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# Gating in Iodate-Modified Single Cardiac $Na^+$ Channels

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**Summary.** Elementary $Na^+$ currents were recorded at 19°C during 220-msec lasting step depolarizations in cell-attached and inside-out patches from cultured neonatal rat cardiocytes in order to study the modifying influence of iodate, bromate and glutaraldehyde on single cardiac $Na^+$ channels.

Iodate (10 mmol/liter) removed $Na^+$ inactivation and caused repetitive, burst-like channel activity after treating the cytoplasmic channel surface. In contrast to normal $Na^+$ channels under control conditions, iodate-modified $Na^+$ channels attain two conducting states, a short-lasting one with a voltage-independent lifetime close to 1 msec and, likewise tested between $-50$ and $+10$ mV, a long-lasting one being apparently exponentially dependent on voltage. Channel modification by bromate (10 mmol/liter) and glutaraldehyde (0.5 mmol/liter) also included the occurrence of two open states. Also, burst duration depended apparently exponentially on voltage and increased when shifting the membrane in the positive direction, but there was no evidence for two bursting states. Chemically modified $Na^+$ channels retain an apparently normal unitary conductance (12.8 ± 0.5 pS). Of the two substates observed, one of them is remarkable in that it is mostly attained from full-state openings and is very short living in nature; the voltage-independent lifetime was close to 2 msec. Despite removal of inactivation, open probability progressively declined during membrane depolarization. The underlying deactivation process is strongly voltage sensitive but, in contrast to slow $Na^+$ inactivation, responds to a voltage shift in the positive direction with a retardation in kinetics. Chemically modified $Na^+$ channels exhibit a characteristic bursting state much shorter than in DPI-modified $Na^+$ channels, a difference not consistent with the hypothesis of common kinetic properties in noninactivating $Na^+$ channels.

**Key Words** removal of $Na^+$ inactivation · iodate · bromate · glutaraldehyde · DPI 201-106 · $Na^+$ channel kinetics

## Introduction

Voltage-gated $Na^+$ channels mediate the transmembrane $Na^+$ influx during membrane depolarization in heart muscle and are essential for cardiac excitation under physiological conditions. They share with $Na^+$ channels in other excitable tissues common elementary properties including a voltage-dependent inactivation, which is conventionally termed as h-process in the Hodgkin-Huxley terminology and effectively controls the $Na^+$ conductance. $Na^+$ inactivation is well-established as a vulnerable process (Rojas & Armstrong, 1971), which may be selectively removed by a group of chemicals capable of reacting with certain amino acids or by proteolysis of the cytoplasmic channel surface. Another family of modifiers comprising naturally occurring toxins like batrachotoxin (BTX) and scorpion toxin or plant alcaloides (for review *see* Hille, 1984) remove $Na^+$ inactivation by interacting with a channel-associated binding site. Organic compounds, for example the diphenylpiperazinyl indole derivative DPI 201-106, may exert the same effect (Kohlhardt, Fröbe & Herzig, 1986).

Removal of $Na^+$ inactivation turned out to be a valuable tool to define the $Na^+$ channel properties in greater detail. The most important result from single-channel experiments is that noninactivating $Na^+$ channels resemble kinetically in many aspects other ionic channels like $Cl^-$ or several $K^+$ channels intrinsically devoid of voltage-dependent inactivation. This, simultaneously, emphasizes the dominating role of $Na^+$ inactivation in terminating the open state. Attempts to identify the amino acid residues structurally involved in this gating process were less successful. Most of the chemicals experimentally employed including N-bromoacetamide (Oxford, Wu & Narahashi, 1978), aldehydes (Nonner, Spalding & Hille, 1980) or chloramine-T (Ulbricht & Stoye-Herzog, 1984) are not specific enough to react with only one species of amino acid. They may influence the channel function in a rather complex fashion as suggested by the blocking potency, which is additionally exerted by chloramine-T (Huang, Tanguy & Yeh, 1987).

Iodate belongs to a group of oxidant protein modifiers (Gorin & Godwin, 1966) and is known from earlier $Na^+$ current measurement in voltage-clamped axons to irreversibly eliminate $Na^+$ inactivation when applied internally (Stämpfli, 1974). The

main goal of the present experiments was to analyze the effect of iodate and related agents on the single-channel level in order to characterize the elementary properties of chemically modified $Na^+$ channels. It will be shown that chemically modified cardiac $Na^+$ channels possess some characteristic properties including two open states not common, for example, with the gating in DPI-modified cardiac $Na^+$ channels.

## Materials and Methods

Elementary $Na^+$ currents were recorded with an L-M/EPC 5 amplifier in cell-attached and inside-out patches from cultured neonatal rat cardiocytes by employing the standard patch-clamp technique (Hamill et al., 1981). The cell culture combined methods as elaborated by Mark and Strasser (1966), and by Blondel, Riojeu and Cheneval (1971), and was already described in detail (Kohlhardt et al., 1986). Briefly, the cardiocytes were seeded either immediately after disaggregation or after storage at 8°C for up to three days in CMRL medium (supplemented with 10% fetal calf serum). Short-time (18–24 hr) cultured cardiocytes were washed and equilibrated for 5 min in modified Tyrode solution (solution A) before exposing them to isotonic KCl solution (solution B). Under the latter environmental conditions, their resting potential was close to 0 mV which, consequently, abolished their tendency to beat spontaneously. A major fraction of $K^+$-depolarized cardiocytes responded even to low external $Ca^{2+}$ concentrations (i.e., 0.1–0.2 mmol/liter) with a granulation of their cytoplasm. By using a nominally $Ca^{2+}$-free (residual concentration 20–30 $\mu$mol/liter) solution, this complication, which indicates a $Ca^{2+}$ intolerance, can be avoided and the cardiocytes retained their normal morphology for 1–2 hr or longer.

$Na^+$ channel activity was triggered by rectangular membrane depolarizations of 220 msec in duration at a rate of 0.33 Hz from a holding potential between −110 and −130 mV. The command impulse was delivered from a conventional stimulator. The holding potential was adjusted to a level where the largest number of activity sweeps (i.e., records with openings) without superpositions and the smallest number of blank sweeps (i.e., records without detectable openings) could be obtained. The minimum number of functioning $Na^+$ channels in a patch was two. The recordings were filtered (8-pole Bessel filter) at 1 kHz, digitized by a microcomputer at 5 kHz and stored on floppy discs.

The patch-clamp recordings were corrected for a residual capacity transient and for the leakage current. Open time and closed time were analyzed according to the 50% unitary current method (Colquhoun & Sigworth, 1983) from nonoverlapping events. Mean open time was calculated from $\bar{t}_{open} = \Sigma_i n_i T_i/n$ (Fenwick, Marty & Neher, 1982), where $T_i$ is the duration of $n_i$ open channels and $n$ means the number of events. Histogram analysis was based on an unweighted fit, i.e., each bin was considered to be of identical significance. To fit late and, therefore, rare events correctly, a commonly accepted procedure was used: several bins were lumped to give a larger bin containing a certain minimum of events, in the present analysis arbitrarily chosen to be four. The frequency distribution of open and closed times yielded $\tau_{open}$ and $\tau_{closed}$, respectively, and resulted from the best fit of the probability density functions according to the least squares ($X^2$) method.

Burst analysis was based on the closed-time distribution (Colquhoun & Sigworth, 1983) found in an individual patch. As will be described below, modified $Na^+$ channels may attain at least two closed states, a very short-lasting one with a lifetime close to 0.5 msec and a longer-lasting one with a several-fold larger lifetime. To distinguish closed events within bursts from those between bursts, a critical time was chosen, which is a multiple of $\tau_{closed(1)}$, but smaller than $\tau_{closed(2)}$, and was calculated from the geometric mean value of both closed-time constants. As the ratio $\tau_{closed(2)}/\tau_{closed(1)}$ was rather small and only took values of approximately 6, an unambigious burst definition may be difficult, thus, leading to a notorious error in burst duration. It was, therefore, important to see that the closed-time distribution within bursts was monoexponentially distributed, a result which is expected for theoretical reasons and suggests that this error in burst definition seems to be small. Burst duration is given as $\tau_{burst}$ or $\bar{t}_{burst}$.

To minimize the error in the open-time analysis as arising from the dead time (0.18 msec) in the present recording conditions, some of the data were corrected for unresolved events by applying the procedure introduced by Neher (1983).

Whenever possible, the data are given as mean ± SEM.

### Solutions (Composition in mmol/liter)

(A) Modified tyrode solution: NaCl 137; $CaCl_2$ 0.1; $MgCl_2$ 5; Na pyruvate 5; glucose 20; HEPES 10; pH 7.4. (B) Isotonic KCl solution: KCl 140; $CaCl_2$ 0.1 (or nominally 0); $MgCl_2$ 5; Na pyruvate 2.5; glucose 20; HEPES 10; pH 7.4. (C) $K^+$aspartate solution: K aspartate 120 (or 130); $MgCl_2$ 5; NaCl 2.5; Na pyruvate 2.5; glucose 20; HEPES 10; EGTA 2; pH 7.4. (D) Pipette solution: NaCl 200; $CaCl_2$ 0.2; $MgCl_2$ 5; HEPES 10; pH 7.4.

Purified $KJO_3$ (10 mmol/liter) or $KBrO_3$ (10 mmol/liter) (analytical grade; Merck, Darmstadt) was added to solution C containing 120 mmol/liter $K^+$ aspartate just before use. Freshly diluted, purified glutaraldehyde was added to solution C containing 130 mmol/liter $K^+$ aspartate. Racemic DPI 201-106 was freshly dissolved in HCl and diluted in pipette solution to give a final concentration of $3 \times 10^{-6}$ mol//liter. The compound was kindly provided by Sandoz, Basle.

## Results

### The Manifestation of the Modifying Iodate Effect

A characteristic feature of $Na^+$ channels in cultured neonatal cardiocytes is their less pronounced tendency to reopen (Kohlhardt, Fichtner & Fröbe, 1988). At −50 mV, for example, channel activity is restricted to only a few milliseconds (Fig. 1, left part) as the time-dependent open probability, $P_o(t)$, rapidly declines during membrane depolarization. In analyzing the decay kinetics of reconstructed macroscopic $Na^+$ currents, current relaxation to 0.5% of peak $I_{Na}$ was found to be accomplished within 15.1 ± 1.6 msec ($n$ = 6; −50 mV). A quite

different activity pattern occurred after treating the cytoplasmic membrane surface with iodate (10 mmol/liter). Patch excision into an iodate-containing medium (Fig. 1, right part) evoked repetitive, burst-like openings including the appearance of late channel openings several tens of milliseconds after the onset of the command impulse. Consequently, the number of openings during membrane depolarization may rise significantly. Clamping back the membrane to the holding potential effectively terminated channel activity. Spontaneous openings at the holding potential were never observed.

Pronounced changes in $P_o(t)$ were observed. Reconstructed macroscopic $Na^+$ currents showed a significantly retarded decay phase. In two out of six experiments at $-50$ and $-20$ mV, two kinetically different decay components became detectable (Fig. 2) suggesting that the decrease in $P_o(t)$ may proceed with second order kinetics. An alternative explanation would be a heterogeneous channel population differing in $P_o(t)$.

Iodate-modified $Na^+$ channels can attain at least two closed states. A systematic histogram analysis revealed that the closed-time distribution could be best fitted in about 50% of the iodate experiments by three exponentials. At $-50$ mV, for example, $\tau_{closed(1)}$ was close to 0.5 msec, $\tau_{closed(2)}$ amounted to about 3 msec and $\tau_{closed(3)}$ may attain values between 50 and 70 msec. However, $\tau_{closed(3)}$ is not necessarily indicative of an ultralong closed state since the ultralong closed time is ambiguous when, as in the present experiments, more than one channel is operating in the patch. Ultralong closed times may, therefore, alternatively reflect gaps between two individual $Na^+$ channels. This hypothesis was tested in three patches at $-50$ mV in a stochastic analysis according to

$$L_{(N)} = \left( \frac{1}{1 + \left( \frac{N-1}{N} \right) \cdot \frac{MOT}{MC}} \right)^n$$

(Colquhoun & Hawkes, 1983). In this equation, $N$ means the number of channels whose likelihood to open sequentially is estimated, MOT and MC refers to mean open and mean closed time and $n$ refers to the number of events in the activity sweep. In the presence of ultralong gaps, repetitive openings, with a likelihood of 50% or more, reflect the activity of two individual channels. Late openings as demonstrated in Fig. 1 (right part) could, therefore, well be due to a late-activating channel not operating under control conditions.

Iodate treatment significantly influenced the open-state kinetics. Open-time histogram analysis

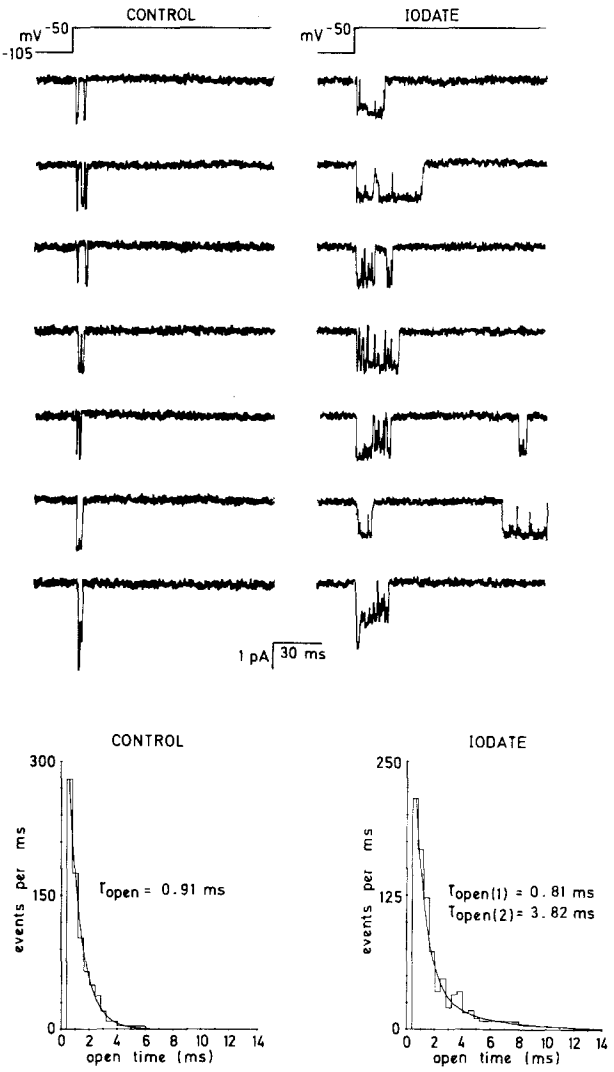



**Fig. 1.** Upper part: Recordings of elementary $Na^+$ currents under control conditions (left) and after patch excision into an iodate-containing (10 mmol/liter) solution (right). Selected sweeps were from an ensemble of 425 records collected prior to and of 435 records collected after iodate treatment. Lower part: Open-time histograms prior to (left) and after (right) the treatment with iodate with time constants as indicated. Patch 342CA/IO; $-50$ mV

revealed consistently a bimodal event frequency distribution, a result which fundamentally contrasts with the monoexponential distribution obtained under control conditions (*see* Fig. 1). $\tau_{open(1)}$ was close to $\tau_{open}$ in normal $Na^+$ channels whilst $\tau_{open(2)}$ increased several-fold; at $-50$ mV, for example, $\tau_{open(1)}$ amounted to $0.87 \pm 0.1$ msec and $\tau_{open(2)}$ to $2.54 \pm 0.14$ msec ($n = 5$). This suggests either two conductive states or two classes of channels, one of them having a normal conductive state and the other class having a prolonged conductive state.

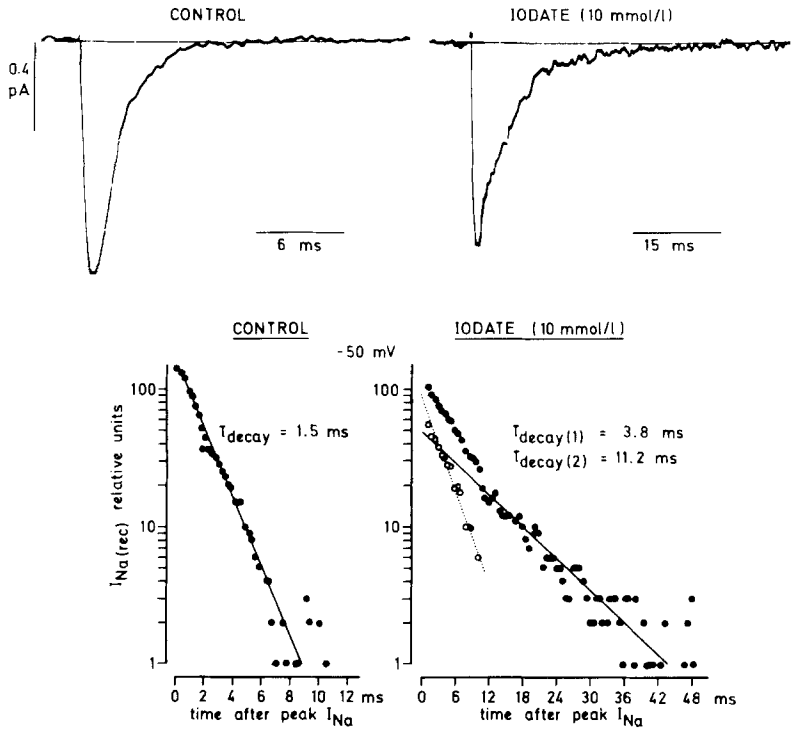



**Fig. 2.** Changes of $I_{Na}$ decay kinetics in the presence of iodate. Upper part: Reconstructed macroscopic $Na^+$ currents as averaged from an ensemble of 425 records under control conditions and of 435 records in the presence of iodate. Note the differences in time scale. The reduction in peak $I_{Na}$ under iodate is due to the run-down induced by patch excision. Lower part: Semilogarithmic plots of $I_{Na}$ decay *versus* time after peak current. Patch 342CA/IO; −50 mV

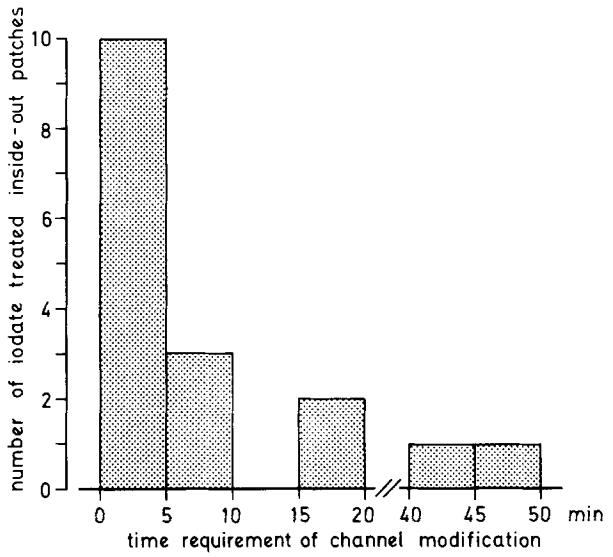



**Fig. 3.** Time requirement for the iodate-induced $Na^+$ modification in 17 inside-out patches. Zero time refers to patch excision in the iodate-containing solution

Two classes could conceivably arise from a differential channel sensitivity to iodate in that a fraction in the multi-channel patch is suspected to preserve normal properties. This idea was tested in a biased open-time analysis. It was based on $P_o(t)$ found in normal $Na^+$ channels and neglected, at −50 mV, events occurring during the first 20 msec of membrane depolarization. Consequently, iodate-resistant, normally gating $Na^+$ channels should be eliminated. Nevertheless, such biased open-time histograms still could be best fitted by the sum of two exponentials and yielded the same values for $\tau_{open(1)}$ and $\tau_{open(2)}$ as in the unbiased analysis. A coexistence of normal with modified $Na^+$ channels is, therefore, unlikely.

Modified $Na^+$ channel activity occurred in most of the experiments rapidly, i.e., within 5 min after iodate exposure (Fig. 3), and developed in an all-or-none fashion. The conductive state was abruptly prolonged from one depolarization to the other as found in analyzing $\bar{t}_{open}$ from individual activity sweeps which were continuously triggered at a rate of 0.2 Hz after excising the patch in the iodate-containing solution. Iodate experiments in cut-end axons, however, have revealed that the retardation of $I_{Na}$ decay kinetics requires a longer time to appear (Stämpfli, 1974). This discrepancy may be easily explained with methodological differences. In the present experiments, the cytoplasmic $Na^+$ channel surface is freely accessible for iodate whilst, in the cut-end axons, and iodate diffusion through the cytoplasm may well be a time-dependent, rather slow process.

It was, therefore, a surprising result that the fast response to iodate was not always the rule. In two inside-out patches, an exposure time of 43 and 48 min, respectively, was required until the $Na^+$ channels switched into the modified activity mode. A virtual iodate resistance was obtained in three other experiments where normal channel activity persisted during an iodate treatment of 60, 63 and 75

min. Nonresponding, late responding and rapidly responding experiments are comparable in that the patches contained at least three functioning $Na^+$ channels. Moreover, the channels shared under control conditions the same biophysical properties as judged at −50 mV from open-state and reopening kinetics and the unitary current size. Whether heterogeneous molecular properties could be underlying the divergent iodate responses remains to be elucidated.

Bromate (10 mmol/liter) exerted the same modifying effect when applied to the cytoplasmic membrane surface. At −50 mV, conventional and biased open-time histogram analysis revealed a bimodal event frequency distribution with values for $\tau_{open(1)}$ of 0.85 ± 0.05 msec ($n$ = 3) and for $\tau_{open(2)}$ of 2.8 ± 0.19 msec ($n$ = 3).

More interesting is the effect of glutaraldehyde as this protein reagent differs from iodate and bromate in the chemical mode of action. Although external treatment is likewise effective, glutaraldehyde (0.5 mmol/liter) was internally applied in order to create comparable experimental conditions. The chemical-induced kinetic changes including the occurrence of two conductive states are not distinguishable from the iodate effect. The experiment demonstrated in Fig. 4 still merits a comment because a $Na^+$ channel switched already under control conditions into an apparently modified activity mode. One out of 518 repetitive (frequency 0.5 Hz) membrane depolarizations triggered repetitive, burst-like activity (*see* Fig. 4, left part, record 3) showing about a threefold prolonged open time (2.44 msec) when compared with $\bar{t}_{open}$ (0.79 msec) of the whole ensemble. A similar activity mode has been reported in adult myocardium (Patlak & Ortiz, 1985) and skeletal muscle (Patlak, Ortiz & Horn, 1986) and is suggestive for a spontaneous failure of inactivation (Kohlhardt, Fröbe & Herzig, 1987). Interestingly, $\bar{t}_{open}$ during this episode was close to $\tau_{open(2)}$ (3.06 msec) found after channel modification with glutaraldehyde.

Despite the existence of two open states, chemically modified $Na^+$ channels may only attain a single bursting state, at least during a 220-msec lasting membrane depolarization. Burst-time histograms could be best fitted by a single exponential and gave values for $\tau_{burst}$ (at −50 mV) of 4.4 ± 0.5 msec with glutaraldehyde ($n$ = 3), 4.5 ± 0.5 msec with iodate ($n$ = 3) and 5.1 ± 1.0 msec with bromate ($n$ = 2).

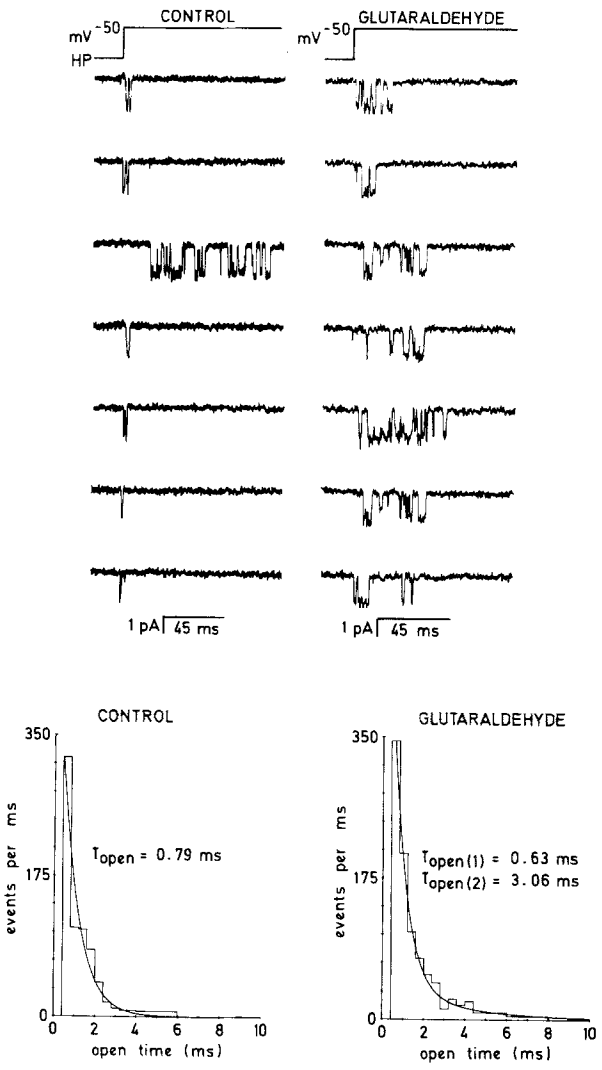



**Fig. 4.** Upper part: Recordings of elementary $Na^+$ currents prior to (left) and after (right) patch excision into a glutaraldehyde-containing (0.5 mmol/liter) solution. Note record 3 in the control registrations. Selected sweeps were from an ensemble of 518 records collected under control conditions and of 663 records in the presence of glutaraldehyde. Lower part: Open-time histograms prior to (left) and after (right) glutaraldehyde treatment with time constants as indicated. Patch 456CA/IO; −50 mV

## Conducting Properties in Iodate-Modified $Na^+$ Channels

Elementary currents passing chemically modified $Na^+$ channels clearly sense changes in membrane potential. Current-voltage relationships were analyzed under asymmetrical ionic conditions between −50 and +20 mV and showed in this range an apparent linear shape (Fig. 5). The unitary channel conductance (12.8 ± 0.5 pS; $n$ = 6) agrees fairly well with the conductance of normal $Na^+$ channels in several cardiac preparations including rat heart (15 pS; Patlak & Ortiz, 1985), Purkinje fibers (16 pS; Scanley & Fozzard, 1987) or frog heart (10 pS; Clark & Giles, 1987).

Fully-sized and substate openings may coexist during membrane depolarization. Figure 5 demonstrates the most frequently detectable substate. It represents a fraction of about 10% of an ensemble and also occurred in bursts. The lifetime was indistinguishable from $\bar{t}_{open}$ in the full state. Both states

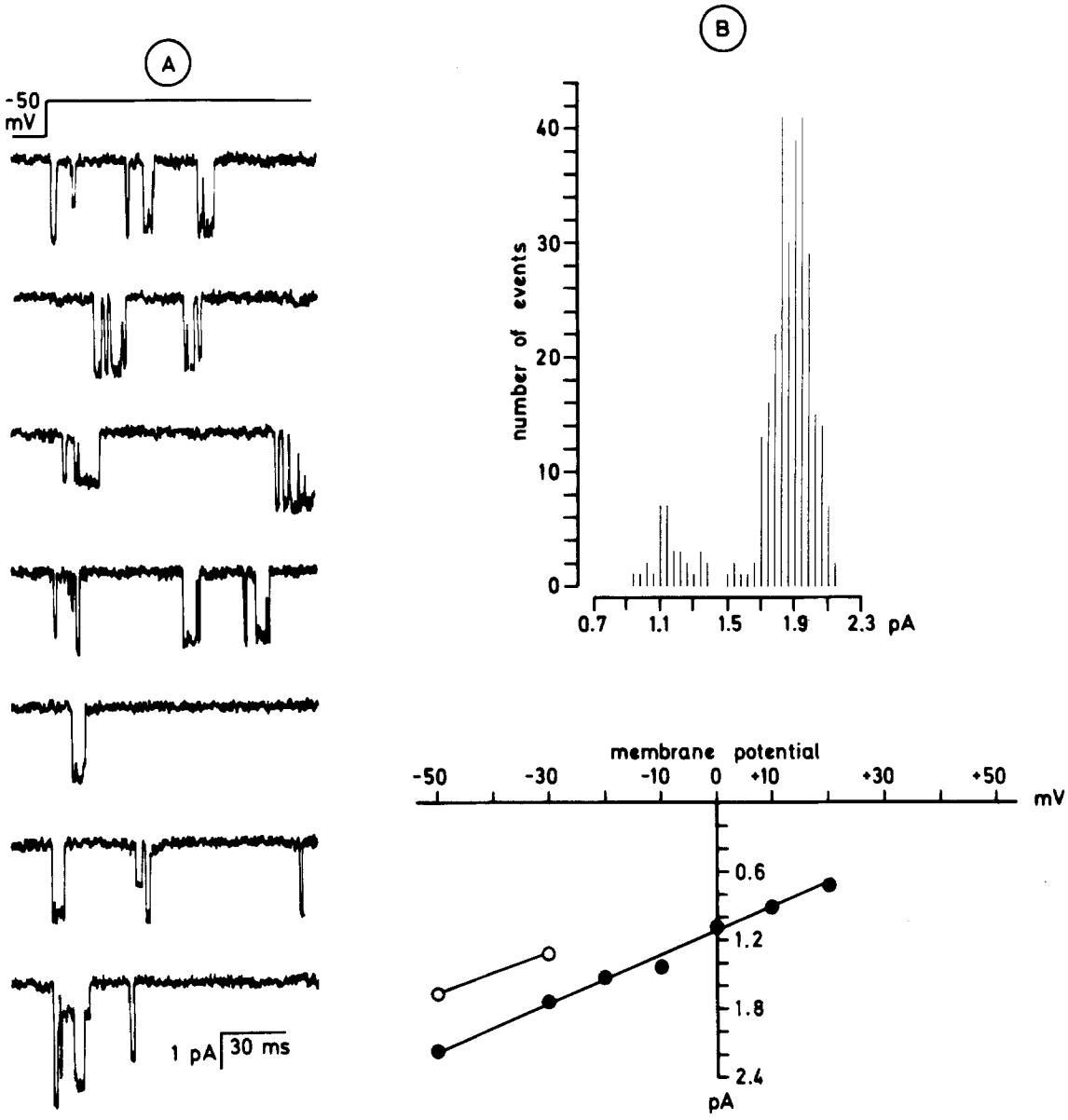



**Fig. 5.** Full-state and substate openings in iodate-modified $Na^+$ channels. (*A*) Selected records of elementary $Na^+$ currents with substate openings in records 1, 3, 6 and 7 at −50 mV. (*B*) Amplitude histogram at −30 mV. (*C*) *IV*-relationship of the full state (filled circles) and of the substate (open circles). Patch 377IO

can be separated by gaps of several tens of milliseconds. In this case, they are unlikely to reflect varying conducting properties of an individual channel but, as found in a stochastic analysis, are due to two separate channels with a calculated likelihood of more than 50%.

Another type of substate is illustrated in Fig. 6. If detectable, it was mostly observed during long-lasting full state openings. Obviously, an individual channel can transitorily leave its fully conducting state. This event is rare (estimated likelihood about 1%) and of short lifetime, as the mean open time of this substate was found to be close to 2 msec. Another peculiarity is the voltage independence of its lifetime.

## Voltage Dependence of $P_o$ and Open State

$P_o$ of iodate-modified $Na^+$ channels is a function of voltage and was found to increase upon shifting of the membrane potential in the positive direction. This resulted from reconstructed $I_{Na}$ by analyzing an arbitrarily chosen portion attained at the end of the 220-msec lasting step depolarization (Fig. 7*A*).

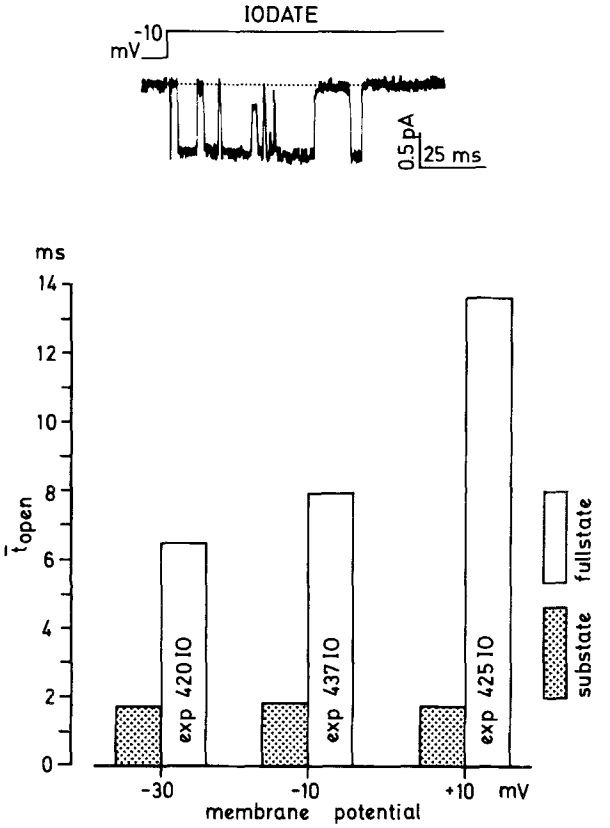



**Fig. 6.** Transitory substate in iodate-modified $Na^+$ channels. Upper part: Selected activity sweep with a well-resolved short-lasting substate. The dotted line symbolizes zero current. Membrane potential was −10 mV. Lower part: Voltage dependence of the lifetime of transitory substate (shaded columns) and of the full state (open columns). For clarity, lifetime was expressed in both bases as $\bar{t}_{open}$. The ratio between the number of resolved transitory substate openings and the number of full-state openings was 0.030 in expt 420IO, 0.005 in expt 437IO and 0.012 in expt 425IO

$I_{Na}$ decay kinetics depended similarly on voltage and became significantly retarded by membrane depolarization as evidenced by the rise in $\tau_{decay}$ (Fig. 7*B*). This relationship is in fundamental contrast to the voltage dependence of $I_{Na}$ decay under normal conditions when $Na^+$ inactivation is operating, since $\tau_h$ is expected to decline in response to a potential shift from −50 to +10 mV. A time-independent, sustained $I_{Na}$ component was not observed.

The open-state kinetics showed the same voltage dependence as $P_o$ and also seem to be exponentially related to membrane potential. It is important to note that the voltage sensitivity was restricted to $\tau_{open(2)}$. In the experiment depicted in Fig. 8, for example, $\tau_{open(2)}$ (corrected for missed events) increased from 1.45 to 4.55 msec between −50 and +10 mV, while $\tau_{open(1)}$ remained almost unchanged and was close to 1 msec. The failing voltage dependence of $\tau_{open(1)}$ is strongly reminiscent of

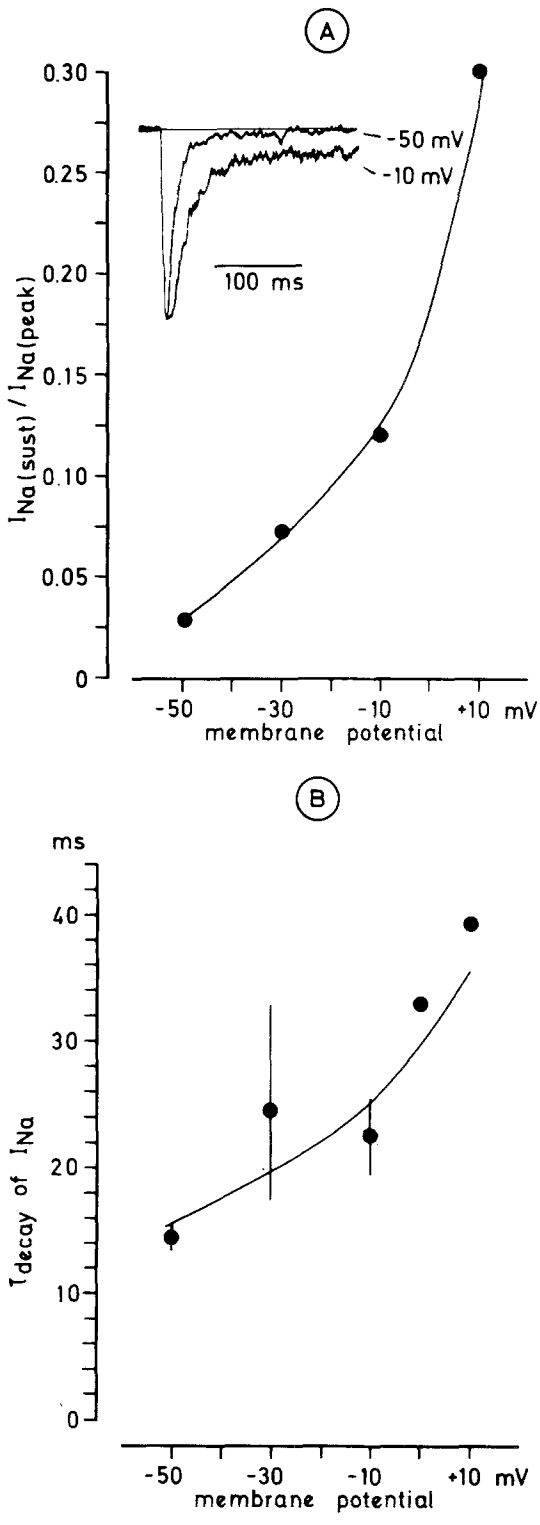



**Fig. 7.** Voltage dependence of $I_{Na}$ in iodate-modified $Na^+$ channels. (*A*) Voltage dependence of a residual fraction of peak $I_{Na}$ (labeled as $I_{Na(sust)}$ and measured 220 msec after onset of the command impulse). The inset shows superimposed and normalized current traces at −50 and −10 mV. The curve relating the current to the voltage was drawn by eye. (*B*) Voltage dependence of $\tau_{decay}$. The data points at −50, −30 and −10 mV are mean values of 5, 3 and 4 inside-out patches; vertical bars indicate SEM. The curve relating $\tau_{decay}$ to voltage was drawn by eye

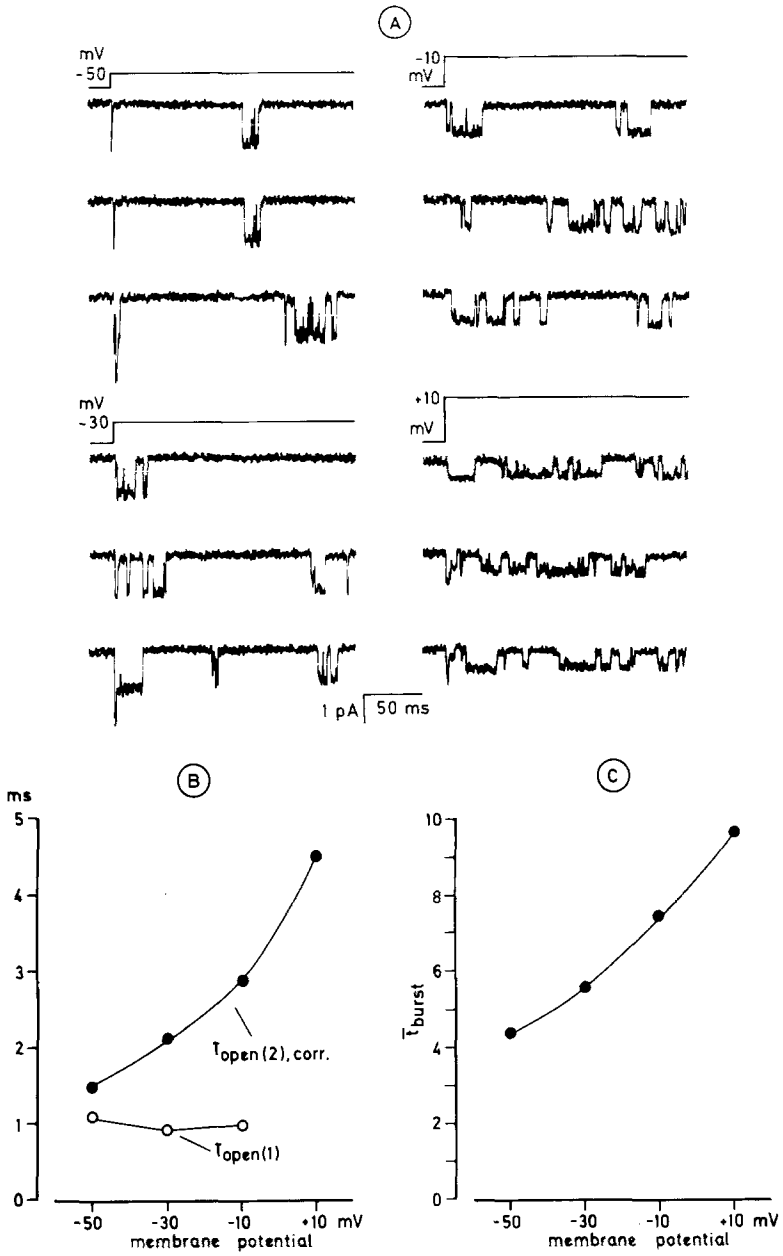



**Fig. 8.** Voltage dependence of open-state kinetics in iodate-modified $Na^+$ channels. (*A*) Selected recordings of elementary $Na^+$ currents at −50, −30, −10 and +10 mV. (*B*) Voltage dependence of $\tau_{open(1)}$ (open circles) and of $\tau_{open(2)}$ (filled circles). $\tau_{open(2)}$ was corrected for unresolved events (*see* Materials and Methods) to avoid major errors in determining the steepness of the voltage dependence. The latter follows the function $y = 3.7 \exp(V/52.7)$. (*C*) Voltage dependence of the burst duration. The data points follow the function $y = 8.5 \exp(V/75)$. Patch 416IO

the open-state properties in normal $Na^+$ channels with intact inactivation (Aldrich, Corey & Stevens, 1983; Kohlhardt et al., 1987; Kirsch & Brown, 1989).

Burst duration also varied as a function of voltage and became increasingly prolonged due to depolarization (Fig. 8*C*). Again, $\bar{t}_{burst}$ seems to be exponentially related to membrane potential, at least between −50 and +10 mV. This is not surprising with respect to the voltage dependence of $\tau_{open(2)}$, but disagrees with results in noninactivating $Na^+$ channels of mouse neuroblastoma cells (Quandt, 1987). In the latter, a lumped analysis comprising several individual patches provided evidence for a relationship with saturation characteristics.

### Different Burst Kinetics In Iodate-Modified and DPI-Modified Channels

Although iodate will finally remove $Na^+$ inactivation and, thus, exerts phenomenologically the same effect as other agents capable of provoking channel modification, modified cardiac $Na^+$ channels do not share common kinetic properties. This became evident from a comparison of iodate-modified channels

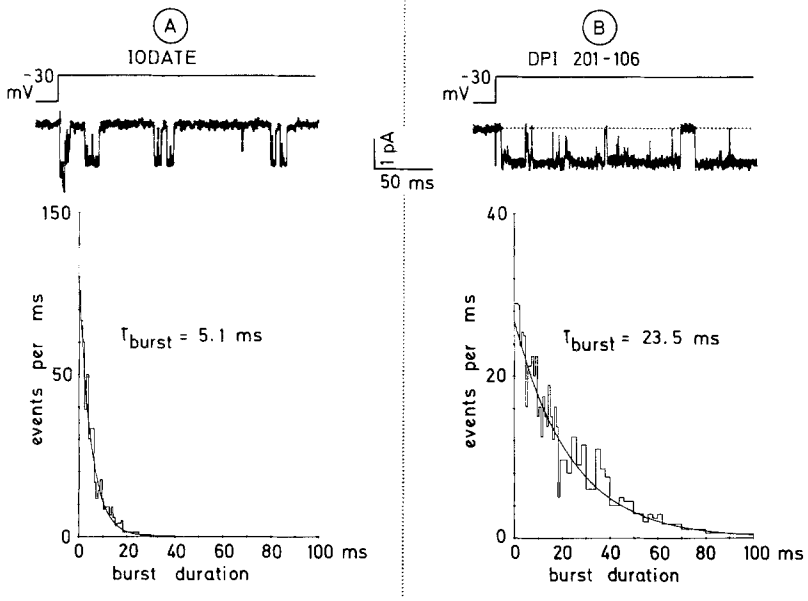



**Fig. 9.** Burst kinetics in iodate-modified $Na^+$ channels (*A*) and in DPI-modified (*B*) $Na^+$ channels. The burst-time histograms yield time constants as indicated. Patch 420IO and 130IO

with DPI-modified channels (Fig. 9). The latter were likewise studied in the inside-out patch configuration. DPI 201-106 ($3 \times 10^{-6}$ mol/liter, externally applied) also caused repetitive, burst-like activity (Kohlhardt et al., 1986), but $P_o$ retained a higher level during membrane depolarization than with iodate because of a significantly shorter duration of the ultralong closed state. This was evident by comparing closed-time histograms. At −30 mV, for example, $\tau_{closed(3)}$ was 10.56 ± 2.1 msec ($n$ = 2) with DPI, but 68.3 ± 2.5 msec ($n$ = 4) with iodate. $\tau_{closed(1)}$ and $\tau_{closed(2)}$, however, were not dependent on the agent employed for channel modification. Moreover, DPI-modified $Na^+$ channels attained the longer-lasting burst state. Burst-time histograms from 220-msec step depolarizations were monoexponentially distributed, $\tau_{burst}$ was 21.2 ± 3.5 msec (−30 mV; $n$ = 2). This exceeds $\tau_{burst}$ in iodate-modified channels by a factor of approximately 3. Obviously, the bursting state may be sensitive to the particular modifying agent, a conclusion not consistent with the hypothesis that its only kinetic effect is a removal of the inactivation process.

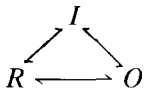
## Discussion

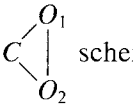
Iodate-modified cardiac $Na^+$ channels share many kinetic properties with other noninactivating $Na^+$ channels. Analogously to BTX (Quandt & Narahshi, 1982), deltametrin (Chinn & Narahshi, 1986), N-bromoacetamide (Patlak & Horn, 1982) or DPI 201-106 (Kohlhardt et al., 1986), iodate, too, induced repetitive, burst-like activity and simultaneously prolonged the open state as another manifestation of an increased open probability. The strongest evidence for removal of inactivation seems to be the fundamentally altered voltage dependence of the open state. The iodate effect may be the final result of a cleavage of S-S bonds (Gorin & Godwin, 1966) in the $Na^+$ channel protein and, in this respect, is remarkable as it developed under experimental conditions not fitting the pH optimum of this chemical reaction. Iodate-modified $Na^+$ channels terminate their conducting state like other noninactivating $Na^+$ channels by a highly voltage-sensitive transition into a closed, nonabsorbing configuration.

Channel modification by iodate, bromate or glutaraldehyde consistently included the occurrence of two open states. Multiple open states are well established in chloramine-T-modified $Na^+$ channels (Nagy, 1988) and in $Na^+$ channels with enzymatically destroyed inactivation (Quandt, 1987). In contrast, removal of inactivation by BTX (Quandt & Narahashi, 1982; Huang et al., 1984; Keller et al., 1986), deltametrin (Chinn & Narahashi, 1986) or DPI 201-106 (Kohlhardt et al., 1986) allows $Na^+$ channels to attain only a single open state. This suggests a specific influence of modifying agents, which might be related to their molecular mode of action, covalent binding and irreversible structural changes of the channel protein or interaction with a channel-associated binding site. The appearance of two open states is of particular relevance in cardiac $Na^+$ channels as they are proven to have a single conducting state under normal conditions, i.e., in the presence of operating inactivation. This means that iodate and the other chemicals would introduce a second open state with properties just mentioned,

i.e., long lasting and highly voltage dependent. However, it cannot be excluded that normal $Na^+$ channels may also reach the latter but with a likelihood, which could be too small to be detectable even in open-time histograms based on a large event ensemble (500–1000 events in the present experiments).

$Na^+$ channels can be modeled, in the simplest form, by the reaction scheme




where $R$ combines the three closed states ($C_1$, $C_2$, $C_3$; Horn & Vandenberg, 1984), $O$ means the open and $I$ is the inactivated, absorbing (Aldrich et al., 1983) configuration. Accordingly, modifying agents may be supposed to reduce critically the transition rate from $O$ to $I$, which would enable the $Na^+$ channel to cycle repetitively between $O$ and $R$. In fact, a $C$–$C$–$O$ reaction scheme sufficiently describes the gating in BTX-modified $Na^+$ channels (Keller et al., 1986). The highly voltage-dependent $O$-state in BTX- and DPI-modified $Na^+$ channels is consistent with the hypothesis that removal of inactivation demasks this intrinsic property. Gating in chemically modified $Na^+$ channels, however, is more complicated because of the existence of two open states, $O_1$ and $O_2$. Clearly, the $C$–$C$–$O$ reaction is specifically related to the BTX (and probably to the DPI) modification but cannot be considered to represent generally the gating in noninactivating $Na^+$ channels. Whether a  scheme or another conceivable Markovian reaction will be the valid model awaits clarification in a maximum likelihood analysis. Nevertheless, it becomes evident that iodate, bromate and glutaraldehyde exert a more complex influence, i.e., the transition from $O$ to $I$ appears unlikely to be the only vulnerable or sensitive reaction in the presence of these chemicals. The approximately threefold longer bursting state in DPI-modified $Na^+$ channels observed in the present experiments provides another argument that a reduced exit rate from $O$ to $I$ does not necessarily represent the only response to a modifying agent. Recent observations in neuronal $Na^+$ channels with chloramine-T, sea anemona toxin and scorpion toxin agree with this conclusion as they revealed quite individual, modifier-dependent transition rates (Nagy, 1988).



Numerous studies in neuronal preparations and skeletal muscle have confirmed that slow $Na^+$ inactivation can survive the removal of fast $Na^+$ inactivation (Oxford et al., 1978) since $I_{Na}$ retains its characteristic sigmoidal dependence on holding potential in the presence of modifying agents. Fast and slow $Na^+$ inactivation are, therefore, proposed not to be coupled with each other. Slow $Na^+$ inactivation controls under normal conditions a comparatively small fraction of $Na^+$ conductance and gives rise to the multiexponential $I_{Na}$ decay, but is unlikely the process which determines the decline of $P_o$ of chemically modified cardiac $Na^+$ channels during membrane depolarization. Slow $Na^+$ inactivation operates also in heart muscle with a time constant ($\tau_{h(2)}$) having the same voltage dependence as the time constant of fast $Na^+$ inactivation, $\tau_{h(1)}$ (Brown, Lee & Powell, 1981), i.e., $\tau_{h(2)}$ declines with membrane depolarization. The decay kinetics of reconstructed $I_{Na}$ in iodate-modified $Na^+$ channels, however, were found to be inversely related to voltage. The same increasing retardation in $I_{Na}$ decay on shifting the membrane potential in the positive direction was previously reported in neuronal $Na^+$ channels with enzymatically destroyed inactivation (Quandt, 1987). This deactivation seems to be an inherent channel property not identical with slow $Na^+$ inactivation and is evidently demasked by removal of fast $Na^+$ inactivation.

To consider the possible site of action of iodate, easily accessible amino acids, which constitute the cytoplasmic channel surface, are the most attractive candidates since, for physicochemical reasons, iodate and related chemicals barely penetrate this superficial region and are unlikely to find a reactant buried in the core of the $Na^+$ channel protein. Cytoplasmically localized amino acid chains supposed to be adjacent to the domains of the alpha-subunit and to form connecting loops (Catterall, 1986) may be another target. Iodate, as well as bromate, reacts preferentially with S-S bonds between cysteine molecules (Gorin & Godwin, 1966), while glutaraldehyde, the other chemical modifier studied, reacts with $\varepsilon$-amino groups of lysine, thereby crosslinking them to neighboring amide, guanidyl and imidazole groups in order to form a Schiff base. Other but less likely reactions would be an oxidation of lysine by iodate and a chemical modification of cysteine by glutaraldehyde. Lysine attracts particular interest as a possible target because it is a functionally important constituent of the so-called S4 segment. The S4 segment is found in each of the four repeated domains, which are forming the alpha-subunit of the $Na^+$ channel protein (Noda et al., 1984). It is outstanding among the other five segments by bearing positive charges. The latter are provided by the side chains of arginine and lysine. The S4 segment is, therefore, proposed to act as voltage sensor and to

be basically involved in the channel gating (Catterall, 1986). The modifying efficacy of various amino group specific and cross-linking imidoesters, demonstrated in voltage-clamped frog nerves, has been related to their chemical interference with lysine (Drews & Rack, 1988). But it should be emphasized that several other protein reagents, which are lacking this apparently selective affinity for lysine, are likewise effective in eliminating $Na^+$ channel inactivation. N-bromoacetamide, for example, prefers methionine, cysteine and cystine (Means & Feeney, 1971) as reactants, which do not contribute to the structure of the S4 segment. It is rather speculative to propose that chemically modified amino acids may allosterically influence other amino acids forming the S4 segment or other parts of the alpha-subunit of the channel protein involved in gating. Tosteson, Auld & Tosteson, (1989) synthesized a 22-amino acid peptide with the same sequence as the S4 segment forming in lipid bilayers a voltage-gated channel. Highly interesting, this cationic channel is devoid of inactivation and displays repetitive activity. The assumption is, therefore, not plausible that chemical modification of lysine or other constituents of the S4 segment eliminates, in native $Na^+$ channels, fast $Na^+$ inactivation.

The hypothesis cannot be definitely rejected that $Na^+$ channels could also sense interacting influences arising from the surrounding lipid matrix. Arachidonic acid is a constituent of the latter and shares with other long-chain fatty acids the susceptibility to oxidants such as iodate and bromate. Thus, oxidized arachidonic acid could be suspected to play a role in the development of channel modification. However, the reactive bonds of long-chain fatty acids are in distinct distance from the membrane surface and remain, therefore, unaccessible for such lipophobic chemicals. This excludes $Na^+$ channel modification as an event which occurs secondarily to lipid oxidation in the vicinity.

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