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ACCELERATED COMMUNICATION

Sodium Channel Comodification with Full Activator Reveals Veratridine Reaction Dynamics

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SUMMARY

Veratridine association and dissociation rates were determined at single sodium channels in outside-out patches of cultured ventricular myocytes obtained from late-fetal rat hearts. In single cardiac sodium channels depolarized from −110 to −30 mV, intracellular veratridine induced a long lasting (τ = 0.48 sec) open state with small current amplitude (−0.3 pA, i.e., ¼ of normal) and frequent closing transitions, giving it a burstlike appearance, in agreement with reports on other types of sodium channel. Veratridine-associated and veratridine-free states of a single sodium channel were monitored by comodifying it with an allosteric activator, BDF 9145 (1 μM), that induced a burst with normal open channel current amplitude (−1.2 pA at −30 mV) upon veratridine dissociation. Veratridine and BDF 9145 interacted with reciprocal synergism at the single sodium channel such that veratridine-induced bursts (called P-bursts for partially activated) alternated with BDF 9145-induced bursts (called F-bursts for fully activated) many times following a single depolarization to −30 mV. P-bursts and F-bursts within such trains of bursts had exponentially distributed durations. The reciprocal time constant for F-bursts, τ_F^{-1}, increased linearly with veratridine concentration (0.3–30 μM), whereas τ_P was insensitive. We conclude, therefore, that P-bursts reflect veratridine occupancy and F-bursts reflect the veratridine-free state; if veratridine and BDF 9145 bind to a sodium channel simultaneously, veratridine exerts conformational dominance, i.e., retains its property to reduce channel conductance. For the single cardiac sodium channel activated (i.e., deprived of inactivation) by BDF 9145, we have determined a veratridine association rate constant k_1 = 4.3×10^6 M^{-1} sec^{-1}, dissociation rate constant k_{-1} = 2.2 sec^{-1} and equilibrium dissociation constant K_D = 5.1×10^{-7} M (20°, −30 mV membrane potential).

Ionic channels of excitable membranes are opened or activated by endogenous (e.g., acetylcholine, glutamate, γ-aminobutyric acid) and many exogenous ligands (e.g., ceveratrum alkaloids, dihydropyridines, cromakalim). Recent work at the single-channel level has shown that veratridine, a commonly used pharmacological activator of the voltage-dependent sodium channel in nerve, muscle, and heart cells (1–3), induces a long lasting (~1-sec) open state with low conductance (¼ of normal) and frequent closing transitions giving it a burstlike appearance (4–6). An important, yet unresolved, question is whether the duration of this burst reflects the lifetime of the veratridine-sodium channel complex or whether the true lifetime is significantly longer because of a long-lived inactivated veratridine-bound state that has been proposed on the basis of macroscopic sodium current recording in frog node of Ranvier (7). The problem is thus the lack of distinction between two closed channel states, one activator bound and the other activator free. Here we directly monitor both veratridine-associated and veratridine-free states of a single cardiac sodium channel by comodifying it with an allosteric activator, BDF 9145, that imposes the normal open channel conductance upon veratridine dissociation. Rather than assuming the normal inactivated or resting conformation, two closed conformations, upon veratridine dissociation, the sodium channel is thus pharmacologically forced to assume an open conformation that has a higher conductance and is, therefore, distinguishable from the veratridine-induced open state. In the comodified sodium channel, individual veratridine binding reactions will be shown to appear as partially blocking events similar to the fully blocking events induced by saxitoxin in single batrachotoxin-activated sodium channels (8). Our results suggest that the veratridine-induced burst can in fact be equated with the lifetime of the drug-channel complex (τ = 0.45 sec) and reveal a microscopic asso-

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ABBREVIATIONS: BDF 9145, 4-(3-(4-((4-cyanomethoxyphenyl)phenylmethyl)-1-piperazinyl)-2-hydroxypropoxy)-1*H*-indole-2-carbonitrile; DPI 201-106, 4-(3-(4-diphenylmethyl-1-piperazinyl)-2-hydroxypropoxy)-1*H*-indole-2-carbonitrile; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

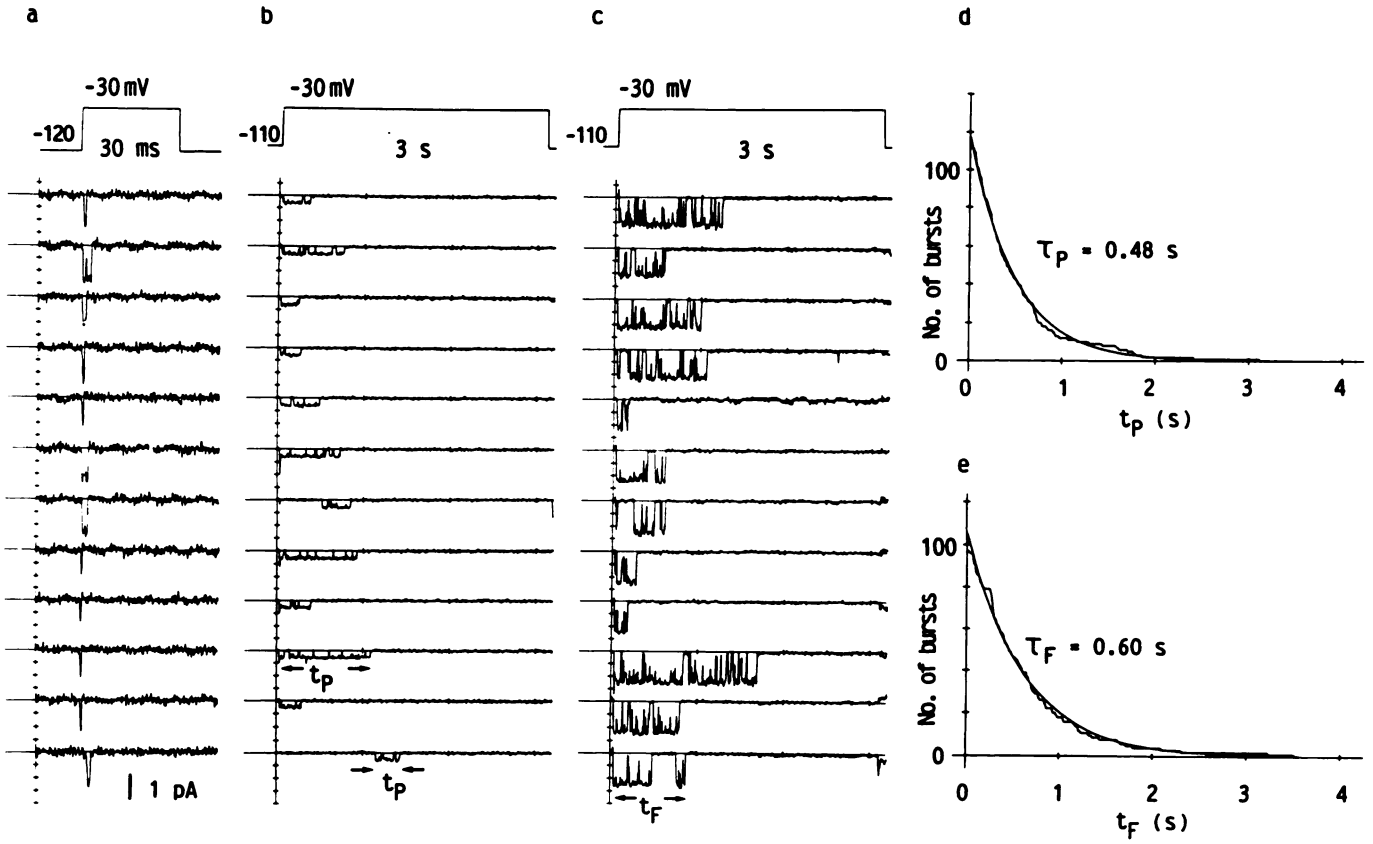


Fig. 1. Activation of single sodium channels (normal openings) (a) by veratridine (250 μM intracellularly) (b) or BDF 9145 (1 μM extracellularly) (c) in three different outside-out patches from cultured late-fetal rat heart cells. Note slower time scale in b and c. Successive activity-containing traces during recording periods of 3.8 (a), 8.2 (b), and 8.3 min (c) with pulsing rates of 0.33 Hz (a) or 0.1 Hz (b and c). Sampling rates: a, 2 kHz; b and c, 0.5 kHz. Filter: a, 1 kHz; b and c, 0.2 kHz. Partially activated sodium channel currents induced by veratridine (P-bursts) started usually within the first few milliseconds of depolarization, occasionally late. Fully activated sodium channel currents induced by BDF 9145 (F-bursts) started always within the first few milliseconds after the voltage step. Cumulative probability histograms for P-burst duration, t_P, (d, two patches) and F-burst duration, t_F, (e, eight patches) were monoexponential with the time constants shown.

TABLE 1

Intraburst gating kinetics in the presence of veratridine, BDF 9145, or both

Drug	Type of burst[a]	N_p[b]	t_o[c]	N_o[d]	t_c[e]	N_c[f]	P_o[g]
			msec		msec		%
Veratridine (250 μM)	P	2	71 ± 87	848	7.9 ± 7.8	740	90
Veratridine + BDF 9145[h]	P	3	84 ± 85	1019	5.1 ± 9.5	1300	94
BDF 9145 (1 μM)	F	5	49 ± 58	1133	5.4 ± 7.2	1139	90
Veratridine + BDF 9145[i]	F	3	55 ± 63	876	6.7 ± 7.4	850	89

[a] P, partially activated (veratridine-induced) sodium current burst; F, fully activated (BDF 9145-induced) sodium current burst.
[b] Number of patches.
[c] Mean open time ± standard deviation.
[d] Number of evaluated openings.
[e] Mean closed time ± standard deviation.
[f] Number of evaluated closings.
[g] Open probability, $t_o/(t_o + t_c)$.
[h] BDF 9145 (1 μM) combined with 0.3, 1, or 30 μM veratridine.
[i] BDF 9145 (1 μM) combined with 0.3 or 1 μM veratridine.

ciation rate constant of 4.3×10^6 M^{-1} sec^{-1}. Comodification of single-ion channels with a partial and an allosteric full activator may be a generally useful method to analyze microscopic reaction dynamics of conformationally dominant ion channel activators.

Materials and Methods

Methods for cell culturing, recording, and data acquisition have been described in detail (9). Briefly, ventricular myocytes were isolated from late-fetal Wistar albino rats and cultured for 4–12 hr. Single sodium channel currents were recorded from excised (outside-out) patches (10). Intracellular medium in the pipette was (mM) CsCl, 120; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 11; glucose, 10; HEPES, 10; CsOH, ~28 (pH 7.3). Extracellular bath solution was (mM) NaCl, 137; KCl, 5.4; $MgCl_2$, 2; $CaCl_2$, 0.1; glucose, 10; HEPES, 10; NaOH, ~3.2 (pH 7.3). The temperature was 20°. Veratridine (Sigma) was dissolved as the hydrochloride and BDF 9145 (synthesized at Beiersdorf AG, Hamburg, FRG) was dissolved in dimethyl sulfoxide (1 mM; final concentration, 1 μM). Within a series of analogs of DPI 201-106, BDF 9145 induced the most marked prolongation of the action potential in guinea pig ventricular

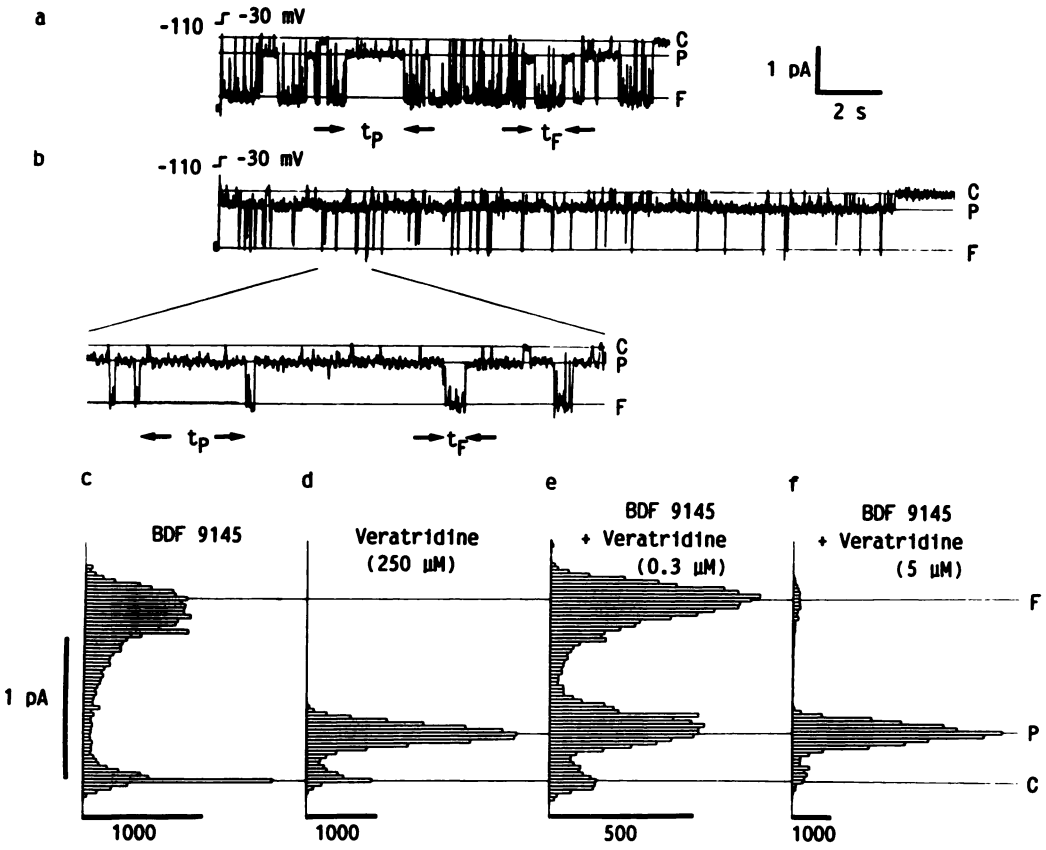


Fig. 2. Current through a single cardiac sodium channel activated by depolarization from −110 to −30 mV in the combined presence of BDF 9145 (1 μM) and 0.3 μM (a) or 5 μM veratridine (b) (two different patches). P-bursts of duration t_P alternate with F-bursts of duration t_F, resulting in a total burst duration of 14 sec in a and 22 sec in b. c–f, Amplitude histograms of all 0.5-kHz data points (horizontal calibrations give data points/bin) obtained during cumulative burst durations of 40 sec with BDF 9145 in eight patches (c), 28 sec with 250 μM veratridine in two patches (d), 16 sec with 0.3 μM veratridine and BDF 9145 in one patch (e), and 40 sec with 5 μM veratridine and BDF 9145 in one patch (f). C refers to closed channel current level.

muscle (by 140 msec at 3 μM under experimental conditions described in Ref. 11). We have chosen BDF 9145 in order to delay sodium channel inactivation markedly. Veratridine was applied intracellularly (pipette solution) because the delayed onset during extracellular application in the whole-cell recording configuration[1] and earlier work on squid axon (12) suggest an intracellular site of action at the sodium channel. Capacitance and leak resistance currents were subtracted using averaged empty traces. Burst durations were evaluated manually by cursor on the computer screen.

Results and Discussion

Fig. 1 compares openings of normal single sodium channels with modified openings induced by veratridine or BDF 9145 in outside-out patches from rat cardiac myocytes. Following a step depolarization to −30 mV, brief and occasionally repetitive elementary currents occurred during the initial 5 msec of the depolarization, confirming previous work (13–15). The mean open channel amplitude was −1.2 pA in 50 well resolved openings in two patches. In the presence of veratridine, which is known to act preferentially on activated sodium channels (6, 16), normal openings were converted into long lasting bursts with a much lower open channel amplitude (−0.3 pA), as has been observed in other types of sodium channel (4–6). Occasionally, the burst appeared late during the depolarization (Fig. 1b). In the presence of BDF 9145, normal openings were replaced by prolonged bursts with an open channel amplitude of −1.2 pA (Fig. 1c). BDF 9145 thus eliminated sodium channel inactivation for hundreds of milliseconds without, in contrast to veratridine, affecting open channel amplitude, similar to the closely related DPI 201-106 (15, 17). Veratridine acted as a partial activator, BDF 9145 as a full activator of the Na channel, on the basis that they induced a high probability open state of subnormal and normal single-channel conductance, respectively, and we call veratridine-induced bursts P-bursts and those induced by BDF 9145 F-bursts.

Intraburst gating kinetics were analyzed by determining mean open and closed times ($\bar{t}_o$, $\bar{t}_c$) using 50% of open channel current level as transition criterion. These data and open probability, $\bar{t}_o/(\bar{t}_o + \bar{t}_c)$, are presented in Table 1. Open and closed times scattered widely, and their distributions could not be fitted by single exponentials. Furthermore, the 0.2-kHz filter (necessary to identify the low amplitude bursts induced by veratridine) prevented detection of many closings in the millisecond range. The mean values presented in Table 1, therefore, do not characterize transitions from single open or closed states.

When activated by step depolarization to −30 mV in the presence of both veratridine and BDF 9145 (1 μM), the single sodium channel responded with a continuous series of alternating P- and F-bursts that ended after 14 sec with 0.3 μM veratridine and after 22 sec with 5 μM veratridine (Fig. 2, a and b). If veratridine and BDF 9145 were binding independently to the same activated sodium channel, the maximum possible burst duration would only be ~3 sec, corresponding to the maximum induced by BDF 9145 (Fig. 1e), which initiated somewhat longer bursts than veratridine (Fig. 1d). Thus, the bursts shown in Fig. 2 cannot be explained by independent binding of veratridine and BDF 9145 to the same sodium channel. Veratridine and DPI 201-106, which is closely related to BDF 9145, have previously been shown to produce supraadditive effects on cardiac action potential duration (18). We suggest that veratridine and BDF 9145 interact with reciprocal synergism such that BDF 9145 binding increases the association rate of veratridine and vice versa. The train of alternating

[1] Unpublished observations.

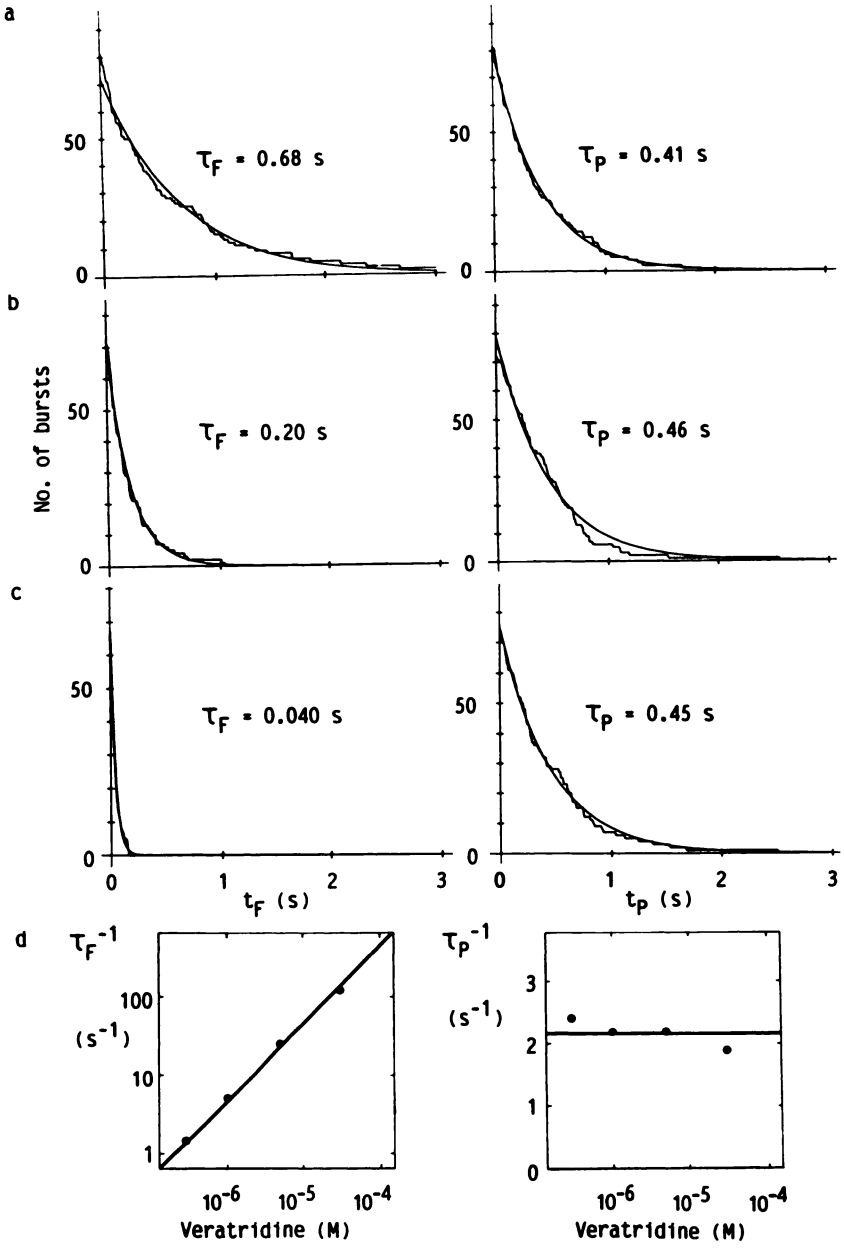
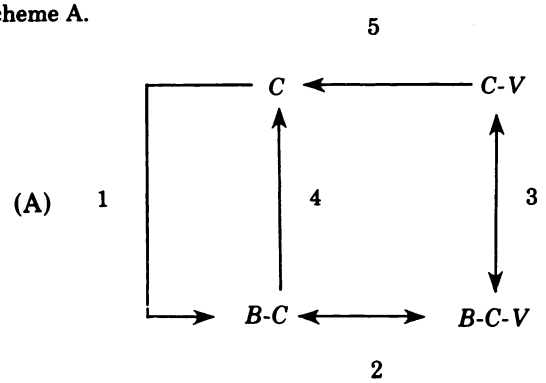


Fig. 3. Cumulative probability histograms showing mono-exponential distributions of t_P and t_F during trains of bursts elicited in the combined presence of BDF 9145 and 0.3 μM (seven patches) (a), 1 μM (two patches) (b), or 5 μM veratridine (one patch) (c). Time constants, τ, of fitted curves are indicated. τ_F^{-1} increases linearly with veratridine concentration (slope factor, 4.3 × 10^6), whereas τ_P^{-1} is unaffected (d). Data for BDF 9145 plus 30 μM veratridine were obtained from one patch and two burst trains (15 and 56 sec) with a filter setting of 0.4 kHz.

F- and P-bursts is, therefore, interpreted to be the result of several successive BDF 9145 binding periods overlapping and alternating with veratridine binding periods, as depicted in scheme A.



The sodium channel, *C*, binds BDF 9145, *B*, if activated by depolarization to −30 mV (step 1); initial binding of veratridine, *V*, is negligible at the low concentrations used in comodification experiments. The resulting noninactivating channel conformation, *B-C*, has a high affinity for veratridine, resulting in additional and reversible binding of veratridine to give the ternary complex *B-C-V* (step 2). BDF 9145 may dissociate from and reassociate with the veratridine-bound conformation *C-V* (step 3). Dissociation of BDF 9145 from *B-C* (step 4) or of veratridine from *C-V* (step 5) terminates the train of bursts. As proven later, veratridine dominates sodium channel conformation during the periods when both ligands are bound simultaneously. F- and P-bursts would thus directly monitor the veratridine-free and veratridine-associated state, respectively. Several tests, based on the stochastic analysis of drug binding to single-ion channels (19), were performed to validate this interpretation.

If the sodium channel was modified by both veratridine and

BDF 9145, it displayed two open states, one corresponding to the partially activated veratridine level P (Fig. 2d) and the other to the fully activated BDF 9145 level F (Fig. 2c). Transitions between the partially and fully activated sodium channel states define the burst durations t_P and t_F, as shown in Fig. 2, a and b. Ninety-three percent of 346 evaluated P-bursts at different veratridine concentrations were enclosed by direct transitions between the fully open and partially open states. In the remainder, the transition was via a brief (msec) closed state, which was added to t_P.

The evaluation of intraburst gating kinetics is shown in Table 1. Comodification did not seem to markedly alter the gating of P-bursts or F-bursts. The open probability remained high (94% for P-bursts, 89% for F-bursts), thus allowing accurate determinations of t_P and t_F. We cannot exclude subtle changes in intraburst kinetics effected by comodification, however, because of the complex gating kinetics within both P-bursts and F-bursts and because of the heavy filtering (0.2 kHz).

If, within a train of bursts, P-bursts reflect veratridine occupancy and F-bursts reflect the veratridine-free state, P-bursts and F-bursts should have exponentially distributed durations, t_P and t_F, at any given veratridine concentration. Furthermore, the reciprocal time constant for F-bursts, τ_F^{-1}, should increase linearly with veratridine concentration, whereas τ_P should be insensitive. All these predictions were quantitatively fulfilled over a 100-fold range of veratridine concentration (0.3–30 μM) (Fig. 3). If veratridine dominates the conductance level, τ_P and τ_F should remain unchanged with different BDF 9145 concentrations at constant veratridine. In a single experiment with 1 μM veratridine and BDF 9145 raised from 1 to 10 μM, τ_P was 0.37 sec (51 bursts) and τ_F was 0.21 sec (51 bursts). These values are similar to those at 1 μM BDF 9145 (τ_P = 0.46 sec and τ_F = 0.20 sec (Fig. 3), in line with the prediction.

Because veratridine dominates sodium channel conductance during comodification, it is difficult to get information on BDF 9145 reaction dynamics. We consider two possibilities. As stated in scheme A, BDF 9145 may dissociate and reassociate (invisibly) during P-bursts, with F-burst reflecting part of the dwell time of BDF 9145 at the sodium channel. Alternatively, veratridine binding may stabilize the binding of BDF 9145 such that the same molecule of BDF 9145 that modified the sodium channel during the initial depolarizing step remains bound until the end of the train of many F-bursts and P-bursts (elimination of step 3 in scheme A). If there is reassociation of BDF 9145 during P-bursts, resulting in a subsequent F-burst, the probability of an F-burst to follow a P-burst should increase with BDF 9145 concentration. Among 23 burst trains observed with 1 μM veratridine and 1 μM BDF 9145, six trains ended with a P-burst (as in Fig. 2b). In contrast, all 27 trains observed in a different patch at 1 μM veratridine and 3 μM BDF 9145, and all 22 trains of another patch with BDF 9145 raised to 10 μM, ended with an F-burst (as in Fig. 2a). Although the number of data is small, these observations suggest that BDF 9145 may dissociate and reassociate during veratridine binding periods.

Could the results of Fig. 2 be explained by simple competition for the same site between veratridine and BDF 9145? In this case, F-burst duration would reflect time of occupancy by BDF 9145 and should be independent of, rather than inversely related to (Fig. 3), veratridine concentration. Another argument against competitive (and for an allosteric synergistic) interaction is provided by the prolonged trains of bursts themselves. Neither veratridine nor BDF 9145 alone induced repetitive bursts late after the start of a depolarizing pulse (Fig. 1), as would be required if the burst trains of Fig. 2 were the result of repetitive and competitive binding of the two drugs.

We have, thus, determined association and dissociation rates of veratridine at the single sodium channel by comodifying the channel with a second allosteric activator that imposes a high conductance conformation during the veratridine-free state. Veratridine-induced bursts in the absence of the coactivator (Fig. 1b) and those enclosed by BDF 9145-induced F-bursts (Figs. 2 and 3) had an exponentially distributed duration, with time constants agreeing to within 5%. Veratridine-induced bursts thus seem to directly reflect the lifetime of the veratridine-sodium channel complex in heart sarcolemma. There was no evidence of a prolonged veratridine-bound inactivated state. For the single cardiac sodium channel deprived of inactivation by BDF 9145, we have determined a veratridine association rate constant $k_1 = 4.3 \times 10^6$ M^{-1} sec^{-1}, dissociation rate constant $k_{-1} = 2.2$ sec^{-1}, and equilibrium dissociation constant $K_D = 5.1 \times 10^{-7}$ M (20°, −30 mV membrane potential). A very similar estimate of k_1 was obtained by Barnes and Hille (6) (1.6–4.5 × 10^6 M^{-1} sec^{-1}), who counted the fraction of normal sodium channel openings leading to a veratridine modification in neuroblastoma cells.

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