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DIFFERENT EFFECTS OF LOCAL ANESTHETICS ON CALCIUM INFLUX INTO RAT MAST CELLS

KAZUHIKO OHISHI, TAMIKO SUZUKI* and MASAATSU K. UCHIDA
Department of Molecular Pharmacology, Meiji College of Pharmacy, 1-35-23, Nozawa, Setagaya-ku, Tokyo 154, Japan

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Abstract—1. Lidocaine, W49091, procaine and benzocaine inhibited mast cell secretion induced by concanavalin A and A23187.

2. They inhibited Ca influx stimulated by concanavalin A, suggesting that they inactivate Ca channel of mast cells.

3. Lidocaine and procaine inhibited Ca influx stimulated by A23187, but W49091 and benzocaine did not.

INTRODUCTION

Local anesthetics induce conformational change of the sodium channel to block nerve conduction (Seeman, 1972). They also inhibit mast cell secretion (Kazimierczak *et al.*, 1976), but it is uncertain whether they inhibit calcium influx in mast cells by altering the calcium channel as they alter the sodium channel in nerve membrane. We reported previously that lidocaine and mepivacaine inhibit mast cell secretion and that their inhibitory effects increase with increase in pH, indicating that it is as nonionized molecules that they inhibit the secretion and penetrate the mast cell membrane (Suzuki *et al.*, 1984).

There are three types of local anesthetics; tertiary amines, which are present in both ionized and nonionized forms at a physiological pH, neutral local anesthetics, which are always nonionized, and ionized local anesthetics. To examine the interaction between these drugs and the calcium channel, we compared the effects of these local anesthetics on mast cells stimulated by concanavalin A and A23187.

MATERIALS AND METHODS

Isolation of rat mast cells

Purified mast cells were prepared from male Sprague-Dawley rats in Hepes-buffered Tyrode solution as described previously (Suzuki *et al.*, 1982). The purity of the cells in the final preparation was more than 90% and their viability, measured by Trypan blue exclusion, was more than 95%.

Incorporation of ^{14}C-serotonin into rat mast cells

^{14}C-Serotonin was incorporated into rat mast cells by the method of Stechschulte and Austen (1974) with a minor modification. In practice, 1 ml of suspension of purified mast cells (10^6 cells/ml) in Hepes-buffered Tyrode solution containing 0.03% bovine serum albumin, pH 7.4, was incubated with 1 μCi of ^{14}C-serotonin for 1 hr at 37°C, and then the cells were washed three times.

Assay of mediator release

Mediators were released from mast cells by treatment with concanavalin A (20 μg/ml) and phosphatidylserine (50 μg/ml) or with A23187 (10 μM) as described previously (Suzuki *et al.*, 1982, 1983). After incubation, the assay mixture was centrifuged at 2100 *g* for 10 min at 4°C, and mediator in the supernatant was determined as released mediator. Histamine was determined by the method of Shore *et al.* (1959). The radioactivity of ^{14}C-serotonin was measured in a scintillation spectrometer (Packard 4530). Mediator release is expressed as a percentage of the total cell content. Spontaneous release occurring in the absence of a releaser was less than 10%. Cells were pretreated with a test drug for 10 min before adding a releaser. Since A23187 and benzocaine have strong fluorescence and interfere with the determination of histamine, they were removed by a single extraction with diethylether as described previously (Suzuki *et al.*, 1983) before measurement of histamine. Procaine also interferes with histamine determination, but it is not extractable with diethylether. We, therefore, measured the effect of procaine on ^{14}C-serotonin release from mast cells, since procaine did not quench the fluorescence of scintillator containing ^{14}C-serotonin.

Measurement of ^{45}Ca uptake

^{45}Ca uptake was measured in duplicate samples as reported previously (Suzuki and Uchida, 1984). A volume of 100 μl of silicone oil was placed in the bottom of a microfuge tube (capacity 400 μl), and 40 μl of test drug solution in Hepes-buffered Tyrode solution containing ^{45}Ca (0.2 μCi) and 50 μl of cell suspension (2×10^5 cells) were layered on top of the silicone oil. The tubes were equilibrated at 37°C for 10 min, and then 10 μl of releaser solution was added to give final concentrations of 20 μg/ml of concanavalin A with 50 μg/ml of phosphatidylserine or 10 μM A23187. The tubes were incubated further for 10 min and then centrifuged (10,000 *g*, 1 min, 20°C) in a Beckman Microfuge to terminate the reaction. The tubes were placed in a dry ice/acetone bath and the bottom layer containing the cell pellet was cut off. The pellet was dissolved in 1 ml of Triton X-100 (10% w/v) and left overnight at room temperature. The radioactivity in 0.8 ml of the sample was measured in a scintillation spectrometer (Packard 4530).

Lactate dehydrogenase activity

A sample of 150 μl of Hepes-buffered Tyrode solution containing a test drug was placed in a microfuge tube (capacity 1.8 ml). Cell suspension (10^5 cells/50 μl) was added with thorough mixing. The tubes were incubated at 37°C for 30 min and then centrifuged at 1500 *g* for 5 min at 4°C, and lactate dehydrogenase activity in the supernatant was assayed by the method of Lowry *et al.* (1957). A mixture of

*Author to whom all correspondence should be addressed.

100 μl of sample, 5 mg of β-NADH and 0.25 mg of pyruvate in 0.1 M potassium phosphate buffer (pH 7.0) was incubated for 30 min at room temperature and then 20 μl of 2 N HCl was added to destroy excess NADH. Then 200 μl of 12 N NaOH was added and the mixture was allowed to stand for 1 hr at room temperature. After addition of 3 ml of water, the fluorescence was measured in a fluorometer (Hitachi MPF-4). Lactate dehydrogenase activity was assayed in duplicate samples. After treatment with Triton X-100 (1% w/v) for 30 min, the total activity of lactate dehydrogenase was determined. Lactate dehydrogenase activity released in the presence of a test drug was expressed as a percentage of the total activity.

Preparation of calcium-loaded liposomes

A lipid mixture composed of egg yolk phosphatidylcholine, dicetylphosphate and cholesterol (molar ratio, 10:1:0 or 10:1:10) in chloroform was dried in a small test tube in a rotary evaporator and the resulting lipid film was kept under reduced pressure for at least 1 hr to allow complete evaporation of the solvent. The dried lipids were then dispersed in 98 mM $CaCl_2$ containing 6 mM Hepes (pH 7.4) in a Vortex mixer. Then the liposomes were dialysed against 147 mM NaCl containing 6 mM Hepes (pH 7.4) for 1 hr to remove the untrapped calcium marker. The final liposome concentration was 10 mM as phosphatidylcholine. The background level of calcium was less than 6×10^{-5} M.

Calcium flux across liposomal membranes

Because the lipid bilayer prevents calcium flux across them, calcium encapsulated in liposomes was not released in the absence of A23187. When A23187 was added, it carried calcium from the inside to the outside of the liposomal membranes according to the gradient of calcium ion concentration. Calcium flux induced by A23187 from the liposomes in the presence of a test drug was measured at 20°C. Since benzocaine is poorly soluble in the medium, it was added as a solution in chloroform before evaporation of the solvent to obtain a lipid film containing benzocaine. The concentration of calcium was determined with a research pH meter equipped with a calcium electrode (F2110Ca) (Radiometer, Copenhagen, Denmark). With this electrode, a linear Nernstian curve was obtained at concentrations more than 3×10^{-5} M calcium.

Chemicals

Lidocaine was a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan). W49091 was from Astra Pharmaceutical Products (Worcester, Mass, U.S.A.). Benzocaine was purchased from Tokyo Chemical Industry (Tokyo, Japan). Concanavalin A, bovine brain phosphatidylserine, dicetylphosphate, cholesterol and procaine were from Sigma Chemical Co. (St Louis, Mo., U.S.A.). A23187 was from Calbiochem-Behring (La Jolla, Calif., U.S.A.). ^{14}C-Serotonin (^{14}C-5-hydroxytryptamine creatinine sulfate) and ^{45}Ca were from Amersham Int. (Bucks, U.K.) and New England Nuclear (Boston, Mass, U.S.A), respectively. Lactate dehydrogenase from pig heart (EC 1.1.1.27), pyruvate, β-NADH and *o*-phthalaldehyde were from Wako Pure Chemicals (Osaka, Japan). Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was purchased from Dojin Kagaku (Kumamoto, Japan). Egg yolk phosphatidylcholine was prepared as described previously (Suzuki *et al.*, 1984). Silicone oil (TSF440) was provided from Toshiba Silicone Co. (Tokyo, Japan). A23187 and benzocaine were dissolved in dimethylsulfoxide as stock solutions and diluted with Hepes-buffered Tyrode solution before use. The final concentration of dimethylsulfoxide during incubation was never more than 0.4% (v/v) and preliminary experiments showed that at this concentration it did not influence cell responses. Hepes-buffered Tyrode solution had the following composition: 137 mM NaCl, 2.7 mM KCl, 12 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM dextrose and 0.03% bovine serum albumin (pH 7.4).

Statistical analysis

Statistical significance was evaluated by the unpaired Student's *t*-test and $P = 0.05$ was taken as the upper limit of significance.

RESULTS

Effects of lidocaine and W49091 on histamine release

The effects of lidocaine on histamine release induced by concanavalin A and A23187 are biphasic (Fig. 1). Lidocaine (0.6–10 mM) caused dose-dependent inhibition of histamine release induced by concanavalin A and its concentration for 50% inhibition (IC_{50}) was 6 mM. The IC_{50} for A23187-induced histamine release was 10 mM, at which concentration lidocaine inhibited histamine release induced by concanavalin A almost completely. At higher concentrations (30–100 mM), lidocaine did not cause more inhibition, but released histamine. Lidocaine (30 mM) also released lactate dehydrogenase, a cytoplasmic enzyme, in the absence of a releaser, its release increasing with increase in the concentration of lidocaine. This effect of lidocaine may be due to its cytotoxic effect.

The effect of W49091, which is an ionized form of lidocaine, on mast cells was also examined and compared with that of lidocaine. Figure 2 shows that 1–3 mM W49091 did not inhibit histamine release induced by A23187 and scarcely affected that induced by concanavalin A, but that at higher concentrations (10–100 mM) W49091 inhibited histamine release. The IC_{50} values for concanavalin A- and A23187-induced histamine release were 23 mM and 70 mM, respectively. These values were much larger than those of lidocaine.

Effects of procaine and benzocaine on histamine release

The effects of procaine and the related compound benzocaine, which is always in a nonionized form, on histamine release and ^{14}C-serotonin release were examined. Since procaine interferes with the fluorometric assay of histamine, we examined its effect of degranulation by measuring ^{14}C-serotonin release. Our preliminary data showed that the release of

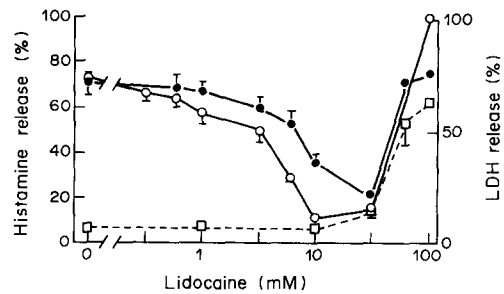

Fig. 1. Effects of lidocaine on histamine release induced by concanavalin A (○) and A23187 (●). Lactate dehydrogenase (LDH) release in the presence of lidocaine, but absence of releaser (□) was expressed as a percentage of the total activity. Values are means ± SE for 3–4 duplicated experiments. When no vertical bar is shown the SE was less than 2%.

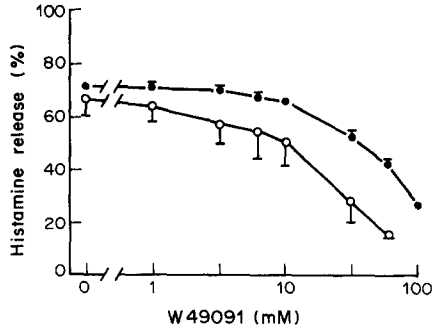

Fig. 2. Effects of W49091 on histamine release induced by concanavalin A (○) and A23187 (●). Values are means ± SE for 2 duplicated experiments. When no vertical bar is shown the SE was less than 2%.

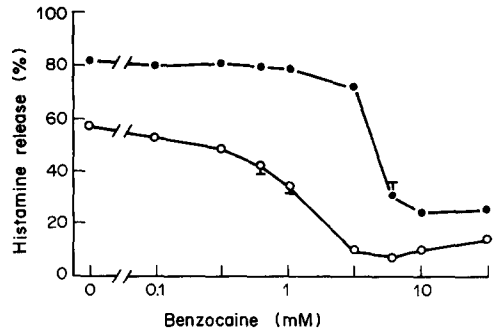

Fig. 4. Effects of benzocaine on histamine release induced by concanavalin A (○) and A23187 (●). Values are means ± SE for 3 duplicated experiments. When no vertical bar is shown the SE was less than 2%.

serotonin was less than that of histamine, but that the two releases are parallel (data not shown). Procaine (0.3–10 mM) inhibited ^{14}C-serotonin release from mast cells induced by concanavalin A (Fig. 3). It is interesting that 10 mM procaine caused 55.0% inhibition of the release induced by concanavalin A, but it did not cause complete inhibition, because at this concentration 22% of ^{14}C-serotonin incorporated into mast cells was released in the absence of a releaser. On the contrary, benzocaine effectively inhibited histamine release and its IC_{50} values for the releases induced by concanavalin A and A23187 were 1.2 mM and 4.8 mM, respectively (Fig. 4).

Effects of local anesthetics on calcium influx into mast cells

We measured calcium influx stimulated by concanavalin A and A23187 in the presence of local anesthetics and compared their effects on calcium influx with their inhibitory effects on mediator release from mast cells. The background binding of calcium to cells in the absence of releaser was 3325 ± 126 cpm/10^6 cells. The ^{45}Ca uptakes stimulated by concanavalin A and A23187 corrected for background binding were 24178 ± 1576 cpm/10^6 cells and 30250 ± 2312 cpm/10^6 cells (means ± SE), respectively.

Lidocaine (1–30 mM) inhibited ^{45}Ca uptake stimulated by concanavalin A and A23187 (Table 1). At 10 mM, ^{45}Ca uptakes stimulated by concanavalin A and A23187 were 29 and 74%, and histamine release % of control release in the absence of lidocaine were 15 and 49%, respectively. W49091 also inhibited ^{45}Ca uptake stimulated by concanavalin A, whereas it did not inhibit ^{45}Ca uptake stimulated by A23187. Like lidocaine, procaine inhibited the ^{45}Ca uptakes stimulated by concanavalin A and A23187 (Table 2). Its inhibitory effect seemed to be less than that of lidocaine; 30 mM procaine enhanced spontaneous release of serotonin, but caused 52% inhibition of A23187-induced serotonin release, because ^{45}Ca uptake was decreased up to 51%. Benzocaine also inhibited ^{45}Ca uptake stimulated by concanavalin A. Benzocaine enhanced rather than inhibited ^{45}Ca uptake stimulated by A23187, but it inhibited histamine

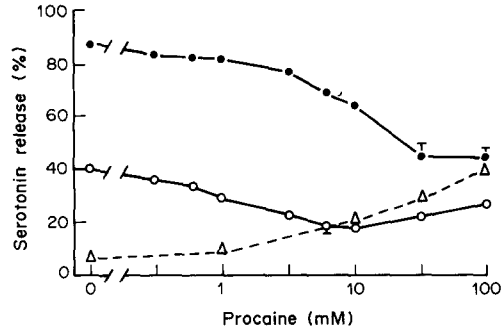

Fig. 3. Effects of procaine on mediator release induced by concanavalin A (○) and A23187 (●). Open triangles show spontaneous mediator release in the absence of releaser. Values are means ± SE for 3 duplicated experiments. When no vertical bar is shown the SE was less than 2%.

Table 1. Comparison of effects of lidocaine and W49091 on ^{45}Ca uptakes stimulated by concanavalin A and A23187

Drug	(mM)	^{45}Ca uptake stimulated by Concanavalin A (% of control)	A23187 (% of control)
Lidocaine	1	87.3 ± 8.8	—
	3	68.1 ± 9.8	106.0 ± 16.4
	10	29.4 ± 4.4	74.3 ± 11.2
	30	—	34.7 ± 5.2
W49091	30	42.2 ± 7.3	96.3 ± 9.2
	60	39.8 ± 6.4	102.8 ± 3.2
	100	—	104.6 ± 3.7

^{45}Ca uptake was corrected for background binding of calcium to cells in the absence of a releaser. Values are means ± SE for 3–4 experiments.

Table 2. Comparison of effects of procaine and benzocaine on ^{45}Ca uptakes stimulated by concanavalin A and A23187

Drug	(mM)	^{45}Ca uptake stimulated by Concanavalin A (% of control)	A23187 (% of control)
Procaine	1	87.9 ± 8.4	—
	3	66.7 ± 6.9	108.5 ± 11.4
	10	31.0 ± 3.8	111.0 ± 13.2
	30	17.8 ± 5.2	51.0 ± 8.8
Benzocaine	0.3	95.1 ± 11.3	—
	1	80.1 ± 12.7	—
	3	54.9 ± 8.8	151.2 ± 8.9
	6	—	163.9 ± 14.0
	10	—	182.9 ± 12.7

^{45}Ca uptake was corrected for background binding of calcium to cells in the absence of a releaser. Values are means ± SE for 3 experiments.

release induced by A23187. These results indicate that local anesthetics inhibit ^{45}Ca uptake stimulated by concanavalin A due to an effect on calcium influx mechanisms activated by concanavalin A. However, they seemed to interact in a quite different manner with mast cells treated with A23187. Tertiary amines (lidocaine and procaine) reduced ^{45}Ca uptake, whereas an ionized local anesthetic (W49091) and a nonionized local anesthetic (benzocaine) rather enhanced calcium uptake. Since A23187 caused entrance of calcium into cells directly, the calcium influx mechanisms activated by concanavalin A are not related to the action site of this drug. Then, we examined the ionophore activity of A23187 by measuring the leakage of calcium from calcium-loaded liposomes in its presence. A23187 (30 μM) released $61.3 \pm 3.8\%$ and $83.5 \pm 1.7\%$ of the total calcium incorporated into liposomes composed of egg yolk phosphatidylcholine, dicetylphosphate and cholesterol at molar ratios of 10:1:10 and 10:1:0, respectively. Lidocaine enhanced calcium leakage from these liposomes, but benzocaine had no effect (data not shown). It is likely that calcium influx caused by A23187 is inhibited only secondarily by tertiary amine local anesthetics that penetrate cytosol, because these drugs do not decrease the ionophore activity of A23187.

DISCUSSION

Previously our group examined the effect of lidocaine on rat mast cells and its pH dependence (Suzuki *et al.*, 1984). Lidocaine inhibits histamine release from mast cells and it seems that this effect is due to the nonionized form of lidocaine. We, therefore, compared the effect of ionized and nonionized local anesthetics with that of lidocaine. Procaine, another tertiary amine, also inhibited mediator release from mast cells induced by concanavalin A or A23187. Lidocaine and procaine inhibited mediator release induced by concanavalin A more effectively than that by A23187. This difference may be due to a difference in the amounts of mediator released by the two releasers and to the fact that A23187 was somewhat cytotoxic (Siraganian *et al.*, 1975). The effect of W49091 was much less than that of lidocaine, since ionized molecules hardly penetrate the membrane. Its potency in blocking nerve conduction, however, may be the same as that of lidocaine, when it is injected into the axon (Frazier *et al.*, 1971; Narahashi *et al.*, 1971). Benzocaine also inhibited histamine release induced by concanavalin A and A23187. Since concanavalin A, like antigens or anti-IgE receptors, dimerizes IgE receptors on the surface membrane (Foreman, 1980), calcium influx activated by concanavalin A should occur *via* the innate calcium channel of mast cells. Calcium influx mechanisms activated by concanavalin A were inhibited by all the local anesthetics tested, indicating that these drugs can inactivate the innate calcium channel of mast cells. The effects of local anesthetics on mast cells can be explained in part by their calcium channel blocking actions; for instance D-600, a calcium channel antagonist, inhibited histamine release from rat mast cells by preventing calcium influx (Suzuki *et al.*, 1982). But, their inhibitory actions on histamine release induced by A23187 cannot be explained by their effects on calcium influx. Local anesthetics enhanced calcium efflux across the liposomal membrane by A23187 rather than inhibiting it. Moreover, they did not affect the u.v. spectrum of A23187, indicating that they do not change the conformation of A23187, as described by Fewtrell and Gomperts (1977). For instance, W49091 and benzocaine inhibit histamine release after sufficient calcium influx to trigger histamine release. Thus W49091 and benzocaine may block steps at which intracellular calcium triggers histamine release. Since local anesthetics inhibit calmodulin (Tanaka and Hidaka, 1981; Volpi *et al.*, 1981) and calmodulin inhibitors inhibit histamine release at low concentration but enhance the release at high concentration (Douglas and Nemeth, 1982; Suzuki *et al.*, 1983), these local anesthetics may inhibit calmodulin and calmodulin-dependent processes thus blocking histamine release from mast cells. Furthermore, lidocaine inhibits assembly, and enhances disassembly of microtubules in rabbit brain (Haschke *et al.*, 1974). Cholchicine and vinblastin, inhibitors of microtubule assembly, also inhibit mediator release from mast cells (Lagunoff and Chi, 1980). Thus it is also possible that lidocaine and other local anesthetics inhibit microtubule assembly of mast cells.

SUMMARY

The effects of local anesthetics on anaphylactic mediator release and calcium influx were studied in rat mast cells. Lidocaine, W49091, procaine and benzocaine inhibited mediator release induced by concanavalin A, decreasing calcium flux stimulated by concanavalin A. The inhibitory effect of W49091, an ionized form of lidocaine, was much less than that of lidocaine, because it cannot be transferred across the surface membrane. Benzocaine, an analogue of procaine that is nonionizable and only slightly soluble in water, was effective. Lidocaine and procaine also inhibited mediator release and calcium influx induced by A23187. W49091 and benzocaine did not decrease the amount of mediator released by A23187. Moreover drugs like W49091 and benzocaine that cannot penetrate the cytosol did not inhibit calcium uptake by A23187. These four drugs may inactivate calcium channels of mast cells and thus inhibit mediator release induced by concanavalin A. But, it is likely that calcium influx caused by A23187 is inhibited only secondarily by tertiary amine local anesthetics that penetrate cytosol, because these drugs do not decrease the ionophore activity of A23187 across a lipid bilayer. Moreover it seems that W49091 and benzocaine inhibit mediator release when there is enough intracellular calcium to trigger mast cell secretion.

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