bottom traces). These observations suggest, but do not prove, that OXZ acts on the Ca^{2+} channels.

3.7. Effects of OXZ on the $[Ca^{2+}]_i$ increase induced by S(−)-BAY K 8644

S(−)-BAY K 8644, an L-type Ca^{2+} channel agonist in cardiac and smooth muscle [22], was found to produce rapid and sustained increases in fluorescence intensity when applied to quin2-loaded PC12 cells in KRH, with a maximum effect at 10^{-7} M (Fig. 7A, left panel). This was inhibited almost completely by pretreatment of the cells with 10^{-6} M OXZ for 5 min (Fig. 7A, right panel). Since such changes were not observed in EGTA–KRH or with unloaded cells, it was evident that S(−)-BAY K 8644 caused an increase in $[Ca^{2+}]_i$, which was inhibited by OXZ. The inhibition by OXZ was concentration-dependent and significant at 10^{-7} M (Fig. 7B). OXZ also effectively

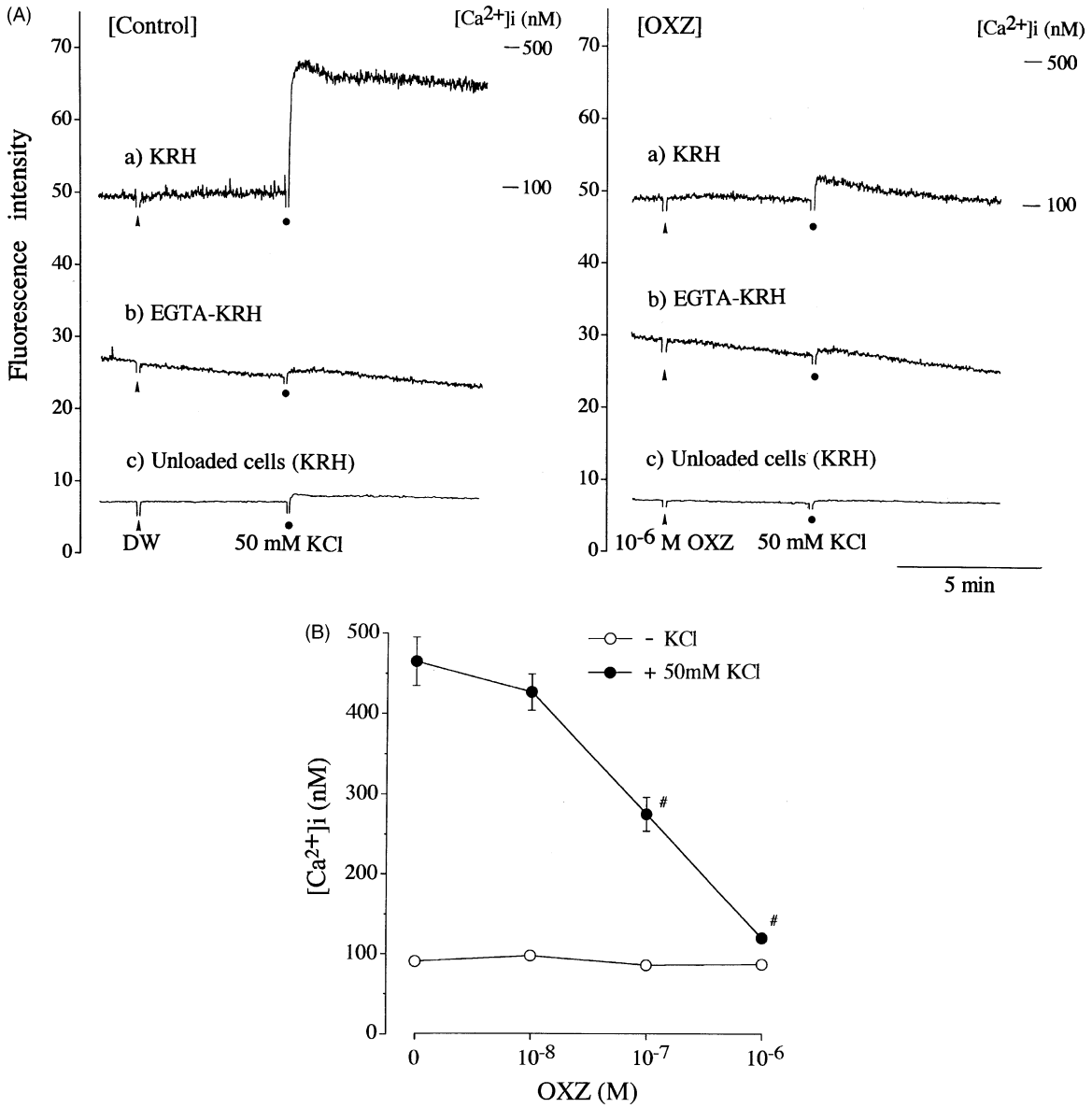

Fig. 4. Effects of low concentrations of OXZ on the $[Ca^{2+}]_i$ increase in PC12 cells depolarized by high K^+. The quin2-loaded cells (10^6 cells/mL), equilibrated in a fluorometer cuvette at 37°, were preincubated for 5 min with various concentrations of OXZ, and then 50 mM KCl was added. Details of the experimental procedure and the calculation for $[Ca^{2+}]_i$ are described in "Section 2". (A) Examples of the fluorescence recordings. Left: Control experiments. The quin2-loaded cells in KRH or EGTA–KRH, or the unloaded-cells in KRH were preincubated for 5 min with 20 μL of distilled water (DW, arrowheads), and then 50 mM KCl was added (closed circles). Right: Pretreatment with 10^{-6} M OXZ (arrowheads). (B) Concentration-dependent decrease of $[Ca^{2+}]_i$ by OXZ. Open and closed symbols represent the basal $[Ca^{2+}]_i$ and the maximum $[Ca^{2+}]_i$ after addition of KCl, respectively. Each point represents the mean ± SEM (N = 5). OXZ showed no significant effect on basal $[Ca^{2+}]_i$. Key: (#) significantly lower than the control value at 0 μM OXZ ($P < 0.01$).

decreased the elevation of $[Ca^{2+}]_i$ levels by S(−)-BAY K 8644 (Fig. 7C).

4. Discussion

OXZ exhibited biphasic effects on $[Ca^{2+}]_i$ levels of PC12 cells, leading to cell death or inhibition of DA release by K^+-depolarization in a concentration-dependent manner.

First, cytotoxicity exhibited by high OXZ concentrations of 6×10^{-5} M in KRH may be due to the rapid influx of a large amount of extracellular Ca^{2+}, since LDH leakage was (a) dependent upon the presence of extracellular Ca^{2+}, even at concentrations much lower than the physiological level of 1.2 mM, (b) inhibited by non-selective metal Ca^{2+} channel antagonists, and (c) preceded by a rapid and sustained increase in $[Ca^{2+}]_i$ over 1 μM. OXZ produced only a small increase in quin2 fluorescence in the absence of extracellular Ca^{2+} (in EGTA–KRH), suggesting that the release of Ca^{2+} from intracellular pools may not be critical. Moreover, OXZ at concentrations of 3×10^{-6} to 3×10^{-5} M increased $[Ca^{2+}]_i$ levels 2- to 4-fold but produced no LDH leakage, indicating that a certain level of $[Ca^{2+}]_i$ is

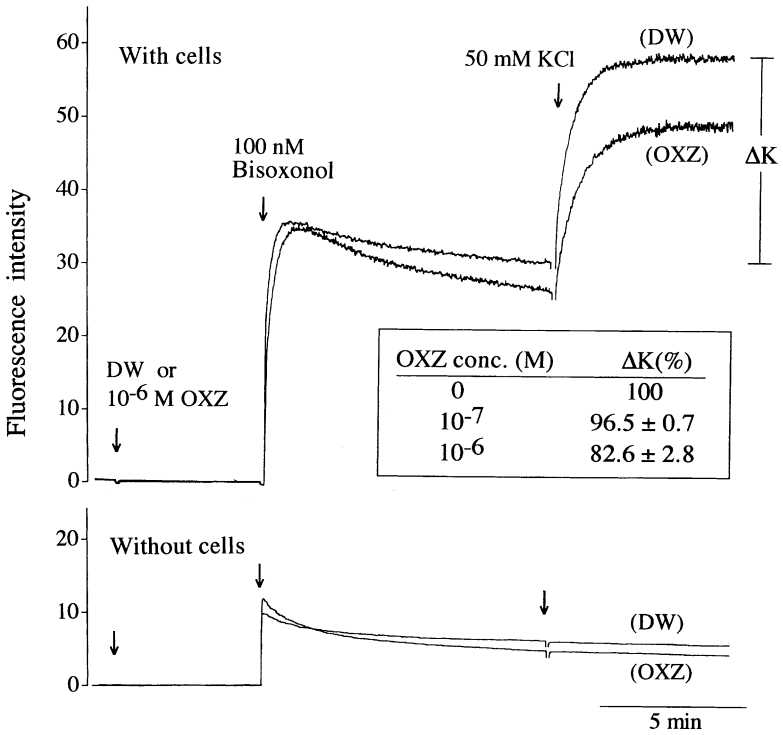

Fig. 5. Effects of OXZ on the membrane potential change induced by high KCl in PC12 cells, examined using bisoxonol. Top recordings: PC12 cells (10^6 cells/mL, in KRH) were preincubated with OXZ in a fluorometer cuvette at 37° for 5 min, bisoxonol (100 nM) was then added, and after an equilibration period of 10 min, 50 mM KCl was added. Typical recordings with 10^{-6} M OXZ and the control (DW, distilled water) are shown. Increases in fluorescence intensity following KCl addition (ΔK, as percent of the paired controls, the mean ± SEM, N = 5) are shown in the box. Bottom recordings: the control experiments without cells.

necessary for cell death. The results of our previous study with red blood cells [9], in which a rapid uptake of $^{45}Ca^{2+}$ preceded hemolysis by OXZ, also supported the suggestion of Ca^{2+} influx into PC12 cells. Ca^{2+} is well known as a mediator for toxic cell death in various cellular systems [23,24]. OXZ may act as a Ca^{2+}-ionophore at high concentrations.

Other LAs had no or only weak (in the case of dibucaine) cytotoxicity. The strong cytotoxicity of OXZ may be due to the characteristic chemical nature of OXZ as discussed below. However, it is unlikely that the cytotoxic action observed at such high concentrations is the primary cause of the toxicity of OXZ *in vivo*.

Second, at non-cytotoxic concentrations below 10^{-6} M, OXZ inhibited the $[Ca^{2+}]_i$ increase as well as the DA release in K^+-depolarized cells, both in a parallel and in a concentration-dependent manner. These observations suggested a causal relationship between them, since DA release by K^+-depolarization in PC12 cells is known to be triggered by the influx of Ca^{2+} through voltage-dependent L-type Ca^{2+} channels [21]. However, similar observations might be attained if OXZ suppressed the depolarization. As found by the bisoxonol method, OXZ appeared to affect K^+-depolarization itself only minimally. This method, however, measures influx of the dye indirectly [18]. Confirmation of these findings by electrophysiological methods is required.

Further experiments with the quin2 method revealed the following: (a) OXZ was both protective and post-effective against the K^+-induced $[Ca^{2+}]_i$ increase, indicating that OXZ interferes with activated as well as resting states of Ca^{2+} channels; (b) by comparison with L-type Ca^{2+} channel antagonists, the order of the inhibitory potency was nifedipine > OXZ ~ verapamil > diltiazem. The effects of OXZ were additive to those of Ca^{2+} channel blockers, although the additive effects were blurred when Ca^{2+} channel blockers were given after OXZ. These observations suggested, but did not prove, that OXZ acted on the L-type Ca^{2+} channels with higher affinity than the blockers. This must be verified by binding experiments; (c) OXZ (10^{-7} to 10^{-6} M) inhibited the $[Ca^{2+}]_i$ increase induced by 10^{-7} M S(−)-BAY K 8644, an L-type Ca^{2+} channel agonist of the dihydropyridine type [22], in a concentration-dependent manner—the inhibition being nearly complete at 10^{-6} M OXZ. In this case also, OXZ was effective with both pre- and post-treatments. These observations strongly suggest that OXZ interacts with L-type Ca^{2+} channels.

Comparison with the effect of other LAs on $[Ca^{2+}]_i$ levels was difficult, since most LAs other than bupivacaine interfered with the fluorescence of quin2. However, in preliminary studies, the DA release induced by K^+-depolarization was inhibited by 10^{-5} to 10^{-4} M dibucaine > tetracaine > bupivacaine > lidocaine (decreasing potency) but not by

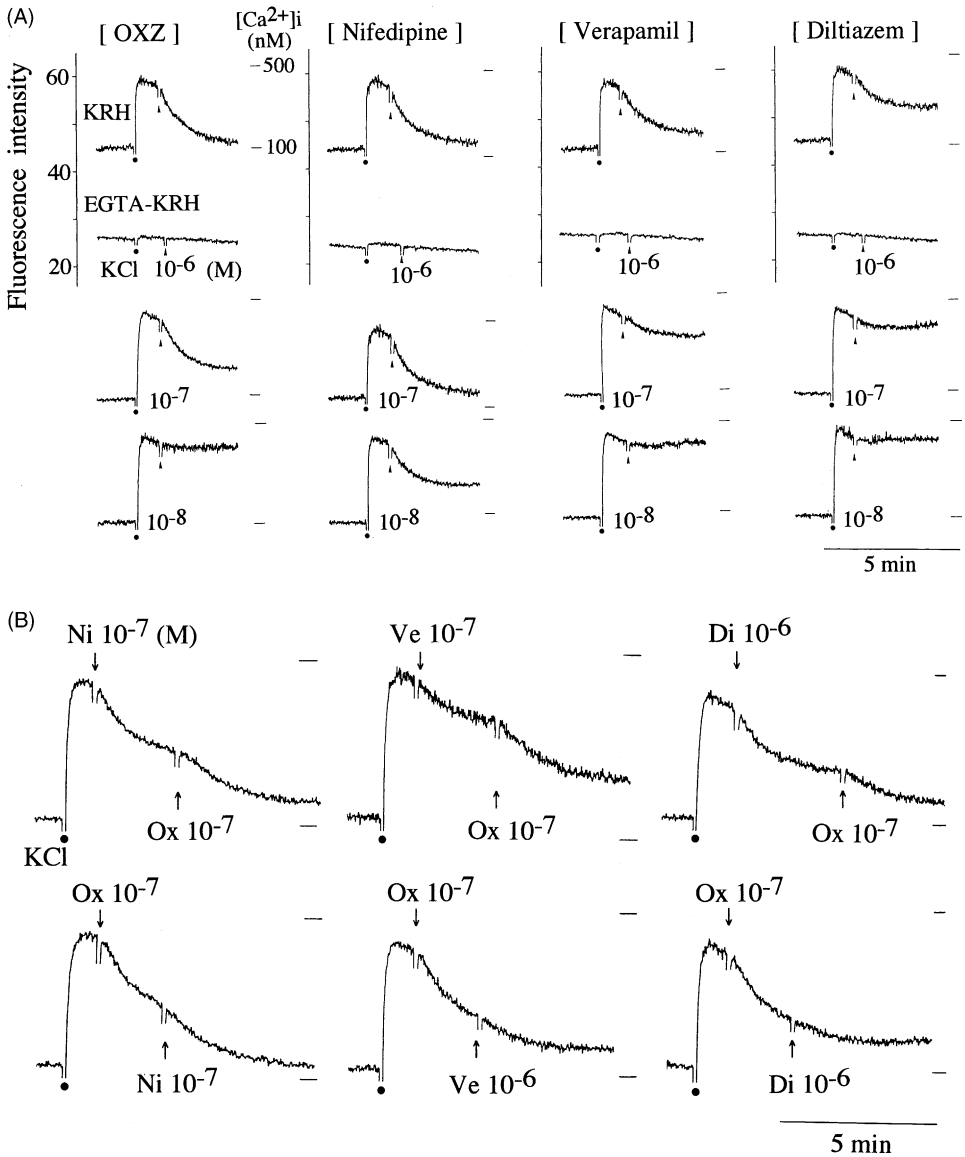

Fig. 6. Comparison of the effects of OXZ and some Ca^{2+} channel blockers on the increased $[Ca^{2+}]_i$ levels in K^+-depolarized PC12 cells. (A) Comparison of the potency of each drug. The experimental procedure was the same as that shown in Fig. 4, except that the drugs (arrowheads) were added after the addition of 50 mM KCl (closed circles). At the highest drug concentration (10^{-6} M), changes in fluorescence intensity in EGTA–KRH were examined simultaneously. Representative recordings from 3 to 5 experiments are shown. (B) Additive effects of OXZ and Ca^{2+} channel blockers. Data were selected from the experiments with various combinations of drug concentrations. Ox, OXZ; Ni, nifedipine; Ve, verapamil; and Di, diltiazem.

procaine, with the $[Ca^{2+}]_i$ increase being inhibited by bupivacaine. These observations suggested that blocking of L-type Ca^{2+} channels may be a general characteristic of most LAs that differ only in their potency.

Third, the inhibition of Ca^{2+} channels by OXZ may occur at anesthetic doses. OXZ is clinically applied only orally to relieve stomach pain [3], in a dose of, for example, 15–40 mg/day, divided into 3–4 administrations, but its effective local concentration at the site of action is unknown. In experimental animals, OXZ has highly potent LA activity, e.g. OXZ concentrations that cause anesthesia in the rabbit cornea are 0.0005% (11 μM) by topical application and 0.0001% by infiltration, the potency being 4000 and 5000 times those of procaine, 500 and 2500 times those of cocaine, and 8 and 10 times those of dibucaine, respectively [1]. Therefore, OXZ concentrations below 1 μM, that inhibited the Ca^{2+} channels in the present study, may not be far from the anesthetic concentrations.

LAs affect various membrane functions at their anesthetic concentrations other than the blockade of Na^+ channels, e.g. inhibition of K^+ or Ca^{2+} currents [25–27], nitrendipine binding to brain and cardiac membranes [28], binding of agonists to the receptors [29], signal transducing systems [30], and membrane enzymes [31,32]. LAs also interfere with the nicotinic acetylcholine receptors [33–35]. Since both nicotinic and muscarinic receptor agonists reportedly induce catecholamine release accompanied by an increase in $[Ca^{2+}]_i$ in PC12 cells

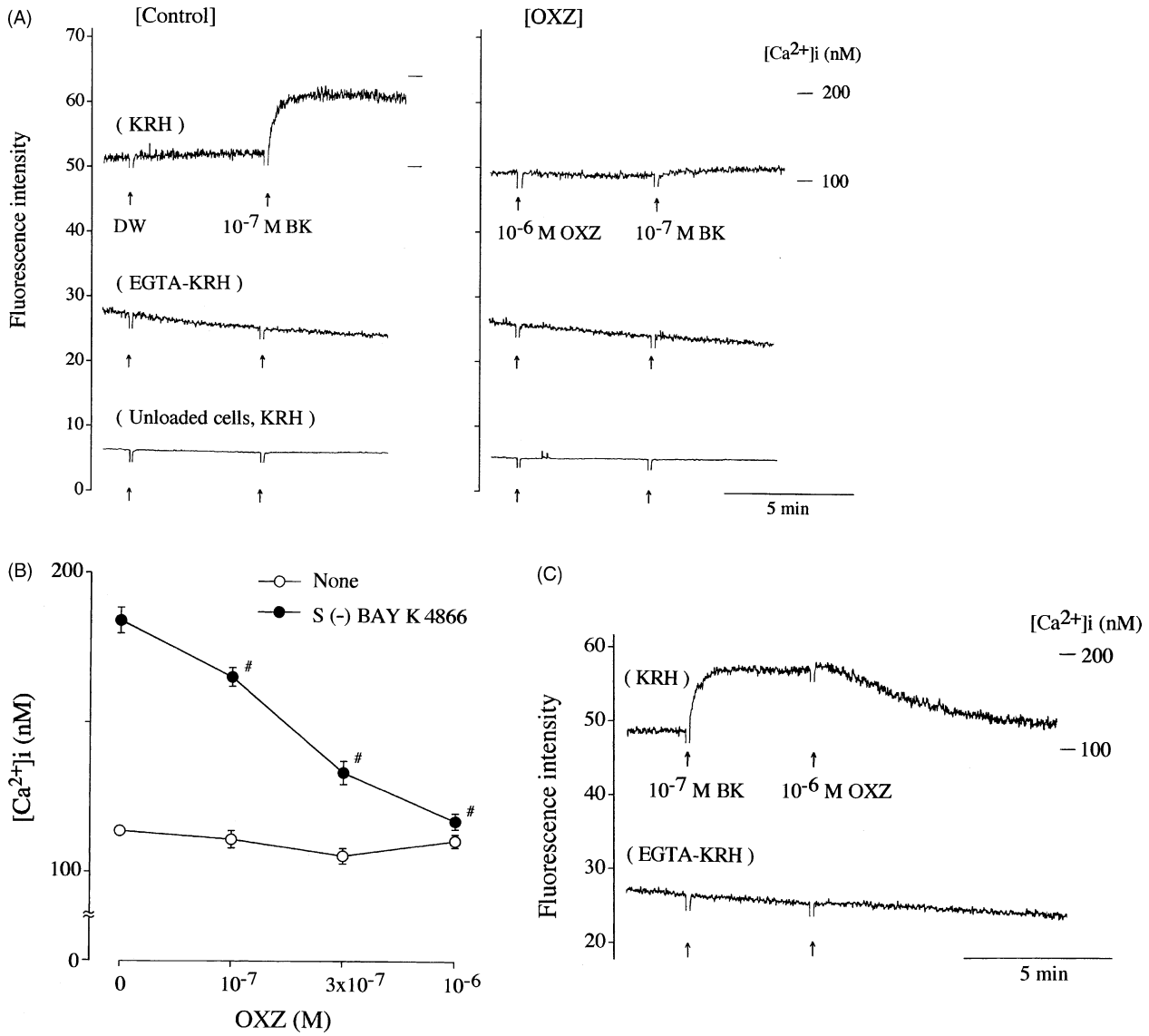

Fig. 7. Effects of OXZ on S(−)-BAY K 8644-induced increase in $[Ca^{2+}]_i$ in PC12 cells. The experimental procedure was the same as that shown in Fig. 4A, except that quin2-loaded PC12 cells were stimulated with 10^{-7} M S(−)-BAY K 8644 (BK) instead of KCl. (A) Representative recordings from control (left panel) and OXZ-pretreatment (right panel) experiments. BK was added to the quin2-loaded cells suspended in KRH (top) or in EGTA–KRH (middle), or to the unloaded cells in KRH (bottom). (B) Concentration-dependent effects of OXZ on the BK-induced increase in $[Ca^{2+}]_i$. Open and closed symbols indicate $[Ca^{2+}]_i$ levels before and after the addition of 10^{-7} M BK, respectively (means ± SEM, N = 4–5). Key: (#) significantly different from the control ($P < 0.01$). (C) Effects of OXZ post-treatment on the elevated $[Ca^{2+}]_i$ levels induced by 10^{-7} M BK. Typical results from 3 similar observations are shown.

[36,37], we attempted to test the effect of OXZ on this receptor-operated system. However, nicotine, methacholine, carbamylcholine, anabasin, 1,1-dimethyl-4-phenylpiperazinium, and muscarine at concentrations up to 10^{-4}–10^{-3} M produced no or only a slight increase in $[Ca^{2+}]_i$. Thus, the effects of OXZ on this receptor system still remain to be examined. For this purpose, other methodology or more sensitive subclones of PC12 cells may be necessary.

These non-specific actions of LAs are thought to contribute to spinal and epidural anesthesia as well as the toxic side-effects on the brain and heart [6]. The present findings in PC12 cells suggest an additive mechanistic basis for the potent local anesthetic and toxic actions of OXZ. For example, the circulatory disruption induced by toxic intravenous doses of OXZ in experimental animals [2] may be potentiated by blockade of the L-type Ca^{2+} channels located in the heart and peripheral vascular system. Since $[Ca^{2+}]_i$ controls various cellular activities, the spectrum of actions of OXZ would be much broader. In addition, the $[Ca^{2+}]_i$-increasing action of OXZ, manifested by concentrations just above those exhibiting Ca^{2+} channel blockade, may further complicate the actions of OXZ. Clinically, however, oral administration of OXZ is considered relatively safe [3].

Finally, the Ca^{2+}-dependency of OXZ action may be due to its unique chemical structure. As shown in Fig. 8, the OXZ molecule consists of adrenergic (mephentermine)

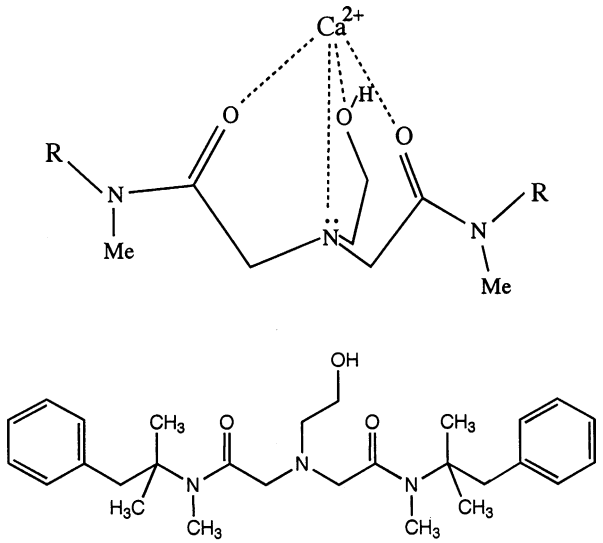

Fig. 8. Chemical structure of OXZ and a possible configuration for interaction with Ca^{2+} (from [8]).

and cholinergic (ethanolamine) components joined via an amide linkage [1]. Sterically, however, OXZ is an amphipathic molecule with a hydrophilic center, composed of three dissociable oxygen atoms branched from one electron-rich nitrogen atom, and two highly hydrophobic side-chains. Such a distinct steric structure cannot be assigned to other LAs. Furthermore, replacement of the alcoholic side-chain with propyl or methyl alcohols has been reported to cause loss of the local anesthetic activity [1]. Accordingly, three symmetrically positioned oxygen atoms appear to provide OXZ with the potential to coordinate with Ca^{2+}, which may lead to hydrophilic pore formation, at high concentrations, within the plasma membrane, while at low concentrations OXZ may interact with channel proteins via hydrophobic and electrostatic binding to block the channel function.

OXZ binding characteristics to Ca^{2+} channels are yet to be elucidated as are the electrophysiological actions of OXZ on Na^+ channels. Further studies are necessary to clarify the mechanism(s) of action of OXZ.

In conclusion, OXZ exhibited biphasic effects on Ca^{2+} movement in PC12 cells. At high concentrations, OXZ acted as a Ca^{2+} ionophore to increase $[Ca^{2+}]_i$, finally leading to cell death. On the other hand, at low concentrations, OXZ inhibited both DA release and the increase in $[Ca^{2+}]_i$ in K^+-depolarized cells. The inhibitory potency against the $[Ca^{2+}]_i$ increase was comparable to that of Ca^{2+} channel blockers, and the inhibitory actions of OXZ and the blockers appeared to be additive. OXZ also had an antagonistic effect on the action of S(−)-BAY K 8644. These observations suggested that OXZ interacts with L-type Ca^{2+} channels. Such mechanisms may participate in and further complicate the understanding of the potent local anesthetic and toxic actions of OXZ. A unique steric structure of the OXZ molecule may be responsible for the disturbance of Ca^{2+} movement.

References

[1] Seifter J, Glassman JM, Hudyma GM. Oxethazaine and related congeners: a series of highly potent local anesthetics. Proc Soc Exp Biol Med 1962;109:664–8.
[2] Glassman JM, Dervinis A, Beckfield WJ, Seifter J. Acute and chronic toxicity of oxethazaine: a highly potent local anesthetic. Toxicol Appl Pharmacol 1963;5:184–200.
[3] Farrar Jr GE, Seifter J. Gastrointestinal dysfunction modified by a topical anesthetic (oxethazaine). Penn Med J 1962;65:1369–72.
[4] Posey Jr EL, Boler K, Posey III L. Inhibition of food-stimulated gastrin release by a topical anesthetic, oxethazaine. Am J Dig Dis 1969;14:797–800.
[5] Posey Jr EL, Boler K, Posey III L. Inhibition of acetylcholine-stimulated gastrin release by the topical anesthetic, oxethazaine. Am J Gastroenterol 1971;55:54–7.
[6] Butterworth IV JF, Strichartz GR. Molecular mechanisms of local anesthesia: a review. Anesthesiology 1990;72:711–34.
[7] Masuda Y, Yoshizawa T, Ozaki M, Tanaka T. The metabolic and hemodynamic effects of oxethazaine in the perfused rat liver. Jpn J Pharmacol 1996;70:243–52.
[8] Masuda Y, Ozaki M, Oguma T. Alteration of hepatic microcirculation by oxethazaine and some vasoconstrictors in the perfused rat liver. Biochem Pharmacol 1997;53:1779–87.
[9] Yasuno R, Oguma T, Masuda Y. Ca^{2+} enhancement of hemolysis induced by the topical anesthetic oxethazaine *in vitro*. Biol Pharm Bull 1998;21:1294–9.
[10] Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 1976;73:2424–8.
[11] Greene LA, Rein G. Release, storage and uptake of catecholamines by a clonal cell line of nerve growth factor (NGF) responsive pheochromocytoma cells. Brain Res 1977;129:247–63.
[12] Greene LA, Tischler AS. PC12 pheochromocytoma cultures in neurobiological research. Adv Cell Neurobiol 1982;3:373–414.
[13] Shafer TJ, Atchison WD. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. Neurotoxicology 1991;12:473–92.
[14] Isom GE, Borowitz JL. PC12 cells. In: Tyson CA, Frazier JM, editors. Methods in toxicology, vol. 1A. San Diego: Academic Press, 1993. p. 82–93.
[15] Welder AA, Acosta D. Enzyme leakage as an indicator of cytotoxicity in cultured cells. In: Tyson CA, Frazier JM, editors. Methods in toxicology, vol. 1B. San Diego: Academic Press, 1994. p. 46–9.
[16] Tsien R, Pozzan T. Measurement of cytosolic free Ca^{2+} with quin2. Meth Enzymol 1989;172:230–62.
[17] Thomas A. Cell function studies using fluorescent Ca^{2+} indicators. In: Tyson CA, Frazier JM, editors. Methods in toxicology, vol. 1B. San Diego: Academic Press, 1994. p. 287–314.
[18] Capponi AM, Lew PD, Schlegel W, Pozzan T. Use of intracellular calcium and membrane potential fluorescent indicators in neuroendocrine cells. Meth Enzymol 1986;124:116–35.
[19] Hille B. Ionic channels of excitable membranes. 2nd ed. Massachusetts: Sinauer Associates, 1992. p. 83–114.
[20] Chalfie M, Perlman RL. Studies of a transplantable rat pheochromocytoma: biochemical characterization and catecholamine secretion. J Pharmacol Exp Ther 1976;197:615–22.
[21] Virgilio FD, Milani D, Leon A, Meldolesi J, Pozzan T. Voltage-dependent activation and inactivation of calcium channels in PC12 cells: correlation with neurotransmitter release. J Biol Chem 1987;262:9189–95.
[22] Ferrante J, Luchowski E, Rutledge A, Triggle DJ. Binding of a 1,4-dihydropyridine calcium channel activator, S(−)-BAY K 8644, to cardiac preparations. Biochem Biophys Res Commun 1989;158:149–54.
[23] Nicotera P, Bellomo G, Orrenius S. Calcium-mediated mechanism in chemically induced cell death. Ann Rev Pharmacol Toxicol 1992;32:449–70.

[24] Gregus Z, Klaassen CD. Mechanism of toxicity. In: Klaassen CD, editor. Casarett and Doull's toxicology: the basic science of poisons. New York: McGraw-Hill, 1996. p. 35–74.
[25] Palade PT, Almers W. Slow calcium and potassium currents in frog skeletal muscle: their relationship and pharmacological properties. Pflügers Arch 1985;405:91–101.
[26] Carmeliet E, Morad M, Van der Heyden G, Vereecke J. Electrophysiologial effects of tetracaine in single guinea-pig ventricular myocytes. J Physiol (London) 1986;376:143–61.
[27] Josephson IR. Lidocaine blocks Na, Ca and K currents of chick ventricular myocytes. J Mol Cell Cardiol 1988;20:593–604.
[28] Bolger GT, Marcus KA, Daly JW, Skolnick P. Local anesthetics differentiate dihydropyridine calcium antagonist binding sites in rat brain and cardiac membranes. J Pharmacol Exp Ther 1986;240:922–30.
[29] Hisayama T, Takayanagi I, Kumagai N, Kubo H. Interaction of 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride, ryanodine and procaine with muscarinic cholinergic M_2 receptor sites in smooth muscle. J Pharmacol Exp Ther 1989;249:646–51.
[30] Tan Z, Dohi S, Ohguchi K, Nakashima S, Nozawa Y. Local anesthetics inhibit muscarinic receptor-mediated activation of extracellular signal-regulated kinases in rat pheochromocytoma PC12 cells. Anesthesiology 1999;91:1014–24.
[31] Henn FA, Sperelakis N. Stimulative and protective action of Sr^{2+} and Ba^{2+} on (Na^{+}–K^{+})-ATPase from cultured heart cells. Biochim Biophys Acta 1968;163:415–7.
[32] Gordon LM, Dipple ID, Sauerheber RD, Esgate JA, Houslay MD. The selective effects of charged local anaesthetics on the glucagon- and fluoride-stimulated adenylate cyclase activity of rat liver plasma membranes. J Supramol Struct 1980;14:21–32.
[33] Ryan SE, Baenziger JE. A structure-based approach to nicotinic receptor pharmacology. Mol Pharmacol 1999;55:348–55.
[34] Gentry CL, Lukas RJ. Local anesthetics non-competitively inhibit function of four distinct nicotinic acetylcholine receptor subtypes. J Pharmacol Exp Ther 2001;299:1038–48.
[35] Papke RL, Horenstein BA, Placzek AN. Inhibition of wild-type and mutant neuronal nicotinic acetylcholine receptors by local anesthetics. Mol Pharmacol 2001;60:1365–74.
[36] Rabe CS, Delorme E, Weight FF. Muscarine-stimulated neurotransmitter release from PC12 cells. J Pharmacol Exp Ther 1987;243:534–41.
[37] Taylor SC, Peers C. Three distinct Ca^{2+} influx pathways couple acetylcholine receptor activation to catecholamine secretion from PC12 cells. J Neurochem 2000;75:1583–9.