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Local Anesthetics: Comparison of Effects on Batrachotoxin-Elicited Sodium Flux and Phosphoinositide Breakdown in Guinea Pig Cerebral Cortical Synaptoneurosomes

YUKIO NISHIZAWA, FABIAN GUSOVSKY, and JOHN W. DALY

Laboratory of Bioorganic Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

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SUMMARY

Local anesthetics inhibited the sodium influx and the inositol phosphate accumulation elicited by the sodium channel activator batrachotoxin in guinea pig cortical synaptoneurosomes. Inhibitory effects of local anesthetics on sodium influx correlated with inhibitory effects on binding of a tritiated batrachotoxin analog to sodium channels in synaptoneurosomes. There was also a correlation between inhibitory effects on sodium influx and on inositol phosphate accumulation; most local anesthetics inhibited sodium influx at concentrations similar to those required for inhibition of inositol phosphate accumulation. Indeed, euprocin, bupivacaine, lidocaine, and certain analogs were nearly equipotent with respect to inhibition of sodium influx and inositol phosphate accumulation. Local anesthetics also inhibited inositol phosphate accumulation that was induced by carbamylcholine through both a tetrodotoxin-sensitive and a tetrodotoxin-insensitive pathway. Certain local anesthetics, such as dibucaine, inhibited the tetrodotoxin-sensitive pathway with higher potency than for the tetrodotoxin-insensitive pathway, while others, such as quinacrine, inhibited tetrodotoxin-sensitive and tetrodotoxin-insensitive pathways with equal potency. Diphenhydramine and chlorpromazine appeared to inhibit carbamylcholine-elicited phosphoinositide breakdown through blockade of muscarinic cholinergic receptors rather than because of local anesthetic activity or inhibitory effects on phospholipase C.

Local anesthetics are known to affect the functions of a variety of membrane proteins such as (Na^+-K^+)-ATPase (1, 2). (Ca^{2+}-Mg^{2+})-ATPase (3, 4), adenylate cyclase (5, 6), guanylate cyclase (7), calmodulin-sensitive enzymes (8, 9), phospholipase A_2 (10), and the channels responsible for increases in cellular permeability to Na^+, K^+, or Ca^{2+} ions (11–16). The effects of these agents on voltage-sensitive sodium channels have appeared to be fundamental to the local anesthetic activity on neurons (17). No effects of local anesthetics on phospholipase C appear to have been reported although quinacrine, an agent with potent local anesthetic activity, is known to inhibit both phospholipase A_2 and C (18). Local anesthetics inhibit not only stimulus-evoked opening of sodium channels, but also opening of channels elicited by agents, such as batrachotoxin. Such local anesthetic activity appears to be correlated with inhibitory effects of drugs on binding of a radioactive BTX analog to sites on the sodium channel (19, 20). Recently, it has been shown that agents that enhance sodium channel function, such as BTX, veratridine, pumiliotoxin B, and scorpion venom, can induce phosphoinositide breakdown in brain synaptoneurosomes (21, 22). TTX, a potent local anesthetic, selective for voltage-dependent sodium channels, was much more potent in blocking sodium flux elicited by BTX, veratridine, or scorpion venom in synaptoneurosomes than in blocking phosphoinositide breakdown, suggesting that a subpopulation of TTX insensitive sodium channels might be primarily involved in regulation of phospholipase C-catalyzed breakdown of phosphoinositides (23). It appeared that comparison of the effects of a series of local anesthetics on sodium flux and phosphoinositide breakdown by BTX might provide further insights into the nature and relationship of sodium channels and phospholipase C activation. Certain local anesthetics, unlike TTX, might prove more potent versus phosphoinositide turnover than versus sodium flux, indicative of selective actions either at a subpopulation of TTX-insensitive channels or at phospholipase C.

We report here the effects of a series of local anesthetics on sodium influx elicited by BTX into synaptoneurosomes and on the phosphoinositide breakdown elicited by BTX. In addition, the effects of local anesthetics on phosphoinositide breakdown induced by carbamylcholine were determined. Remarkably, carbamylcholine caused the stimulation of phosphoinositide breakdown both through apparent activation of sodium channels, which was inhibited by TTX and by the local anesthetics, and through a TTX-insensitive pathway, which was little affected by local anesthetics, except for those with high affinity

ABBREVIATIONS: BTX, batrachotoxin; TTX, tetrodotoxin; HEPES, 4-(2-hydroxymethyl)-1-piperazine ethanesulfonic acid.

for muscarinic receptors. Most local anesthetics, unlike TTX, had a similar potency versus BTX-elicited sodium flux and versus BTX-elicited phosphoinositide breakdown. None appeared to be selective toward the postulated subpopulation of TTX-insensitive sodium channels (23) or toward phospholipase C.

Experimental Procedures

Materials. BTX was isolated as reported previously (24). ^{22}NaCl (25 Ci/mmol) was from Amersham (Arlington Heights, IL) and [^{3}H] inositol (14–17 Ci/mmol), and [^{3}H]*N*-methylscopolamine chloride (80 Ci/mmol) from New England Nuclear (Boston, MA). Carbamylcholine and TTX were from Sigma Chemical Co. (St. Louis, MO). Anion exchange resin, AG1-X8 (formate form) was from Bio-Rad (Richmond, CA), and Hydrofluor, Betafluor, and Filtron X from National Diagnostics (Sommerville, NJ). Local anesthetics were obtained from the following sources: dibucaine, Ciba-Geigy Corp., Ardsley, NY; tetracaine, quinacrine, and diphenhydramine, Sigma; euprocin, Schering AG, Berlin; bupivacaine, Sterling-Winthrop Research Institute, Rensselaer, NY; dimethisoquin, Smith-Kline French Laboratories, Philadelphia, PA; piperocaine, Eli Lilly, and Co., Indianapolis, IN; prilocaine and etidocaine, Astra Pharmaceutical Products, Inc., Worcester, MA; cocaine, Merck Sharp and Dohme, West Point, PA; pyrilamine, K & K Laboratories, Plainview, NY; pheniramine, Mann Research Laboratories, Inc., New York, NY; promethazine, Wyeth Laboratories, Inc., Philadelphia, PA. Diphenhydramine methiodide was prepared by methylation of diphenhydramine (25). QX-572, QX-314, and lidocaine were provided by Dr. L.-Y. M. Huang, formerly of the National Institute of Mental Health, Bethesda, MD and chlorpromazine by Dr. A. A. Manian, formerly of the National Institute of Mental Health.

Synaptoneurosomes. Cerebral cortical synaptoneurosomes were prepared by the method of Hollingsworth *et al.* (26). Brain cortex of Hartley guinea pigs was homogenized in 7–10 volumes of Krebs-Henseleit buffer (pH 7.4) using a glass homogenizer. The homogenate was centrifuged at 1000 × *g* for 15 min. The resulting pellet was resuspended in an appropriate volume of buffer and used for assay.

[^{3}H]Batrachotoxinin A benzoate binding assay. Incubations were carried out with 50 nM [^{3}H]BTX A benzoate, 1 μM TTX, 0.03 mg of scorpion venom (*Leiurus*), and 400 μg of synaptoneurosome protein in a final volume of 250 μl of buffer, pH 7.4, containing 130 mM choline chloride, 50 mM HEPES buffer, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 5.4 mM KCl as described (19). After 30 min at 37°, incubations were terminated by dilution of the reaction mixture with 3 ml of wash buffer and filtration through Whatman GF/C filters. Filters were washed with three 3-ml portions of wash buffer and placed in scintillation vials. Hydrofluor (4 ml) was added, and radioactivity was determined by liquid scintillation counting (see Ref. 19 for further details).

Phosphoinositide breakdown. Phosphoinositide breakdown was measured as previously described (27). In brief, the synaptoneurosomes (about 50 mg of protein) were suspended in 14 ml of fresh Krebs-Henseleit buffer containing 200 μCi [^{3}H]inositol (1 μM). Aliquots of the synaptoneurosome suspension (300 μl) were distributed in 5-ml tubes and incubated at 37° for 60 min. After the incubation, LiCl (final concentration 10 mM) was added, and 10 min later BTX (1 μM) or carbamylcholine (2 mM) alone or with local anesthetics over a range of concentrations were added. The synaptoneurosome suspension was then incubated at 37° for 90 min. After the incubation, the suspension was centrifuged to remove free [^{3}H]inositol and the pellet was resuspended in 1 ml of Krebs-Henseleit buffer, followed by centrifugation. The resulting pellet was mixed with 1 ml of 6% trichloroacetic acid and centrifuged. The supernatant fraction was used for the determination of [^{3}H]inositol phosphate by anion exchange column chromatography (AG1-X8, formate form) as reported by Berridge *et al.* (28). The trichloroacetic acid supernatant was added to a column and the column was washed five times with 3 ml of distilled water to elute [^{3}H] inositol. [^{3}H]Inositol monophosphate was then eluted with 1 ml of 200 mM ammonium formate/100 mM formic acid and the radioactivity was measured by liquid scintillation spectroscopy after 7 ml of Hydrofluor were added. The trichloacetic acid precipitate was used for the measurement of incorporation of [^{3}H]inositol into lipids. The pellet was suspended in 0.5 ml of the mixture of 1 M KCl/10 mM inositol and methanol (1:1). Chloroform (0.5 ml) was added to the suspension and lipids were extracted by shaking for 5 min. An aliquot of the chloroform layer (0.2 ml) was transferred to a scintillation vial and evaporated to dryness. Radioactivity in lipid fraction was measured after 4 ml of Betafluor were added. The results were calculated as counts per minute of inositol phosphate/10,000 cpm of lipids (27). IC_{50} values for local anesthetics were determined from dose-dependent inhibition curves for one to three experiments (Table 1).

Sodium influx. The influx of $^{22}Na^+$ induced by BTX was measured by a method based on that of Tamkun and Catterall (29). Synaptoneurosomes were resuspended at a concentration of 1–2 mg/ml in a buffer containing 50 mM HEPES (pH 7.4 adjusted with 50 mM Tris), 130 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 1 mg/ml of bovine serum albumin. Aliquots of 100 μl were incubated with 1 μM BTX at 37° for 10 min in the presence or absence of local anesthetics in a range of concentrations. The $^{22}Na^+$ influx was initiated by adding 150 μl of influx buffer containing ^{22}NaCl (1.3 μCi/ml), 2.66 mM NaCl, 50 mM HEPES, 128 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 1 mg/ml of bovine serum albumin. Local anesthetics were present over a range of concentrations in the 150 μl of influx buffer. After 10 sec incubation at 37° the influx was terminated by adding 4 ml of an ice-cold wash buffer containing 5 mM HEPES, 163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 1 mg/ml of bovine serum albumin, and the synaptoneurosomes were collected on a Gelman GN-6 filter (0.45 μm pore size) and washed twice with 4 ml of ice-cold wash buffer. The filter was then solubilized in Filtron X and the radioactivity was measured by liquid scintillation counting. BTX-induced influx of ^{22}Na into the synaptoneurosomes through voltage-dependent sodium channels was obtained by subtraction of the influx in the presence of 5 μM TTX from that in the absence of TTX. IC_{50} values were determined from dose-dependent inhibition curves for one to three experiments (Table 1).

[^{3}H]*N*-Methylscopolamine binding assay. The binding of [^{3}H] *N*-methylscopolamine was determined as described (30). Synaptoneurosomes were suspended at a concentration of 50 μg of protein/ml in a buffer containing 20mM HEPES (pH 7.4 adjusted with 20 mM Tris), 100 mM NaCl, and 10 mM $MgCl_2$, and aliquots of 2 ml were preincubated at 37° for 10 min, followed by incubation at 37° for 30 min with 1 pmol of [^{3}H]*N*-methylscopolamine in the presence or absence of local anesthetics in a range of concentrations. After the incubation, the synaptoneurosomes were collected on a Whatman GF/C filter and the filter was washed three times with 3 ml of the above buffer (ice-cold). The filter was transferred to a scintillation vial, and the radioactivity was measured after addition of Hydrofluor. Nonspecific binding was determined in incubations with 1 μM atropine. Specific binding represented 98% of total binding under the assay conditions. K_i values were calculated as described (30).

Results

Effects of local anesthetics on sodium influx elicited by BTX. Local anesthetics inhibit the binding of [^{3}H]BTX-A 20α-benzoate to sodium channels (19, 20, 31) and block sodium influx through channels activated by BTX in neuroblastoma cells (32–34) and synaptosomes (35). The effects of a variety of local anesthetics of different structural classes on the influx of ^{22}Na induced by BTX into guinea pig cerebral cortical synaptoneurosomes were determined. The relation between IC_{50} values of 16 local anesthetics for ^{22}Na influx and for the reported IC_{50} values versus [^{3}H]BTX-A 20α-benzoate binding (31) is depicted in Fig. 1. A correlation coefficient of $r = 0.803$ was obtained. The local anesthetics showing the most marked de-

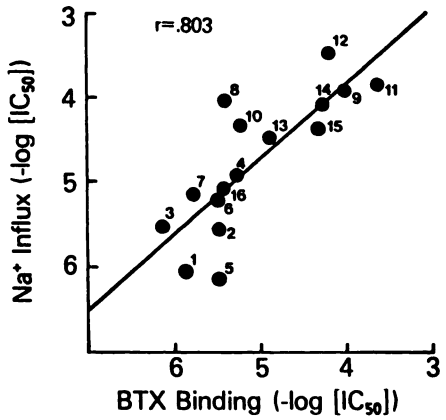

Fig. 1. Relationship between IC_{50} values of local anesthetics versus sodium influx elicited by 1 μM BTX and for the binding of [^{3}H]BTX-A 20α-benzoate to guiea pig cerebral cortical synaptoneurosomes. Binding data are from Ref. 31. Agents tested: **1**, dibucaine; **2**, tetracaine; **3**, euprocin; **4**, bupivacaine; **5**, dimethisoquin; **6**, quinacrine; **7**, phenacaine; **8**, QX-572; **9**, QX-314; **10**, diphenhydramine; **11**, lidocaine; **12**, diphenhydramine methiodide; **13**, piperocaine; **14**, prilocaine; **15**, cocaine; **16**, etidocaine.

viations are dibucaine (**1**), dimethisoquin (**5**), QX-572 (**8**), diphenhydramine (**10**), and diphenhydramine methiodide (**12**). It is remarkable that the correlation is relatively good in spite of the differences in the assay systems involved in measuring inhibition of BTX-elicited sodium flux and inhibition of [^{3}H]BTX-A binding. It should be noted that in synaptoneurosomes the quarternary local anesthetics, namely QX-572 (**8**), QX-314 (**9**), and diphenhydramine methiodide (**12**) are effective in blocking both BTX-elicited sodium flux and BTX-elicited phosphoinositide breakdown (Table 1) suggesting that at least in this preparation such polar compounds do penetrate well to their site(s) of action.

Effects of local anesthetics on phosphoinositide breakdown elicited by BTX. BTX caused the increase in [^{3}H] inositol phosphate accumulation in synaptoneurosomes prelabeled with [^{3}H]inositol and local anesthetics inhibited the inositol phosphate accumulation in a dose-dependent manner. The IC_{50} values of 16 local anesthetics for inhibition of BTX-elicited phosphoinositide breakdown are compared with those for BTX-elicited sodium influx in Fig. 2. A correlation coefficient of $r = 0.879$ was obtained. Euprocin (**3**), bupivacaine (**4**), lidocaine (**11**), and structurally similar local anesthetics, such as etidocaine (**16**), QX-314 (**9**), and QX-572 (**8**), had almost same IC_{50} values for sodium influx and inositol phosphate accumulation, while of the other local anesthetics only cocaine (**15**) inhibited sodium influx at a markedly lower concentration than those required for inhibition of phosphoinositide breakdown (Fig. 2). Local anesthetics had little or no effect on basal levels of phosphoinositide breakdown (data not shown).

Effects of local anesthetics on phosphoinositide breakdown induced by carbamylcholine. The cholinergic agonist carbamylcholine induces phosphoinositide breakdown through muscarinic cholinergic receptors in brain preparations (36) and the increase in inositol phosphate accumulation has been found to be partially inhibited by TTX, a selective blocker of sodium channels (22). The effects of local anesthetics on the accumulation of inositol phosphate stimulated by 2 mM carbamylcholine were determined. Dibucaine caused a biphasic inhibition of inositol phosphate accumulation stimulated by carbamylcholine alone (Fig. 3). However, in the presence of TTX (5 μM), dibucaine inhibited inositol phosphate accumulation only at higher concentrations (Fig. 3). Such concentrations corresponded to the higher concentration range of the biphasic curve (Fig. 3). On the other hand, the inhibition curve of dibucaine for inositol phosphate accumulation elicited by BTX corresponded roughly to the lower concentration range for the biphasic curve. Thus, the estimated IC_{50} value of 0.5 μM for the lower range clearly is similar in magnitude to the IC_{50} value of 2 μM for the BTX-response.

Quinacrine caused a monophasic inhibition of the inositol phosphate accumulation induced by carbamylcholine/TTX

TABLE 1

Inhibitory effects of local anesthetics on BTX-elicited sodium flux, BTX-elicited phosphoinositide breakdown, and binding of [^{3}H]BTX-A 20α-benzoate to sodium channels in guinea pig cerebral cortical synaptoneurosomes

Values are means with SEM from three preparations of synaptoneurosomes or are averages of one or two preparations with each measurement in triplicate.

	Sodium flux		Phosphoinositide Breakdown		BTX Binding
	IC_{50}	n_H[b]	IC_{50}	n_H[b]	IC_{50}[a]
	μM		μM		μM
1. Dibucaine	0.99 ± 0.25	1.1 ± 0.1	2.1 ± 0.5	1.1 ± 0.2	1.4
2. Tetracaine	2.7	0.9	4.5	1.2	3.4
3. Euprocin	2.9	1.6	3.3	1.0	0.74
4. Bupivacaine	13 ± 1.7	0.9 ± 0.2	14 ± 4.7	0.9 ± 0.0	5.4
5. Dimethisoquin	0.71	1.1	3.3	1.0	3.4
6. Quinacrine	6.8 ± 1.7	1.4 ± 0.1	44 ± 11	1.8 ± 0.1	3.3
7. Phenacaine	6.9	0.8	23	0.9	1.7
8. QX-572	92	1.0	83	1.5	3.9
9. QX-314	120	1.0	85	1.1	97
10. Diphenhydramine	45	0.9	100	1.3	6.0
11. Lidocaine	140	1.0	110	1.0	240
12. Diphenhydramine methiodide	330	0.9	720	1.9	64
13. Piperocaine	32	0.8	95	0.8	13
14. Prilocaine	84	0.7	430	0.8	54
15. Cocaine	48 ± 13	1.2 ± 0.1	1400 ± 250	ND[c]	49
16. Etidocaine	8.2	1.0	12	0.9	3.5

[a] Data from Ref. 31.
[b] Hill coefficient.
[c] ND, not determined.

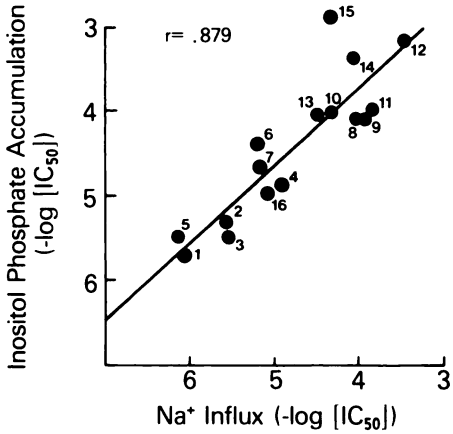

Fig. 2. Relationship between IC_{50} values of local anesthetics versus sodium influx and versus accumulation of inositol phosphate in both cases elicited by 1 μM BTX in guinea pig cerebral cortical synaptoneurosomes. For numbers of agents see legend to Fig. 1.

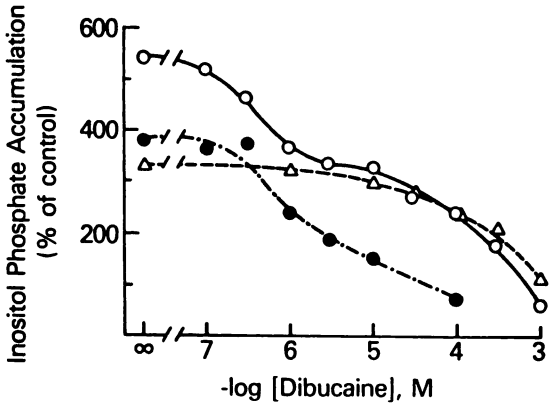

Fig. 3. Effects of dibucaine on accumulation of inositol phosphate elicited by carbamylcholine, carbamylcholine/TTX, or BTX in guinea pig cerebral cortical synaptoneurosomes. The stimulation of inositol phosphate accumulation was induced by 2 mM carbamylcholine alone (○), 2 mM carbamylcholine in the presence of 5 μM TTX (△), or 1 μM BTX (●).

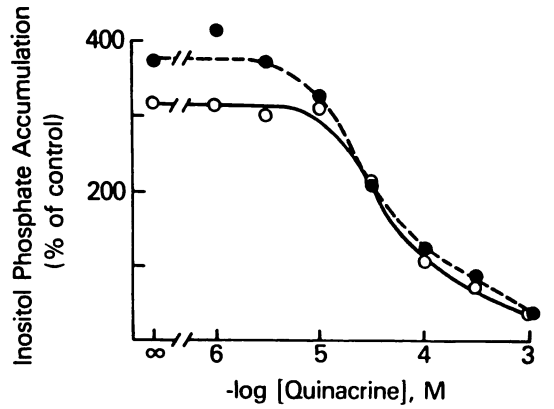

Fig. 4. Effects of quinacrine on accumulation of inositol phosphate elicited by carbamylcholine/TTX or BTX in guinea pig cerebral cortical synaptoneurosomes. The stimulation of inositol phosphate accumulation was induced by 2 mM carbamylcholine in the presence of 5 μM TTX (○) or 1 μM BTX (●).

(Fig. 4). Inhibition occurred over the same concentration range as seen for inhibition of BTX-stimulation of inositol phosphate accumulation.

The effects of local anesthetics on inositol phosphate accumulation induced by carbamylcholine/TTX and by BTX were compared, since selective inhibitory effects on the former response might reflect a selective action of these agents on phospholipase C (Fig. 5). Many local anesthetics, namely dibucaine (**1**), tetracaine (**2**), bupivacaine (**4**), phenacaine (**7**), and etidocaine (**16**), were much more potent as inhibitors of BTX stimulation than as inhibitors of carbamylchoine/TTX stimulation. The majority of the local anesthetics had similar potencies versus BTX stimulation and carbamylcholine/TTX stimulation. However, diphenhydramine (**10**) and its methiodide (**12**) were much more potent versus carbamylcholine/TTX stimulation. In addition, pheniramine (**19**), which is structurally similar to diphenhydramine, and two phenothiazines, namely chlorpromazine (**17**) and promethazine (**20**), also caused selective inhibition of inositol phosphate accumulation induced by carbamylcholine/TTX (Fig. 5). Pyrilamine (**18**), another antihistamine related in structure to diphenhydramine, was only slightly more potent versus the carbamylcholine/TTX stimulation than versus the BTX stimulation. Most of these agents are known to have ancilliary activity as muscarinic antagonists. The affinity of such compounds for cholinergic receptors was ascertained through effects on binding of the muscarinic antagonist [^{3}H]*N*-methylscopolamine to synaptoneurosomes. The order of potency as inhibitors of [^{3}H]*N*-methylscopolamine binding was promethazine (IC_{50}, 28 nM) > chlorpromazine (110 nM) > diphenhydramine (1.1 μM) > pheniramine (5.2 μM) > pyrilamine (21 μM). The potency as muscarinic antagonists of these agents and certain of the local anesthetics does appear to correlate with their ability to block the inositol phosphate accumultion elicited by carbamylcholine/TTX (Fig. 6) and not with a selective blockade of phospholipase C, since they had lesser effects on BTX-elicited phosphoinositide breakdown (Fig. 5).

Discussion

The effects of local anesthetics on sodium influx and phosphoinositide breakdown elicited by BTX were compared. BTX binds to sodium channels and enhances the activation process

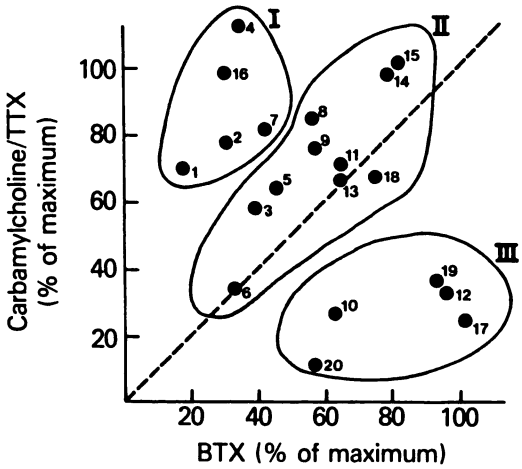

Fig. 5. Relationship between inhibitory effects of local anesthetics versus BTX- and versus carbamylcholine/TTX-elicited accumulation of inositol phosphate in guinea pig cerebral cortical synaptoneurosomes. The stimulation of inositol phosphate accumulation was induced by 1 μM BTX or 2 mM carbamylcholine in the presence of 5 μM TTX. Agents were added at 100 μM. For identification of agents 1 to 16 see legend to Fig. 1; **17**; chlorpromazine; **18**, pyrilamine; **19**, pheniramine; **20**, promethazine.

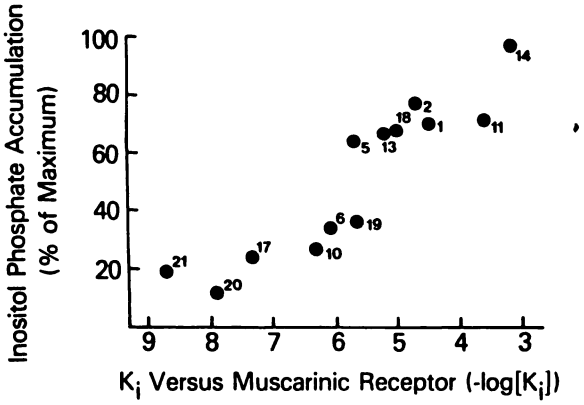

Fig. 6. Relationship between K_i values of local anesthetics versus muscarinic receptor binding and their inhibitory effects on carbamylcholine/TTX-elicited accumulation of inositol phosphate in guinea pig cerebral cortical synaptoneurosomes. The stimulation of inositol phosphate accumulation was induced by 2 mM carbamylcholine in the presence of 5 μM TTX. Agents were added at 100 μM. For identification of agents 1 to 20 see legend to Fig. 5; **21**, atropine. K_i values of the agents 1, 2, 5, 6, 11, 13, and 14 are from Ref. 41.

of sodium channels resulting in the stimulation of sodium influx (17). The inhibitory effect of local anesthetics on BTX binding and BTX-elicited sodium flux has been proposed to be due to an allosteric enhancement of dissociation of BTX from the sodium channels (17). Indeed, the inhibitory effects of 16 local anesthetics, for which structural variations were marked, on sodium influx elicited by BTX in synaptoneurosomes closely correlated with the effects on the binding of [^{3}H]BTX-A 20α-benzoate to synaptoneurosomes (Fig. 1). Correlations between intrinsic local anesthetic activity or inhibition of BTX-induced depolarization of synaptoneurosomes and inhibition of [^{3}H]BTX-A binding have been previously reported (19, 20). A rather close correlation also pertains for the comparison of potencies of the 16 local anesthetics as inhibitors of BTX-elicited sodium flux and of BTX-elicited phosphoinositide breakdown (Fig. 2). Effects of various local anesthetics on phosphoinositide breakdown have not been previously investigated. Cocaine and tetracaine have been reported recently to inhibit BTX-elicited phosphoinositide breakdown in mouse cerebral cortical slices (37).

In the case of BTX elicited phosphoinositide breakdown in synaptoneurosomes, inhibitory effects of local anesthetics might be due either to effects on BTX-elicited activation of sodium channels or to direct effects on the function of phospholipase C. TTX, a potent local anesthetic with great specificity for the voltage-dependent sodium channels, had been shown to inhibit BTX-elicited phosphoinositide breakdown in synaptoneurosomes (22, 23). However, 100-fold higher concentrations of TTX were required to block phosphoinositide breakdown (IC_{50}, 2 μM) than to block BTX-elicited sodium flux (IC_{50}, 20 nM). Such a marked difference in potencies of local anesthetics versus batrachotoxin-elicited sodium influx and batrachotoxin-elicited inositol phosphate accumulation did not pertain (Fig. 2). Indeed, certain local anesthetics, such as euprocin (**3**), bupivacaine (**4**), lidocaine (**11**), and analogs or derivatives of lidocaine, namely etidocaine (**16**), QX-572 (**8**), and QX-314 (**9**), inhibited both sodium influx and inositol phosphate accumulation with almost same potency (Fig. 2). Other local anesthetics did have lower IC_{50} values for sodium influx than those for inhibition of inositol phosphate accumulation, but the difference was much less than 10-fold (Fig. 2) unlike the 100-fold difference that pertains for TTX (23). Cocaine (**15**), a relatively weak local anesthetic, was the most selective versus sodium channel flux, showing a 22-fold greater potency versus sodium flux than versus phosphoinositide breakdown. A recent report compared potencies for cocaine and tetracaine versus inhibition of BTX-elicited phosphoinositide breakdown in mouse cerebral cortical slices and versus veratridine-stimulated guanidine influx through sodium channels in mouse synaptosomes (37). Cocaine was slightly more potent and tetracaine significantly more potent versus phosphoinositide breakdown than versus guanidine influx (37).

The observation that TTX inhibits BTX-elicited sodium influx at much lower concentrations than those required for inhibition of BTX-elicited inositol phosphate accumulation suggested the presence of TTX-insensitive sodium channels closely linked to phosphoinositide breakdown (23). The majority of channels, namely the TTX-sensitive channels, would then not be closely related with phosphoinositide breakdown. If this hypothesis is correct, then the present study indicates that none of the local anesthetics selectively affect the purported TTX-insensitive sodium channels closely related with phosphoinositide breakdown. Indeed, cadmium ions remain the one channel blocking agent that appear relatively selective toward inhibition of phosphoinositide breakdown elicited by sodium channel agents (23). Another possible explanation for the poor correlation between inhibition of BTX-elicited sodium flux by tetrodotoxin and inhibition of phosphoinositide breakdown (23) is that phosphoinositide breakdown elicited by BTX might require only a very small influx of sodium ions and that a concentration of TTX that nearly completely inhibits sodium influx is needed in order to inhibit phosphoinositide breakdown. However, if this hypothesis is correct, then all of the local anesthetics, like TTX, should be much more potent versus sodium influx than versus phosphoinositide breakdown and this is not the case.

One of the local anesthetics, namely quinacrine, is well known to have inhibitory effects on phospholipases, including phospholipase C (18). However, quinacrine inhibited sodium influx elicited by BTX at concentrations lower than those inhibiting inositol phosphate accumulation. Possible direct effects of quinacrine and the other 15 local anesthetics on phospholipase C were investigated by using a cholinergic agonist, carbamylcholine, to directly activate muscarinic receptors coupled to phospholipase C systems in the synaptoneurosomes. Local anesthetics were found to inhibit inositol phosphate accumulation induced by carbamylcholine. However, the inhibitory action of dibucaine on carbamylcholine stimulation was biphasic (Fig. 3), suggesting that two mechanisms are present for the inhibitory effect of dibucaine.

Inositol phosphate accumulation elicited by carbamylcholine has been found to be partially inhibited by TTX (22), and it has been suggested that the signal transduction mediated by muscarinic receptors in part is linked to sodium channels (38, 39). In order to eliminate contributions of sodium channel activation, phosphoinositide breakdown was stimulated by a combination of carbamylcholine (2 mM) and TTX (5 μM). Inhibition of the carbamylcholine/TTX response by dibucaine was monophasic and corresponded to the second phase of inhibition of the carbamylcholine-response by the higher concentrations of dibucaine (Fig. 3). It would appear likely that the first phase of the inhibition of the carbamylcholine response

by lower concentrations of dibucaine represents inhibition of sodium channels, while the inhibition at higher concentrations of dibucaine of the carbamylcholine response is due to direct inhibitory effects on activation of phospholipase C.

On the other hand, quinacrine inhibited the inositol phosphate accumulation elicited by BTX and the carbamylcholine/TTX combination with almost same IC_{50} (Fig. 4). Quinacrine also inhibited the inositol phosphate accumulation stimulated by norepinephrine at the same concentration range (data not shown). Inhibition of inositol phosphate accumulation of quinacrine appears likely to reflect direct effects on activation of phospholipase C.

Comparison of potencies of local anesthetics on BTX-elicited phosphoinositide breakdown with potencies versus carbamylcholine/TTX suggests that several of the 16 local anesthetics affect sodium channels at concentrations significantly lower than those that would appear to cause any direct inhibition of phospholipase C. This group, designated group I (Fig. 5), consists of dibucaine (**1**), tetracaine (**2**), bupivacaine (**4**), phenacaine (**7**), and etidocaine (**16**). It would appear that such local anesthetics inhibit BTX-elicited phosphoinositide breakdown principally through blockade of sodium channels.

The majority of the local anesthetics, designated group II (Fig. 5), have similar potencies versus BTX-elicited phosphoinositide breakdown as versus carbamylcholine/TTX-elicited breakdown. With such compounds, inhibition of BTX-elicited breakdown appears likely to be due to both direct effects on phospholipase C and effects on sodium channels.

The last group of compounds, designated group III (Fig. 5), consists of diphenhydramine (**10**), its methiodide (**12**), chlorpromazine (**17**), and close structural analogs, namely pheniramine (**19**) and promethazine (**20**). These compounds selectively inhibited inositol phosphate accumulation induced by carbamylcholine/TTX and are known to have antagonist activity at cholinergic receptors. All were potent inhibitors of binding of the muscarinic ligand [^{3}H]*N*-methylscopolamine (Fig. 6). It would, thus, appear likely that rather than directly inhibiting phospholipase C, the compounds of group III inhibit responses to carbamylcholine/TTX primarily by blockade of muscarinic receptors. Such blockade of muscarinic receptors may also play a role in the inhibition of quinacrine (**6**) of carbamylcholine responses, since quinacrine has been reported to have an IC_{50} of 5 μM versus binding of [^{3}H]*N*-methylscopolamine (40) and a K_i of 0.8 μM versus binding of [^{3}H]quinuclidinyl benzilate (41).

In conclusion, local anesthetics inhibit BTX-elicited sodium influx at concentrations comparable to those required to inhibit BTX-elicited phosphoinositide breakdown. Thus, unlike TTX, which was relatively ineffective versus BTX-elicited phosphoinositide breakdown, local anesthetics appear to interact with similar potency at the TTX-sensitive pathways in synaptoneurosomes that are responsible for most of BTX-elicited sodium flux and with the TTX-insensitive pathways that are closely associated with sites of phosphoinositide breakdown. Carbamylcholine stimulated phosphoinositide breakdown both through a sodium channel-mediated pathway that was blocked by TTX and through a pathway involving receptor-mediated activation of phospholipase C. Many of the local anesthetics blocked pathways involving sodium channels and those involving receptor activation of phospholipase C with similar potency, indicating that for these local anesthetics direct effect on phospholipase C may confound any interpretations. However, several local anesthetics, namely dibucaine, tetracaine, bupivacaine, phenacaine, and etidocaine do appear relatively selective for sodium channels. No local anesthetics were found, including quinacrine, which appear to have selective inhibitory actions on phospholipase C.

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Send reprint requests to: Yukio Nishizawa, Laboratory of Bioorganic Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.