MOLECULAR AND STRUCTURAL BASIS OF RESTING AND USE-DEPENDENT BLOCK OF SODIUM CURRENT DEFINED USING DISOPYRAMIDE ANALOGUES

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ABSTRACT The effects of disopyramide (Norpace®) and 14 closely related structural analogues on the Na current of voltage clamped squid axons were examined to determine which physico-chemical properties and which changes in the structure of the Norpace molecule can alter the nature of its sodium channel blocking actions. Conventional voltage clamp technique for internally perfused giant axons was used. Axons were exposed to 100 μM concentrations via the internal perfusion solution, and the actions of the 15 analogues to produce resting and use-dependent block of Na current were assessed. The roles of Na ions and the activation and inactivation processes in the development of and recovery from use-dependent block of Na current induced by the Norpace analogues were also examined. The results indicate that for both mono-tertiary and bis-tertiary amines the potency to produce use-dependent block was proportional to molecular weight, whereas the correlation between potency to produce resting block and molecular weight was significant only for bis-tertiary amines. The mono- were more potent than the bis-compounds. However, comparisons between compounds having similar molecular weights and/or pK_a values indicate that other factors also can influence blocking potency. For compounds within each homologous mono- or bis-tertiary amine series, hydrophobicity as estimated from log P values (P = octanol/water partition coefficient) was found to influence the potency to produce use dependent block of Na current. Use-dependent block was extant in axons internally exposed to pronase to remove the inactivation process, which indicates that inactivation is not an obligate condition for development of use-dependent block of Na current. An important role for the activation process in the development of use-dependent block of Na current is suggested by the finding that, in general, the voltage dependence of Na current activation parelleled that of use-dependent block. However, the potential dependence of use-dependent block produced by less hydrophobic but not by more hydrophobic compounds was shifted in the hyperpolarizing direction by removing Na^+ from the external solution. Compounds with intermediate hydrophobicities altered the time course of Na current during its activating and inactivating phases. This finding can be explained by the kinetics of association and dissociation of drug molecules with channel receptor sites during the development and relaxation of use-dependent block rather than by postulating any major effect of drug to alter channel gating kinetics. In summary, a comprehensive study of the structure–activity relationship of the Norpace molecule was achieved and the implications of the findings with respect to several factors believed to influence drug potency for resting and use-dependent block of the Na current in squid axon are examined and discussed.

INTRODUCTION

The ability to block Na channels in axonal and cardiac sarcolemmal membranes is shared by most local anesthetics and many cardiac antiarrhythmic drugs. The blocking action of these compounds can be separated into several components to facilitate the study of the involved interactions between drug and channel. The strength of block and therefore the potency of a blocking drug can be influenced by membrane voltage (Yeh and Narahashi, 1976, 1977). The increase in the strength of block induced by a change in membrane voltage has been termed "voltage-dependent block" (Strichartz, 1973). Strength of block can also be influenced by the frequency and duration of repetitive depolarizations during a train of action potentials or voltage clamp pulses. The increase in block induced by repetitive firing has been termed "use-dependent block" (Courtney, 1975). Use-dependent block can conveniently be thought of as having a "depolarization" phase during which block accumulates as drug molecules enter and block more Na channels, and a "resting" phase during which block dissipates as blocker leaves channels. Therefore, the rates of block accumulation and dissipation are important determinants of the kinetics and strength of the use-dependent component of block. The strength and rate of block accumulation can be enhanced by increasing either or both the amplitude or duration of the depolariza-

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tions produced during trains of action potentials or voltage clamp pulses. The extent to which use-dependent block accumulates and is enhanced by increasing the amplitude of the depolarizing pulses is influenced partly by the voltage dependent component of the block, and can be described as the "voltage dependence" of use-dependent block.

Many aspects of the nature of use-dependent block are not yet clarified. The voltage dependence of use-dependent block has been studied extensively in nerve membranes. Strichartz (1973) and Cahalan (1978) demonstrated that the strength of the block of Na current by local anesthetics can increase *e*-fold per 28-mV increase in the amplitude of membrane depolarization. This voltage dependence could arise in two ways: directly in that the electric field may affect the migration of drug molecules to their binding sites, or indirectly if either drug access or binding to the channel blocking site is influenced by the voltage dependence of the activation and/or inactivation of the channel gating (Hille, 1977; Cahalan, 1978; Starmer et al., 1984). However, the voltage dependence of block is not always consistent with either of these possibilities. In addition, the slope and midpoint of the voltage dependence can be influenced by molecular structure and physico-chemical properties of the blocker (Yeh, 1980; Wang et al., 1982). Current flowing through Na channels can also modify the voltage dependence (Cahalan and Shapiro, 1976; Shapiro, 1977; Cahalan and Almers, 1979*a*,*b*; Seyama et al., 1980; Yeh and Vijverberg, 1981). Therefore, the structural and physical requirements of the molecule necessary to produce this dependence remain to be fully clarified.

The dissipation of block during the resting phase after membrane repolarization usually is slower than the accumulation process and varies from several seconds to several minutes depending on the blocking molecule. Factors important in determining the time course of recovery such as molecular weight (or size) (Courtney, 1980; Sada and Ban, 1980; Campbell, 1983), lipid solubility (Hille, 1977), and stereo-specificity and the distribution of the molecule's charged groups have been recognized (Yeh, 1980, 1982). However, the structure–activity relationships of these factors have not yet been explored systematically. Using Norpace and 14 closely related derivatives, we investigated some of the physico-chemical and structural properties of molecules that can affect the use-dependent block of Na currents in voltage clamped, perfused squid giant axons internally exposed to these compounds. Use-dependent block was indeed found to be structure-dependent. Drug molecules with higher degrees of hydrophobicity exhibited only voltage dependent block; the voltage dependence parelleled that for channel activation, and removal of the inactivation process did not alter the character of the use-dependence of block. Drug molecules with lesser degrees of hydrophobicity showed current dependencies making the voltage dependence of block less steep in the range of membrane potential where inward Na current flows. Recovery from use-dependent block was faster for lesser hydrophobic molecules, and the process was accelerated by pulsing frequently to open channels.

METHODS

The experiments were carried out with giant axons from squid, *Loligo pealei,* available at the Marine Biological Laboratory, Woods Hole, MA. After the connective sheath tissue was removed, the giant axon was prepared for internal perfusion using the roller method originally developed by Baker et al. (1961) and modified by Narahashi and Anderson (1967). Axons were perfused internally with a standard internal solution (SIS) and externally with normal artificial seawater (ASW). ASW had the following composition (in millimolars): Na, 450; K, 10; Ca, 50; Cl, 560; and HEPES, 10; the pH was adjusted to 8.0. SIS had the following composition (in millimolars): K, 350; Na, 50; glutamate, 320; F, 50; sucrose, 333; and phosphate, 15; the pH was adjusted to 7.3. To study the inward Na current without interference from K current, 20 mM tetraethylammonium ion (TEA) in the form of TEA fluoride was added to SIS. All structurally related Norpace-like drugs were applied internally by adding drug to SIS to make drug concentrations 100 μM for all experiments. Each experimental protocol used to characterize the block produced by each compound was performed on four or more axons. The compounds were dissolved as 10-mM stock solutions in pure water made sufficiently acidic with 1 N HCl to permit dissolution with vigorous shaking. Values for the pK_a, log of the octanol/water partition coefficient (log *P*), and chemical and structural formulae were supplied from in-house data by the Research Division of G.D. Searle Co., Skokie, IL. Drugs were generously supplied also by the G.D. Searle Company.

The perfused axon was voltage clamped using the axial wire method (Oxford, 1981). The electrodes consisted of a 75 μm platinum–iridium wire and a 100-μM glass pipette. The former was attached "piggyback" on the latter and insulated to within 1.2 cm of the tip of the wire with dental wax. The bare wire was electrolytically coated with platinum black to reduce its impedance to current. A 25-μm platinum–iridium wire was inserted into the glass pipette to reduce the series resistance of the electrode and enhance the frequency response of the circuit monitoring membrane potential. Membrane current was measured with a virtual ground circuit. To reduce axial current flow along the axon membrane, two sets of guard electrodes connected to ground at the voltage clamp amplifier were positioned as close as possible on both sides of the axon membrane. Clamp command pulses were generated using a TTL pulse generator (WP Instruments, New Haven, MA) or a specially modified computer (PDP 11/23, Digital Equipment Corp., Marlboro, MA). Membrane currents were sampled at 20 μs per point by an analog-to-digital converter and data were stored on floppy disks or recorded on 35-mm film. Data on film records were analyzed with the aid of an enlarger; data stored on disk were analyzed off-line with analysis utility programs using the computer.

RESULTS

The approach used to elucidate the structure–activity relationship (SAR) for the ionic channel blocking effects of the Norpace molecule involved comparing the blocking effects of disopyramide (Norpace®) with those of a series of compounds closely related to Norpace stucturally, with those of Norpace, and with those of each other. Fig. 1 shows the molecular structures of Norpace and several of the derivatives employed for the SAR study. Most of the compounds that proved valuable contained either mono- or bis-tertiary amino groups. To facilitate a logical presentation of the results, a compound of each type was selected to serve as the basic prototypic molecule against which the

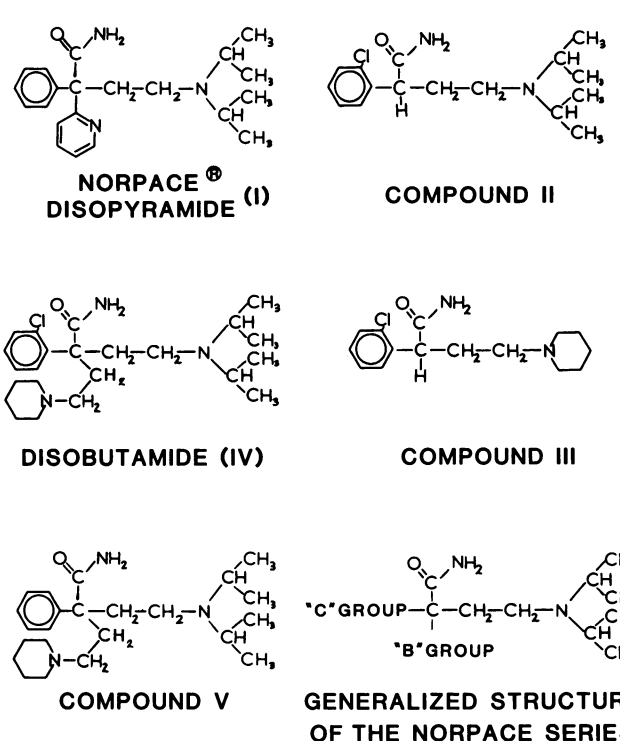


FIGURE 1 The chemical structure of Norpace, four closely related compounds including disobutamide (IV), and the general structure of the series employed for these studies. The general structure shows the diisopropylamino butylamide "backbone" and the positions of the side groups B and C referred to in Table I. Compounds II (SC-33927) and III (SC-33926) are used as reference compounds. Compound IV (disobutamide, i.e., SC-31828) represents the daughter compound resulting from the marriage of compounds II and III. Compound V (SC-30600) contains elements of both Norpace and disobutamide. The compounds I, IV, and V are bis-tertiary amines, whereas compounds II and III are mono-tertiary amines.

effects of specific changes in the molecular structure would be assessed. SC-33927 was selected as the reference mono-tertiary amino compound and SC-31828 was taken for the bis-tertiary amine. The effects of several changes that were identical for each of the reference molecules were also compared. Using this approach the effects of 11 discrete changes in the molecular structure were assessed. The compounds selected for study were specifically chosen to permit assessment of the effects of (*a*) changing the hydrophobicity of the overall molecule or of a specific side group, (*b*) changing the size of the molecule with and without change in hydrophobicity, and (*c*) changing the number of sites in the molecule that are charged at physiological pH, on (*i*) the molecule's ability to produce use-dependent block, (*ii*) the voltage dependence of use-dependent block, and (*iii*) the recovery from use-dependent block.

Fig. 2 shows that SC-31828 (100 μM) decreased the Na but not the K current. Families of membrane currents elicited in response to various step depolarizations before and during internal application of drug are depicted in *A*

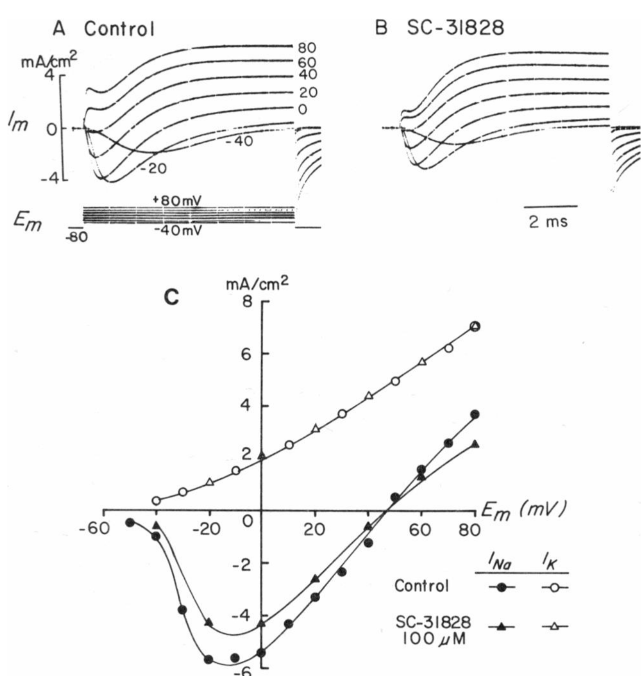


FIGURE 2 SC-31828 (Compound IV) selectively decreased sodium current without affecting the K current at all voltages between −50 and +80 mV. The families of currents obtained during a control (*upper left*) and during exposure to 100 μM SC-31828 (IV) (*upper right*) were obtained using the pulse protocol depicted in *B*. Holding potential was −80 mV and the depolarizing pulses were 8 ms in duration at 0.033 Hz (1/30 s) to minimize effects of any possible use-dependent block component. Effect of SC-31828 on current-voltage curves for I_{Na} and I_K is shown in *C*.

and *B*. The control recordings of membrane current reveal that a transient inward (downward deflection) Na current was flowing during the early (1–2 ms) period of 8-ms step depolarizations. The time-to-peak flow of the Na current shortened as the membrane was depolarized incrementally from −30 to +30 mV. The direction of the Na current transient changed from inward to outward at a potential more positive than +50 mV. The amplitude of the early transient downward deflection of the current trace (relative to the zero level) was used to quantify the Na current component of the membrane current. To assess drug-induced resting block of Na current, step depolarizations from −40 to +80 mV were applied at a frequency of one pulse every 30 s during perfusion with SIS containing drug (100 μM). During exposure to SC-31828, peak Na current was suppressed by ~20%. Fig. 2 *C* shows the current–voltage relations during control and in the presence of 100 μM SC-31828. Depolarizations approximately to or positive to −60 mV evoked Na current (I_{Na}). Peak I_{Na} was maximal at ~−10 mV and its direction of flow reversed at ~+50 mV. From Fig. 2 it can be seen that SC-31828 caused a slight decrease in the amplitude of the Na current without affecting either its time course, voltage dependence of activation, or reversal potential. Thus the resting block of the Na current produced by this compound can be

attributed to a decrease in Na conductance. While the resting block of I_{Na} produced by SC-31828 (100 μM) was not different from that seen in the presence of equimolar amounts of either SC-33926 or SC-33927, the extent of use-dependent block produced by a train of +80 mV depolarizing pulses was more than twofold greater.

Use-dependent Block

The phenomenon of use-dependent block obtained during exposure to SC-31828 is illustrated in Fig. 3. These data indicate that the ability of SC-31828 to block Na current can be enhanced by applying a conditioning train of depolarizing pulses. The pulse protocol is depicted in Fig. 3 (*bottom*). The inward Na current in response to each pulse of a conditioning train of 20 depolarizing pulses to 0 mV applied at 2 Hz (protocol a) incrementally decreased in amplitude relative to the amplitude of first pulse of the train, and after several pulses reached a steady state level. After protocol a, a second train of 20 depolarizing conditioning pulses reaching to +80 mV applied at 2 Hz (protocol b) further decreased Na current. The peaks of the outward-going Na currents decreased in amplitude and reached a steady state level within 20 pulses. Protocol b was more effective than protocol a in producing use-dependent block as evidenced by the response to single test pulses to 0 mV (protocol c). After protocol b, peak Na current in response to the single pulse to 0 mV in protocol *c* (trace *c, top*) was much smaller than the peak of the Na current in response to the 20th pulse to 0 mV during protocol a. This finding indicates that use-dependent block was produced during exposure to SC-31828 and was influenced by the level of the depolarizing potential, being increased as membrane potential was made more positive.

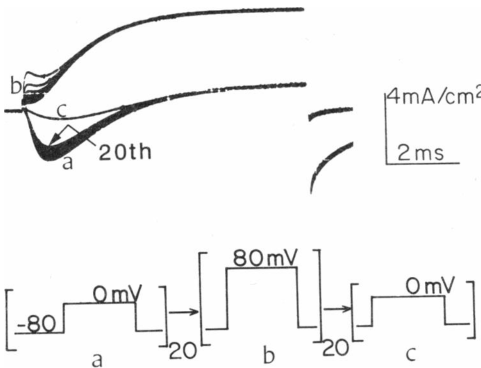


FIGURE 3 SC-31828 exerted a use-dependent blocking effect on I_{Na}. The protocols used to assess block are depicted in the lower panel: protocol *a* consisted of a train of 20 depolarizing conditioning pulses to 0 mV applied at 2 Hz; protocol *b* consisted of a train of 20 depolarizing conditioning pulses to +80 mV applied at 2 Hz, and protocol *c* consisted of a single test pulse to 0 mV. The intervals between protocols *a* and *b* and between *b* and *c* were 500 ms. The effect on the I_{Na} evoked in response to each protocol are indicated by the trace labeled with the letter (near the current trace) corresponding to protocol used. The inward current labeled c was much smaller than the one labeled "20th" even though both were elicited by depolarizing pulses to 0 mV. c was elicited after conditioning by protocol *b* and a by protocol *a*. This result suggests that protocol *b* (i.e., depolarizations to +80 mV) is more effective than protocol *a* (i.e., depolarizations to 0 mV) in producing use-dependent block.

The membrane potential dependence of use-dependent block was studied over a wider range of membrane potentials using the voltage clamp protocol depicted in the inset of Fig. 4. With this protocol, a conditioning train of 30 depolarizing pulses at 2 Hz to several levels of membrane potential (E_c) was applied to produce use-dependent block; each train was followed by a test pulse to 0 mV to assess the extent of the use-dependent block produced during the train. The amplitude of the Na currents associated with the test pulse was normalized to the Na current evoked by a test pulse in the absence of drug (control) (Fig. 4, inset). During perfusion with 100 μM SC-31828 when E_c was negative to −20 mV, the Na currents were ~80% of control (the 20% reduction from control reflects the level of resting block). When E_c was positive to −20 mV, there was a voltage dependent block of Na channels caused by the train of conditioning pulses (E_c) as evidenced by a decrease in the Na current evoked by the test pulses. The peaks of Na currents evoked by the test pulses were decreased from ~80 to 20% of the control level as E_c progressed from −20 to +100 mV. Thus, the blockade of 60% of the Na channels by 100 μM SC-31828 represents the maximal use-dependent component of total block.

Effects of Substituent Groups on I_{Na} Block

Tables I and II summarize the SAR with respect to Na channel blocking actions. To facilitate presenting the SAR of the Norpace molecule, SC-30600 (Fig. 1, compound V) is regarded as the basic reference compound. The molecule is considered to be composed of three components, A, B, and C, attached to a butylamide group at the C-2 carbon. For SC-30600 the A chain is a di-isopropyl tertiary amine

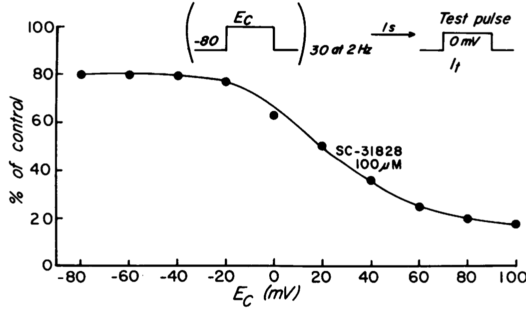


FIGURE 4 Membrane potential dependence of use-dependent block of I_{Na} in the presence of SC-31828. The protocol used to produce use-dependent block is shown in the inset above the curve. The inward Na current remaining after conditioning at 2 Hz to E_c mV was elicited by a test pulse to 0 mV and normalized to the analogous control obtained in the absence of drug. Use-dependent block was observed when conditioning pulses (E_c) were positive to −20 mV, and increased as E_c was made more positive. A resting block of ~20% was observed in the presence of internally applied SC-31828 (100 μM) when holding potential was −80 mV.

TABLE I
CHEMICAL, STRUCTURAL, AND PHYSICAL PROPERTIES OF COMPOUNDS STUDIED

Compound	Searle Co. nomenclature	Chemical formula	Mol wt	A group	B group	C group	Log P	pK_a
I	Norpace	$C_{21}H_{29}N_3O$	339	Unchanged		N	2.71	10.4
II	SC-33927	$C_{16}H_{25}ClN_2O$	297	Unchanged	Absent	Cl	2.83	9.8
III	SC-33926	$C_{15}H_{21}ClN_2O$	281	Absent	$(CH_2)_2-N$	Cl	2.46	9.2
IV	SC-31828	$C_{23}H_{38}ClN_3O$	408	Unchanged	$(CH_2)_2-N$	Cl	4.78	8.2/9.7
V	SC-30600	$C_{23}H_{39}N_3O$	374	Unchanged	$(CH_2)_2-N$		NA	8.3/9.4
VI	SC-34390	$C_{24}H_{39}ClN_2O$	407	Unchanged	$(CH_2)_2-$	Cl	4.23	9.4
VII	SC-35234	$C_{22}H_{30}N_2O$	338	Unchanged	Absent		3.66	9.4
VIII	SC-32684	$C_{30}H_{47}N_3O$	466	Unchanged	$—(CH_2)_2—N(IC_3H_7)_2$		NA	8.7/9.4
IX	SC-32687	$C_{27}H_{41}N_3O$	424	Unchanged	$(CH_2)_2-N$		NA	8.2/9.7
X	SC-31001	$C_{24}H_{42}FN_3O$	408	Unchanged	$—(CH_2)_2—N(IC_3H_7)_2$	F	NA	8.6/9.3
XI	SC-33473	$C_{21}H_{35}N_3O$	346	Unchanged		N	NA	8.3/9.7
XII	SC-34148	$C_{30}H_{37}N_3O$	445	$N-(CH_2)_3-$ $N(CH_3)_2$			NA	NA
XIII	SC-30843	$C_{25}H_{42}N_4O$	415	Unchanged	$-(CH_2)_2-N$ N		NA	7.5/10.0
XIV	SC-31955	$C_{23}H_{42}N_4O$	391	Unchanged	$—(CH_2)_2—N(IC_3H_7)_2$	N	NA	8.8/9.4
XV	SC-32636	$C_{24}H_{49}N_3O$	396	Unchanged	$—(CH_2)_2—N(IC_3H_7)_2$		NA	8.9/9.5

NA, data not available.

group, the B chain contains a piperidine ring, and C is a phenyl group. Most compounds used for the present study had the same di-isopropylamine A group whereas the B chain was typically either removed or modified, and the C group was usually halogenated at the ortho position of the benzene ring, or was attached or annealed to a phenyl ring to form biphenyl or naphthyl groups.

TABLE II
SUMMARY OF BLOCKING EFFECTS ON I_{NA}

Compound	Searle Co. nomenclature	Resting block	Use-dependent block [−20 mV]$_n$ at 2 Hz	Use-dependent block [+80 mV]$_n$ at 2 Hz	Recovery time of I_{Na}
		%	%	%	
I	Norpace	14	1	15	2 min
II	SC-33927	17	1	17	2 s
III	SC-33926	17	2	17	ND
IV	SC-31828	15	0	67	3.5 min
V	SC-30600	2	9	49	1.5 min
VI	SC-34390	49	8	46	4.5 min
VII	SC-35234	49	33	48	3.5 min
VIII	SC-32684	33	44	67	8.2 min
IX	SC-32687	21	28	73	ND
X	SC-31001	2	0	73	ND
XI	SC-33473	5	3	28	ND
XII	SC-34148	20	48	69	2.3 min
XIII	SC-30843	10	3	46	33 s
XIV	SC-31955	5	4	42	ND
XV	SC-32636	8	12	63	ND

ND, data not determined. Resting block, percent inhibition of I_{Na} relative to control occurring after a 10-min rest period preceded by 2 Hz, [−20 mV]$_{20}$. Values are averages of two or three determinations.

The blocking action of the A and B chains of SC-30600 can be considered equivalent for the entire series of compounds because SC-33927 (di-isopropylamine) and SC-33926 (piperidine) were equipotent in blocking Na channels in all respects including resting block, use-dependent block, and the modulatory effect of inward current flow on the voltage dependence of their use-dependent block (see Table II). Modification or substitution of the C ring gave rise to different effects depending on whether the compound being modified was mono-tertiary or bis-tertiary. For the mono-tertiary amines, by comparing the blocking action of SC-35234 to that of SC-33927, it is clear that addition of a phenyl moiety to the C ring to produce biphenyl or naphthyl groups greatly increased the potency to block Na channels. Such modification is expected to increase the molecule's hydrophobicity as evidenced by the higher log P for SC-35234 (see Table I). Fusing a pyrrolidine ring to the piperidine ring (SC-30843 vs. SC-30600) had very little effect on its blocking action. On the other hand, decreasing the distance between the N of the ring structure in the B group and the alpha carbon of the main "backbone" of the molecule decreased the Na channel blocking potency (SC-33473 vs. SC-30600). Comparison of SC-30600 with SC-32684 indicates that addition of the phenyl group to bis-tertiary amines also increases the Na channel blocking potency of compounds containing both the A and B groups. Modifications of the C ring also can affect the Na channel blocking action. For example, insertion of a halogen at the ortho position of the benzene ring producing SC-31828 or SC-31001 modestly increased the Na channel blocking potency when compared with SC-30600. However, neither substituting a nitrogen for the C-2 carbon in the benzyl group of SC-30600 to make

SC-31955 nor saturating the pyridine ring of Norpace to make the piperidine ring of SC-33473 altered I_{Na}. Therefore, this evidence indicates that some but not all changes in the C group can influence the character of the block. Modifications of the C group (SC-30600 vs. SC-31828) also altered the apparent voltage-dependence of use-dependent block of I_{Na} and its sensitivity to the presence of Na^+ in the ASW (see Table III).

Structure Modulates Voltage Dependency of Use-dependent Block

The voltage dependency of use-dependent block varied with most of the compounds tested. Fig. 5 depicts the voltage dependencies of use-dependent block induced by axonal perfusion with several representative compounds. To compare the voltage dependencies of the use-dependent blocks produced by these compounds, the test currents evoked after conditioning trains of step depolarizations to the chosen levels of potential were normalized to the current observed during resting block. Voltage dependency was characterized by the parameters of slope, midpoint, and value for the steady state of the modified Boltzmann equation giving the best fit to the data (Fig. 5 and Table III). Of those compounds represented, the least voltage dependent increase in the degree of use-dependent block was induced by SC-33927. For this drug, a 28-mV increase in the membrane potential caused an *e*-fold increase in the use-dependent block by SC-33927. Similar results were observed with SC-33926 and SC-13957 (Norpace). Changing the pyridine ring in the Norpace molecule to a piperidine ring to form compound SC-33473 resulted in an increase in the sensitivity of the use-dependent block to change in membrane voltage. An 18-mV increase in the membrane potential caused an *e*-fold increase in the use-dependent block. The substitution of a chlorine atom at the ortho position of the benzene ring of SC-30600 to form SC-31828 had little effect on the voltage dependence of block in ASW lacking Na^+. With both compounds a 14–15-mV increase in membrane potential produced an *e*-fold increase in the extent of use-dependent block. However, the degree of current dependence of the block was decreased (see next section). The other extreme in the spectrum of use-dependent block is represented by SC-32684, SC-35234, and SC-34148, which are characterized by steep voltage dependencies of block. An *e*-fold increase in the use-dependent block could be induced by an 8-mV increase in the membrane potential. In addition to the differences in the slope factors, as shown in Fig. 5 (see also Table III), these compounds differed greatly in the midpoints and steady state values for the curves depicting the relation between the use-dependent block and conditioning membrane potential. Midpoints were −28, −5, +25, and +38 mV, respectively for SC-34148, SC-30600, SC-33473, and SC-33927, and the steady state values ranged from 0.08 to ~0.45. The following section addresses these differences.

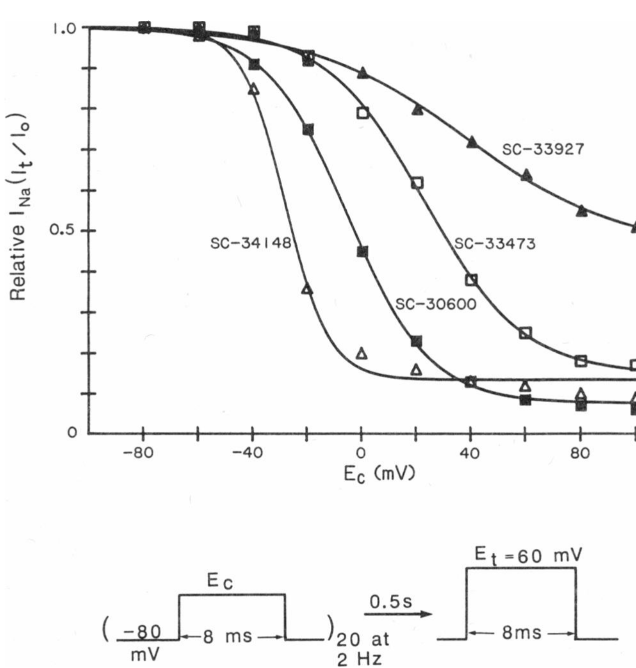


FIGURE 5 Membrane potential dependencies of use-dependent block induced by four compounds selected to illustrate the effect of several changes in the properties of the basic molecule. The protocol used to produce use-dependent block (*bottom, inset*) was essentially the same as shown in Fig. 4 except that the current remaining after the conditioning pulse protocol was assessed with a test pulse to +60 mV and normalized to the current observed when E_c was −80 mV. The axons were externally bathed in Na-free ASW and drug was internally applied (100 μM). The potential dependencies relating the degree of block to the conditioning potential were characterized by the steepness of the slope, midpoint, and the steady state level of each curve (also see Table III). Wide variation in the potential dependencies was observed despite the structural similarity of the selected compounds.

TABLE III
PARAMETERS CHARACTERIZING THE VOLTAGE DEPENDENCE OF USE-DEPENDENT BLOCK OF THE NA CURRENT

Compound	Searle Co. nomenclature	0 Na ASW			450 mM Na ASW		
		K	$E_{0.5}$	B	K	$E_{0.5}$	B
		mV	*mV*		*mV*	*mV*	
II	SC-33927	28	+38	0.45	28	+49	0.73
XI	SC-33473	18	+23	0.15	20	+47	0.29
V	SC-30600	15	−5	0.08	22	+29	0.13
IV	SC-31828	14	−8	0.13	18	+16	0.18
XII	SC-34148	8	−28	0.13	8	−31	0.11

The equation used to evaluate the data shown in Figs. 5 and 6 and generate the values tabulated here is as follows:

$$I_t/I_0 = \frac{1 - B}{\exp\left[(E_c - E_{0.5})/K\right]} + B,$$

where B represents steady-state block, E_c is the conditioning potential, $E_{0.5}$ is the potential at which block is half-maximal, and K is the slope factor (i.e., the change in E_c to produce an *e*-fold change in the block. Values are averages of two or three determinations.

Modification of the Voltage Dependence of Block by Current

Membrane depolarization not only causes Na channels to open, it also initiates the Na current and determines its flow density and direction. The density of current and its direction of flow might affect the blocking action, and manifest as an apparent change in the voltage dependence of block. To demonstrate the characteristic of current dependence, use-dependent block was measured with the axons bathed either in normal ASW containing Na or in ASW containing no Na while internal Na concentration was kept constant. Fig. 6 shows the effect of the presence or absence of Na^+ in ASW on the voltage dependence of use-dependent block in the presence of SC-34148 (*A*) and of SC-30600 (*B*), and indicates that for SC-34148 the voltage dependencies of use-dependent block were similar in Na(−) or Na(+) ASW, suggesting that block had no Na^+ or Na current-dependent component. In addition, the similarity of the voltage dependence of block to that of the activation of the sodium conductance is striking. The midpoints are virtually identical and the slope factors are 8.0 and 6.9 per *e*-fold change in voltage. Thus, use-dependent block caused by SC-34148 appears to be solely attributable to membrane potential; this suggests block is related to the activation process rather than having any component that is affected by Na ions or current flow. Similar results were obtained with SC-32684 and SC-35234.

However, in contrast to SC-34148, use-dependent block induced by SC-30600 was greater at conditioning potentials (E_c) positive to −60 mV when the axon was bathed in the Na-free ASW rather than in ASW containing 450 mM Na^+ (Fig. 6 *B*). This difference in the extent of use-dependent block is related to Na influx since there was no inward Na current flowing through Na channels during Na-free conditions in Na(−)ASW. Indeed, when the axon was bathed in a full Na ASW, the difference was greatest between 0 and 20 mV where the maximal Na influx occurred. Thus, the Na channel block produced by SC-30600 exhibits the characteristic of having a current-dependent component. Table III summarizes some parameters pertaining to the effect of external Na ions supporting inward Na current flow to modulate the voltage dependence of use-dependent block produced by five agents having slightly differing structures when measured in normal ASW and ASW containing no Na. For four of these compounds, when inward current flowed, the voltage dependence was altered in three ways: first, the slopes of the curves defining the voltage dependencies of block became less steep; second, the midpoints of the curves were shifted in the depolarizing direction; and finally, the steady state levels of use-dependent block were reduced. Such effects were not observed with SC-32684, SC-35234, and SC-34148, indicating that these agents do not possess the structural constituents required to enable current flow to alter the voltage dependence of the use-dependent block they can produce.

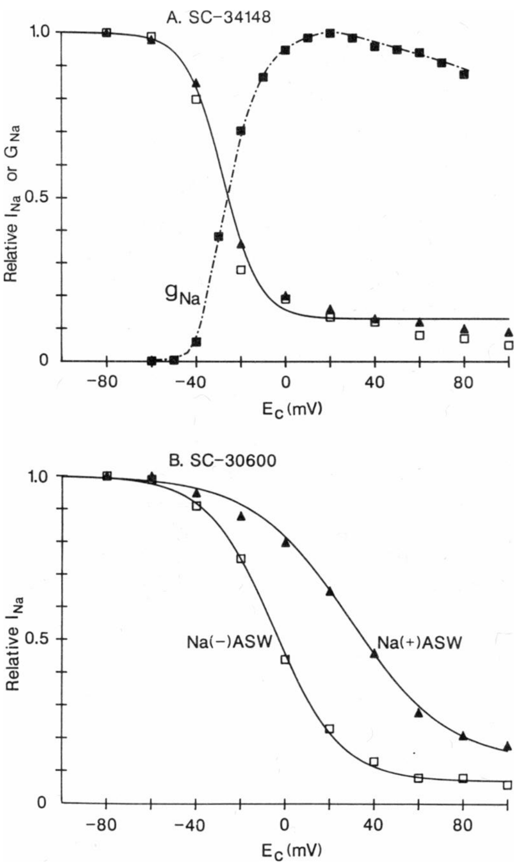


FIGURE 6 The direction and magnitude of Na current modulates the potential dependence of use-dependent block produced by SC-30600 (*B*) but not by SC-34148 (*A*). Both compounds were applied internally (100 μM). The protocol used to produce use-dependent block is illustrated in Fig. 5, *inset*. The characteristics of the current dependence of use-dependent block were assessed in axons bathed either in ASW containing 450 mM Na^+ [Na(+)ASW] or in ASW containing no Na^+ [Na(−)ASW] while the internal Na^+ concentration was kept at 50 mM. The voltage dependence of the Na conductance (g_{Na}) (*dot-dash-dot, filled squares*) is shown in *A* also. In the presence of SC-34148 (*A*) the potential dependence was not changed when Na^+ was deleted from the ASW (*solid line, unfilled squares*) whereas in the presence of SC-30600 (*B*) the curve was shifted in the hyperpolarized direction when Na was removed (*unfilled squares*).

Recovery from Use-dependent Block

Two protocols were used to monitor the time course of recovery from use-dependent block, a "resting" protocol and a "pulsing" protocol. For the resting protocol, membrane potential was held at −80 mV during the recovery or resting period between a conditioning train of pulses and the subsequent test pulse used to elicit the current recovered during the resting period. The pulsing protocol resembled the resting protocol except that during the recovery period a train of test pulses was delivered at either 1 pulse/s or 1 pulse/11 s.

Recovery Characterized by the Resting Protocol. Fig. 7 shows time courses of recovery using the resting protocol. In this experiment, each recovery period was preceded by a train of 20 conditioning pulses to +80 mV for 8 ms at 2 Hz to establish the same starting level of use-dependent block before the initiation of recovery. The time course of recovery was monitored with a test pulse to 0 mV after each recovery period for a family of selected times at the holding potential (−80 mV). Recovery times ranged from several milliseconds to several minutes depending on the period required for complete dissipation of the block induced by the drug added to the internal solution.

In the absence of drug (control), Na current recovered rapidly after a single exponential time course with a time constant of several milliseconds (Fig. 7, *open circles,*). The recovery time constant was similar to that for the recovery from inactivation of I_{Na} induced by a single depolarizing pulse. Thus during control drug-free conditions, a train of depolarizing pulses essentially produced only fast Na inactivation. However, in the presence of drugs producing use-dependent block of I_{Na}, recovery did not follow a single exponential time course; instead, a double exponential time course was observed. The fast phase of recovery had time constants of 3–5 ms at −80 mV, which were similar to that for the control, while the time course of the slow phase was as much as several minutes depending on which drug was added to the internal solution. As illustrated in Fig. 7, the recovery time constants were 2, 92, 236, and 281 s when the drugs used were SC-33927, SC-30600, SC-31828, and SC-34390. The fraction of the recovering I_{Na} associated with the slow phase of recovery during exposure to each of these drugs also varied from drug to drug, being 20, 48, 80, and 90% of the total block induced by trains to +80 mV for the above-mentioned four compounds, respectively. The slow phase can be interpreted to represent recovery from the use-dependent component of block, whereas the fast phase can be interpreted to represent recovery of drug-free channels from fast Na inactivation.

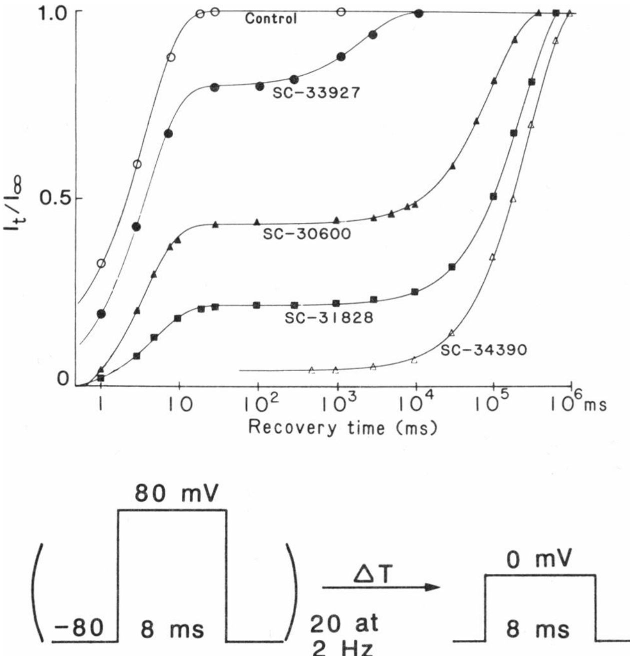


FIGURE 7 Time courses of recovery from I_{Na} inactivation during drug-free control or from use-dependent block during exposure to four different compounds using the resting protocol. Use-dependent block was obtained using a conditioning protocol, which consisted of a train of 20 pulses to +80 mV, each 8 ms in duration, applied at 2 Hz from a holding potential of −80 mV. A 4-ms test pulse to 0 mV was used to monitor the resting recovery of Na current at −80 mV (*bottom inset*). Peak inward Na current (I_t) after various recovery times (Δt) were normalized to that after a long resting period (I_∞). The normalized values I_t/I_∞ were plotted logarithmically as a function of recovery time. The time course of the recovery of the Na current in the absence of drug followed a single exponential with a time constant of 3.6 ms (*unfilled circles*). In the presence of the drugs, Na current recovery time course followed double exponential time courses as follows: SC-33927 (*filled circles*), 3.6 ms and 2 s; SC-30600 (*filled triangles*), 3.7 ms, and 92 s; SC-31828 (*filled squares*), 5.1 ms and 236 s. In the presence of SC-34390 (*unfilled triangles*), the slower time constant was 281 s; the faster component was too small to estimate the time constant.

Recovery Characterized by the Pulsing Protocol. The recovery from the use-dependent block caused by some compounds has been shown to be accelerated by a train of depolarizing pulses that generate inward-going currents (Yeh, 1979; Seyama et al., 1980). This effect represents another manifestation of modulation of use-dependent block by current flow through the Na channel.

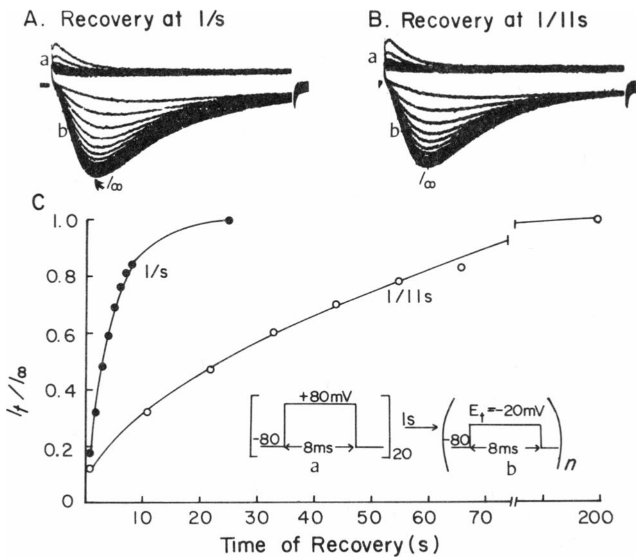


FIGURE 8 The time course of recovery from use-dependent block using a pulsing protocol. Recovery during pulsing was compared at frequencies of 1/s and 1/11 s in the presence of 100 μM SC-31828. In *A* and *B*, Na currents elicited in response to the conditioning protocol *a* (*C, inset*) were labeled *a*, and the inward currents (I_t) in response to the test pulses to −20 mV were labeled *b*. The Na currents recovered to the same steady state level (I_∞). *C* indicates that the percentage of current recovered with each test pulse was essentially the same at both stimulus frequencies despite the 11-fold difference in the interval between pulses.

The pulsing protocol, in contrast to the resting protocol, intermittently opens the channel gating mechanism and apparently allows drug molecules to escape from the blocked channels. Na^{+}, in the act of carrying current, can be thought of as occupying the channel and interfering with drug interaction with the channel blocking site, thereby speeding up the recovery process. SC-31828 was found to exhibit such behavior (Fig. 8). The time courses for recovery (from the use-dependent block produced by SC-31828) were compared when the axon was pulsed at frequencies of 1/s and 1/11 s. The time courses of recovery at these two frequencies were influenced by the pulsing rate during the recovery period, being accelerated by pulsing. However, the percentage of current recovered with each beat was essentially the same at both stimulus frequencies; the recovery time constants expressed in terms of the number of pulses were identical, being four pulses in both cases. In the absence of pulses applied during the recovery period, it took several minutes for I_{Na} to recover from use-dependent block and presumably for the blocked channels to "release" the SC-31828 molecules (Fig. 7). An acceleration of the recovery time course by applying pulses could be demonstrated only for compounds that did not exhibit a major use-dependent component of block at membrane potentials around -20 mV. Such compounds include SC-31828, SC-30600, SC-33473 and SC-33927. In contrast, the recovery rate from use-dependent block caused by SC-34148, SC-32534, and SC-32684, compounds that exhibited a strong voltage dependent block at potentials negative to or at -20 mV, was not accelerated by the pulsing protocol (Table II).

Current Dependence Affects I_{Na} Time Course during Partial Block

Compounds producing a voltage dependent use-dependent block exhibiting current-induced modulation, also concomitantly produced a change in the time course of I_{Na}. Fig. 9 *A* shows an example of this that was observed during recovery from use-dependent block with SC-33473 when examined with a pulsing protocol. Activation kinetics of Na currents evoked by pulses applied while I_{Na} was recovering from use-dependent block (trace *a* in Fig. 9 *A*) appeared to be slowed, reaching the peak inward current more slowly than an inward current evoked after a long rest period (trace *b* in Fig. 9 *A*). Trace *a* actually crossed over trace *b*. This change in activation kinetics could be the result of slow dissociation of drug molecules from use-dependent blocked channels, rather than being due to a true change in gating kinetics. In other words, the kinetics of open gating need not be altered for the inward current to appear to activate more slowly than normal because drug-blocked nonconducting channels become drug-free conducting channels as drug molecules leave the channels during the open gating induced by depolarizing pulses. Such a change in the kinetics of the inward current was minimal in the presence of compounds such as SC-35234, SC-34148, and SC-32684, whose voltage dependent block

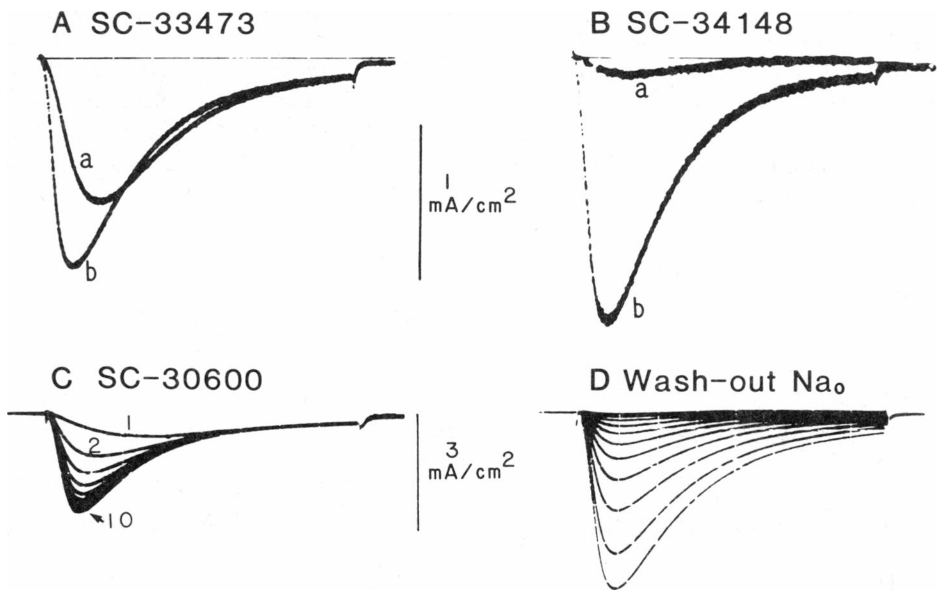


FIGURE 9 Time courses of I_{Na} during resting block (*b*) and after use-dependent block (*a*). The developing and decaying phases of I_{Na} appeared to be slower after use-dependent block than during resting block in the presence of 100 μM SC-33473 (*A*). The I_{Na} time courses during the analogous conditions were less obvious but not much different in the presence of SC-34148 (*B*). *C* shows that the changes in I_{Na} kinetics induced by SC-30600 (labeled 1 to 10) become less dramatic pulse by pulse as recovery from use-dependent proceeds pulse by pulse. The effect of reducing I_{Na} by decreasing the Na concentration in the ASW (*D*) indicates that the changes in time courses shown in *A* and *C* could not have been the result of a problem due to inadequate series resistance compensation because the time-to-peak and decay rate were unaffected by current density.

almost saturated at −20 mV and was not affected by current flow or its direction (Fig. 9 *B*). Fig. 9 *C* illustrates an example of an intermediate case for the change in current kinetics. While the current increased in amplitude, its time to the peak was shortened as depolarizing pulses were applied repetitively to open Na channels and to produce inward current. In contrast to Fig. 9 *C*, 9 *D* shows that the kinetics of the Na current were not changed when I_{Na} was reduced substantially by replacing external Na ions with impermeant ions. This result rules out the possibility that the change in the Na current kinetics during use-dependent block was an artifact arising from an uncompensated residual resistance in series with the axonal membrane.

Role of Inactivation in Use-dependent Block

In addition to causing channels to open, membrane depolarization also causes them to inactivate. Therefore, the effect of membrane depolarization on the use-dependent block could be associated with Na inactivation. To test the role of Na inactivation in producing use-dependent block, the axon was first treated with pronase, which is known to remove Na current inactivation without affecting the activation kinetics (Armstrong et al., 1973; Rojas and Rudy, 1976). Fig. 10 shows that use-dependent block produced with SC-34390, which occurred when inactivation was intact, can still occur in the pronase-treated axon. In addition, the steady state level of use-dependent block was similar, being ~93%. This finding suggests that the inactivation gate is not essential for the production of use-dependent block. Whereas removal of Na inactivation had no effect on the degree of use-dependent block, its rate of onset or development was accelerated. The time constant for the onset of use-dependent block was 0.70 ± 0.01 reciprocal pulses in the pronase-treated axon (Fig. 10 *A*) and 2.45 ± 0.21 reciprocal pulses when inactivation was intact (Fig. 10 *B*). This result suggests that the presence of inactivation gates can actually retard development of use-dependent block.

DISCUSSION

The results of this structure–activity study of the Norpace molecule have permitted characterization of several important aspects of the compound's blocking actions on Na channels in neuronal membrane, including the kinetic nature of the voltage dependence of use-dependent block and the roles for Na ions, current flow, and activation and inactivation gating in regulating the development of and recovery from use-dependent block of the Na current.

Correlations between Channel Blocking Potency and Physico-Chemical Properties and Molecular Weights

Molecular Weight, Size, and Log P. This study confirms that for some, but not necessarily all, molecules, both molecular size and log *P* values can be important determinants of Na channel blocking potency when measured in terms of resting and use-dependent block (Courtney, 1980). The present data (see Fig. 11 *A*) indicate that there is only poor correlation between the potency of compounds in the Norpace series to produce resting block and their molecular weights. In contrast, the analogous correlation for the use-dependent component of block was highly significant (see Fig. 11 *B*). The structure–activity relationship also can be interpreted in terms of changes in physico-chemical properties resulting from structural modification of the molecule. The pK_a value and the partition coefficient (expressed as log *P*) are two parameters frequently correlated with blocking activity. In the present study the effect of any change in pK_a on blocking potency should be minimal because all compounds (whose pK_a values had been determined) have similar pK_a values, all

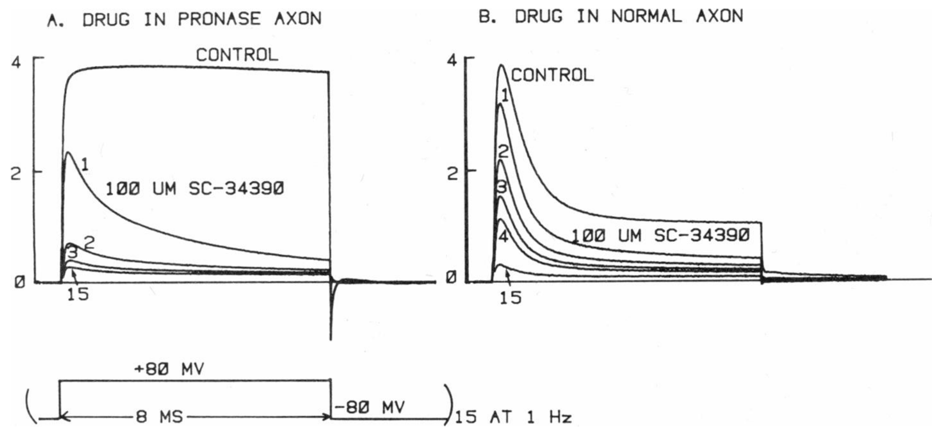
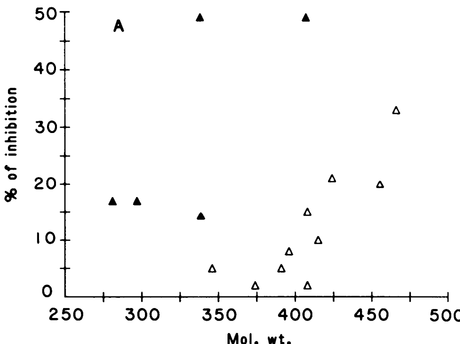
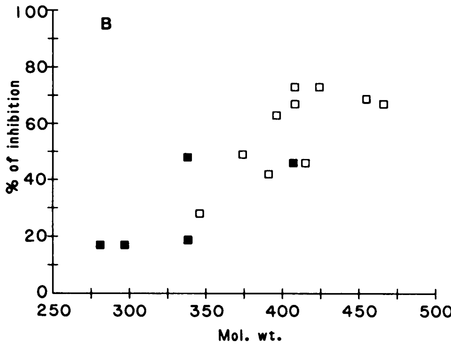


FIGURE 10 Use-dependent block produced by SC-34390 in the presence and absence of Na inactivation. The use-dependent block produced by internal application of 100 μM SC-34390 was compared before (*B*) and after removal of the Na inactivation gate by internal application of pronase (*A*). The onset of use-dependent block was accelerated but the steady state level of block was unchanged by removing inactivation gating.





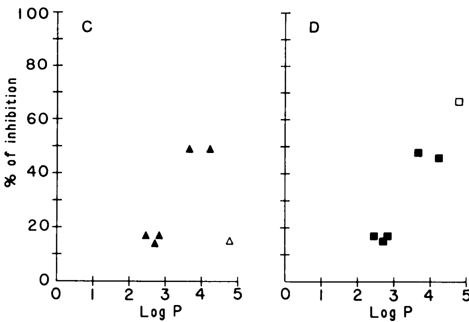


FIGURE 11 The relationship between Na channel blocking potency and molecular weight (*A* and *B*) and log *P* (*C* and *D*). In *A*, resting block is plotted against molecular weight of mono-tertiary amines (*filled triangles*) and of bis-tertiary amines (*unfilled triangles*). The overall correlation between resting block and molecular weight is not significant. However, correlation between resting block and molecular weight of bis-tertiary amines is significant with $r = 0.83$ ($P < 0.01$). In *B*, the use-dependent block is plotted against molecular weight of mono-tertiary amines (*filled squares*) and bis-tertiary amines (*unfilled squares*). The overall correlation between use-dependent block and molecular weight is significant with $r = 0.86$ ($P < 0.05$). The relationship between the log *P* and the potency of blocking Na channels (*C* and *D*). The correlation between Na channel blocking potency and the log *P* values is not significant for the resting block (*C*) but significant for the use-dependent block (*D*) ($r = 0.97$; $P < 0.01$).

being >9.2. The relationship between the blocking potency and the log *P* value of six compounds was plotted for resting block in Fig. 11 *C*, and for use-dependent block in Fig. 11 *D*. For the mono-tertiary amines, the correlation between the extent of resting block and log *P* value is significant. However, the bis-tertiary amine SC-31828 does not similarly conform. In contrast, Fig. 11 *D* indicates that use-dependent block produced by both mono- and bis-tertiary amines correlates well with the value for log *P*. Thus, the "rules" governing development of resting block seem to differ from those governing development of use-dependent block. A similar conclusion was drawn from analyses of a series of lidocaine (Courtney, 1980; Bokesch et al., 1986) and propanolol derivatives (Courtney, 1980).

Hydrophobicity. In addition to molecular weight (i.e., size) and log *P* value, other factors must also be involved in determining the selectivity and potency of channel blocking action (Schwarz et al., 1977). This notion is based on the following observations: first, a similar degree of Na channel block could be brought about by compounds having widely different molecular weights, e.g., compounds I, II, and III, and compounds VI and VII (see table II and Fig. 11, *A* and *B*); second, compounds with similar molecular weights (compounds IV, VI, and X) produced profoundly different degrees of resting block (Fig. 11 *A*) yet caused a lesser difference in use-dependent block (Fig. 11 *B*). SC-31828 and SC-34390, being isosteric isomers, have almost identical molecular size and shape and yet differ in their channel blocking actions, suggesting that molecular size is not a predictable indicator of drug effect, and that other factors, such as those illustrated by the present study, can be important determinants of potency also. For example, the distribution of charge may influence the effective size of the molecule, making it different than that expected from three-dimensional molecular representations by interacting with membrane or channel proteins. This could explain why SC-34390 caused a more potent resting block than SC-31828. Unlike the positively charged (at physiological pH) piperidine group in the SC-31828 molecule, the cyclohexane ring in SC-34390, being an uncharged hydrophobic group, can reside at hydrophobic sites. Being "tucked away," the hydrophobic group would not sterically hinder the molecule from effecting a channel blocking action.

Channel Gating and Voltage Dependency of Use-dependent Block

For many compounds in the Norpace series the voltage dependence of the use-dependent block resembled that of Na activation (Fig. 6). The increase in use-dependent block observed as the membrane became more depolarized can be attributed either to an effect associated with the gating state of Na channels or to an influence of membrane potential directly on the drug-channel interaction. Thus, the voltage dependence of use-dependent block of Na currents could arise from two sources: the drug-receptor binding reaction, because drug molecules move to their binding site under the influence of the electric field and would have voltage dependence directly; or indirectly if drug binding to the channel blocking site depends on the channel gating state because of the voltage dependence of either or both activation and inactivation. If the first

mechanism operates, voltage dependence should be observed only with drug molecules capable of carrying charge. In addition, the voltage dependence for a drug molecule with one net charge should not be steeper than *e*-fold per 24 mV (at 8°C) and the voltage dependence should reflect the location of the channel's drug binding site. With the second mechanism, the voltage dependence of block should reflect the voltage dependence of either Na activation or inactivation.

When the voltage dependence for SC-34148 to cause use-dependent block is compared with that for the Na channel to open, each has three features that are similar: (*a*) the voltage range for channel opening and that for use-dependent block, (*b*) the slopes for both events, i.e., 7–8 mV per *e*-fold increment in block or conductance change, and (*c*) the midpoints for both relations, being between -20 and -30 mV, are strikingly similar. Thus, we could conclude that the use-dependent block caused by SC-34148 derives its voltage dependence primarily from that for the activation of Na channels. Alternatively, in addition to causing channels to open, membrane depolarization also causes them to inactivate. Conceivably, the effect of membrane depolarization on the use-dependent block might be associated with Na current inactivation. However, the voltage dependence of the use-dependent block did not resemble that for I_{Na} inactivation. Na channel inactivation began at a more negative level of membrane potential and saturated at <-20 mV. In contrast, use-dependent block continued to increase as membrane potential increased positive to -20 mV. Furthermore, use-dependent block remained unchanged even after removal of Na inactivation. Thus, for compounds in the Norpace series it appears that use-dependent block and its membrane potential dependence are not closely related to Na current inactivation.

Na Current Flow Modulates Use-dependent Block

Use-dependent block can be affected by current flowing through Na channels in three ways: the apparent voltage dependence of the block can shift, the kinetics of inward Na current can change, and the time course of recovery from use-dependent block can accelerate. Table III indicates that the voltage dependence of use-dependent block became less steep and the membrane potential at which a half maximal voltage-dependent block was achieved was shifted in the depolarizing direction when determined in ASW (containing 450 mM Na) compared with that in Na-free ASW. Both effects may occur because inward going Na current facilitates dissipation of use-dependent block by "forcing" drug molecules out of blocked Na channels. The change in Na current kinetics most likely is an epiphenomenon reflecting the opening of Na gates and the recovery time course of blocked channels. That is, blocked channels gate open permitting egress for the blocking molecules, and thereby provide a kinetically slower source of conducting channels (Courtney, 1975).

Consequently, the pulsing protocol for characterizing recovery opens the activation gates of the drug-blocked channels, and drug, previously trapped by the closed gate, escapes from the channel. Thus, recovery from use-dependent block is facilitated and channel conductance is reestablished. The more hydrophobic of the compounds studied did not exhibit these three features. Presumably binding of these molecules to Na channels was sufficiently strong that they could not be dislodged from their channel binding sites by exposure to Na ions attempting to enter channels gated open by depolarizing pulses.

Activation Gating and Recovery from Use-dependent Block

In addition to being important in the development of use-dependent block, the activation process has also been implicated in the recovery from the use-dependent block. A recent study (Yeh and Tanguy, 1985) has shown that the recovery can indeed be controlled by the activation mechanism. Hyperpolarizing the membrane slowed the recovery time course defined using a resting protocol. In the present study, frequent pulsing to the potentials expected to open the channels accelerated the recovery time course. Thus, opening of Na channels and the subsequent current flow seem to have important roles in the modulation of the time courses for both development of and recovery from the use-dependent block produced by the Norpace series. This action may also have important implications concerning the block of the Na current caused by other antiarrhythmic agents and local anesthetics.

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