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Na^+ Channel Blockade by Cyclic AMP and Other 6-Aminopurines in Neonatal Rat Heart

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Summary. Elementary Na^+ currents were recorded at 19°C in cell attached and inside-out patches from cultured neonatal rat cardiocytes in order to study the effect of cAMP and other 6-aminopurines.

The treatment of the cardiocytes with db-cAMP (1×10^{-3} mol/liter) led to a decline of reconstructed macroscopic peak I_{Na} to 62 ± 7.6% of the initial control value. This reduction in NP_o was mostly accompanied by a decrease in burst activity. Open-state kinetics were preserved even in DPI-modified, noninactivating Na^+ channels. Since the stimulator of the adenylate cyclase, forskolin (1×10^{-6} mol/liter), evoked a similar pattern of response, the NP_o decrease can be considered as the functional correlate of Na^+ channel phosphorylation brought about by cAMP-dependent protein kinase. As found in inside-out patches, cAMP (1×10^{-3} mol/liter) remained effective under cell-free conditions and reduced reconstructed macroscopic peak I_{Na} to about 50% of the initial control value when the absence of Mg-ATP at the cytoplasmic membrane surface prevents phosphorylation reactions. A very similar response developed in the cytoplasmic presence of other 6-aminopurines including ATP (1×10^3 mol/liter), adenosine (1×10^{-4} mol/liter), adenine (1×10^{-5} mol/liter) and hypoxanthine (1×10^{-5} mol/liter). This susceptibility to adenine suggests that cardiac Na^+ channels *in situ* could sense intracellular fluctuations of adenine nucleotides, most likely of ATP.

Key Words single cardiac Na^+ channels · phosphorylation · cAMP · ATP · adenine

Introduction

Phosphorylation represents an important and widespread regulatory principle in ionic channels. As final reaction in a chain of membrane-associated molecular events initiated by cAMP or other second messengers, phosphorylation modulates channel availability and couples channel activity to hormone or neurotransmitter stimulation. Its functional consequence is channel specific and may consist of either an enhanced or a diminished open probability. In contrast to cardiac L-type Ca^{2+} channels, Cl^- channels and many K^+ channels (for review *see* Levitan, 1985), voltage-gated, tetrodotoxin-sensitive Na^+ channels in excitable tissues may be classified to be less sensitive to intracellular cAMP fluctuations. Particularly in healthy atrial and ventricular myocardium, impulse generation and conduction is remarkably resistant to β_1-adrenergic catecholamines or to conditions which are accompanied by a reduced cAMP formation. An excitability largely independent of sympathetic innervation seems actually advantageous for heart function since it would preserve the pattern of impulse propagation over the atria and ventricles despite functionally relevant variations in β_1-adrenoceptor stimulation. During the last decade, however, several studies in partially depolarized myocardium with depressed Na^+ currents (I_{Na}) has led to circumstantial evidence against a frequently proposed principal unresponsiveness of cardiac I_{Na} (for review *see* Catterall, 1988). I_{Na} depression after interventions capable to elevate cAMP (Ono, Kiyosue & Arita, 1989; Schubert et al., 1989) is generally believed to emerge from a phosphorylation reaction of sites located in the intracellular domain between repeats I and II of the α-subunit of the Na^+ channel protein (for review *see* Catterall, 1988).

The present patch-clamp experiments in neonatal rat heart myocytes aimed to further study the influence of cAMP on cardiac Na^+ channels in order to define the biophysical correlate of the phosphorylated state in terms of elementary channel properties. Cell-attached recordings offer the advantage to analyze Na^+ channel activity without dialyzing the cell interior and were, therefore, favored in analyzing the cAMP action. Of particular interest was the question whether cAMP treatment selectively mod-

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ulates open probability or additionally influences open-state kinetics. It will also be shown that cAMP by itself can affect cardiac Na^+ channels by inducing a depression which resides on the adenine group of the molecule.

Materials and Methods

Disaggregated myocytes from newborn, 2–3 day-old rats were short-time cultured for 18–36 hr before using for patch-clamp experiments. Preparation and the handling of the cultured cells were essentially the same as already described in detail earlier (Kohlhardt, Fröbe & Herzig, 1986). To improve patch stability and to avoid errors from spontaneous fluctuations in membrane potential, automaticity as it frequently occurs particularly in rod-shaped myocytes under quasi-physiological conditions (solution A) was abolished by bathing the cardiocytes in an isotonic K^+ saline (solution B and C). In presence of these depolarizing solutions, the resting potential was close to 0 mV but morphological signs of Ca^{2+} intolerance may occassionally occur. The latter can be prevented by using EGTA-buffered solutions. At submicromolar external Ca^{2+} concentrations the cardiocytes retained their normal cytoplasmic structure for several hours and also tolerated the treatment with cAMP, isoproterenol and histamine without detectable morphological changes. The myocytes were kept for 10–15 min in the isotonic K^+ solution before a cell-attached patch-clamp experiment was begun.

Elementary Na^+ currents were triggered by rectangular membrane depolarizations of 70-msec duration at a rate of 0.67 Hz to test potentials between −60 and −40 mV and recorded in the cell-attached or inside-out mode with an L-M/EPC 5 amplifier by applying the standard patch-clamp technique (Hamill et al., 1981). The holding potential was adjusted to a suitable level between −100 and −120 mV in order to minimize both the number of activity sweeps with superpositions and the number of sweeps without activity (blanks). The recordings were filtered (8-pole Bessel filter) at 1 kHz, digitized with a sampling rate of 5 kHz and stored on floppy discs. The dead time was 0.2 msec.

Idealized patch-clamp recordings resulted from the subtraction of capacity and leakage currents. Open time of and gap times between nonoverlapping single events were analyzed according to the 50% unitary current method (Colquhoun & Sigworth, 1983). The probability density functions were based on an unweighted fit, i.e., each bin was considered to be of identical significance. Late and, therefore, rare events were fitted by lumping several bins with a certain minimum of events arbitrarily chosen to be four.

The analysis of repetitive, burst-like channel activity was based on activity sweeps without superpositions showing two or more sequential openings. Gap-time probability density functions revealed a bimodal event distribution with a class of short gaps (in the submillisecond range) which dominated by a factor of 5–6 a class of longer gaps (ranging between 3–4 msec). Despite the dominance of closely timed opening sequences, counting sequential openings will only yield an estimate for reopening in multichannel patches like in the present experiments.

Ensemble averaging yielded the macroscopic I_{Na}. Peak I_{Na} reflects the moment where NP_o (i.e., the product of the number of channels and the open probability) attains its maximum during membrane depolarization and was taken as an index of the latter.

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

Solutions (Composition in mmol/liter)

A: Modified Tyrode solution: NaCl 137; $CaCl_2$ 0.2; $MgCl_2$ 5; Na-pyruvate 5; glucose 20; HEPES 10; pH 7.4. B: Isotonic KCl solution: KCl 140; $MgCl_2$ 5; Na-pyruvate 5; glucose 20; HEPES 10; EGTA 2; pH 7.4. C: Isotonic K-aspartate solution: K aspartate 120; KCl 20; $MgCl_2$ 5; Na-pyruvate 5; glucose 20; HEPES 10; EGTA 2; pH 7.4. D: Pipette solution: NaCl 200 (or 150); $CaCl_2$ 0.02; $MgCl_2$ 1; HEPES 10; pH 7.4.

Compounds

Dibutyryl cyclic AMP (db-cAMP), cyclic AMP (cAMP), forskolin, 1,9-dideoxyforskolin, adenine and hypoxanthine all were purchased from Sigma Chemie (Steinheim, FRG). Isoproterenol was delivered from Boehringer Ingelheim (Ingelheim, FRG) and histaminehydrochloride from Merck AG (Darmstadt, FRG). Racemic DPI 201-106 was a gift of Sandoz, Basle (Switzerland). DPI 201-106 was freshly dissolved in HCl and diluted in pipette solution to give a final concentration of 3×10^{-6} mol/liter. Forskolin and 1,9-dideoxyforskolin were dissolved in ethanol. An appropriate amount of this stock solution was diluted in solution B or C to give a final forskolin or 1,9-dideoxyforskolin concentration of 1×10^{-6} mol/liter, the ethanol concentration being 0.1%. All other compounds were freshly dissolved in solution B or C just before use.

Results

A first series of cell-attached experiments dealt with the influence of db-cAMP on Na^+ channel properties. Although the channels remained in their natural environment, a run-down of channel activity frequently occurred during the very early stage and sometimes continued to develop within the first 10 min of patch lifetime until NP_o reached a new steady state. As an experience with a large number of untreated control patches, this steady state can be expected to be maintained during a long period of time (60 min or longer) provided no morphological signs of cell damage will appear. The experimental protocol, therefore, basically included an initial equilibration period of 10 min before patch-clamp recordings were collected. Consistent with the db-cAMP-induced depression of macroscopic I_{Na} in whole-cell clamped adult myocytes (Ono et al., 1989), the superfusion of the cultured cardiocytes with 1×10^{-3} mol/liter db-cAMP rapidly depressed I_{Na}. Within 3 min, a mean decrease of reconstructed peak I_{Na} to 62 ± 7.6% ($n = 7$) of the initial control value developed. Apart from one exception, I_{Na} decay kinetics remained unaffected.

Figure 1 illustrates that the current depression reflects a decline in NP_o. Without changes in unitary current size, the db-cAMP treatment consistently reduced the fraction of activity sweeps including the number of activity sweeps with superpositions.

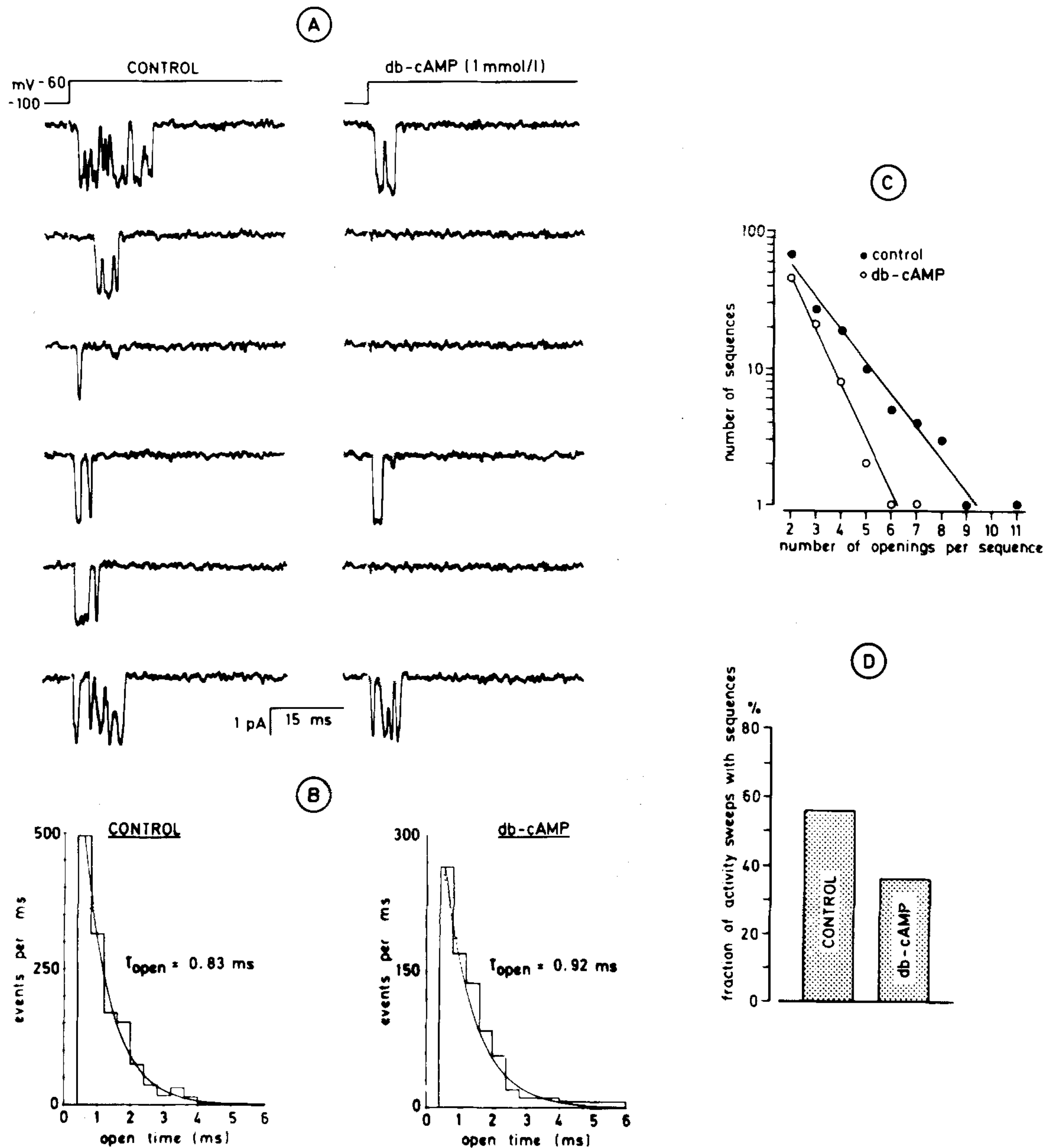

Fig. 1. The response of cardiac Na^+ channels to db-cAMP treatment of a cardiocyte. (*A*) Consecutive recordings of elementary Na^+ currents before and after 1×10^{-3} mol/liter db-cAMP. (*B*) Open-time probability density functions as based on 658 events under control conditions and on 383 events with db-cAMP. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 1022\exp(-t/0.00083)$ and by $N(t) = 484\exp(-t/0.00092)$, respectively. (*C*) Frequency distributions of the number of openings per sequence under control conditions (filled circles) and after db-cAMP treatment. (*D*) Changes in the fraction of activity sweeps with sequential openings after db-cAMP treatment. The analysis was based on 525 records before and on 530 records after db-cAMP. Patch 516CA; −60 mV

Consequently, the fraction of blank sweeps rose. In the experiment demonstrated in Fig. 1, for example, the blank fraction rose from 16 to 39% at the expense of the activity sweep fraction which declined from 84 to 61%. A similar pattern of response was found in four other cell-attached patches. Open-state kinetics were preserved since the monoexponential open-time distribution which is typical for cardiac Na^+ channels (Kunze et al., 1985; Patlak & Ortiz, 1985; Kohlhardt, Fröbe & Herzig, 1987) as well as the duration of the open state did not change; in five cell-attached patches, the ratio $\tau_{open(db\text{-}cAMP)}/\tau_{open(control)}$ amounted to 1.01 ± 0.06.

Nevertheless, db-cAMP can influence channel

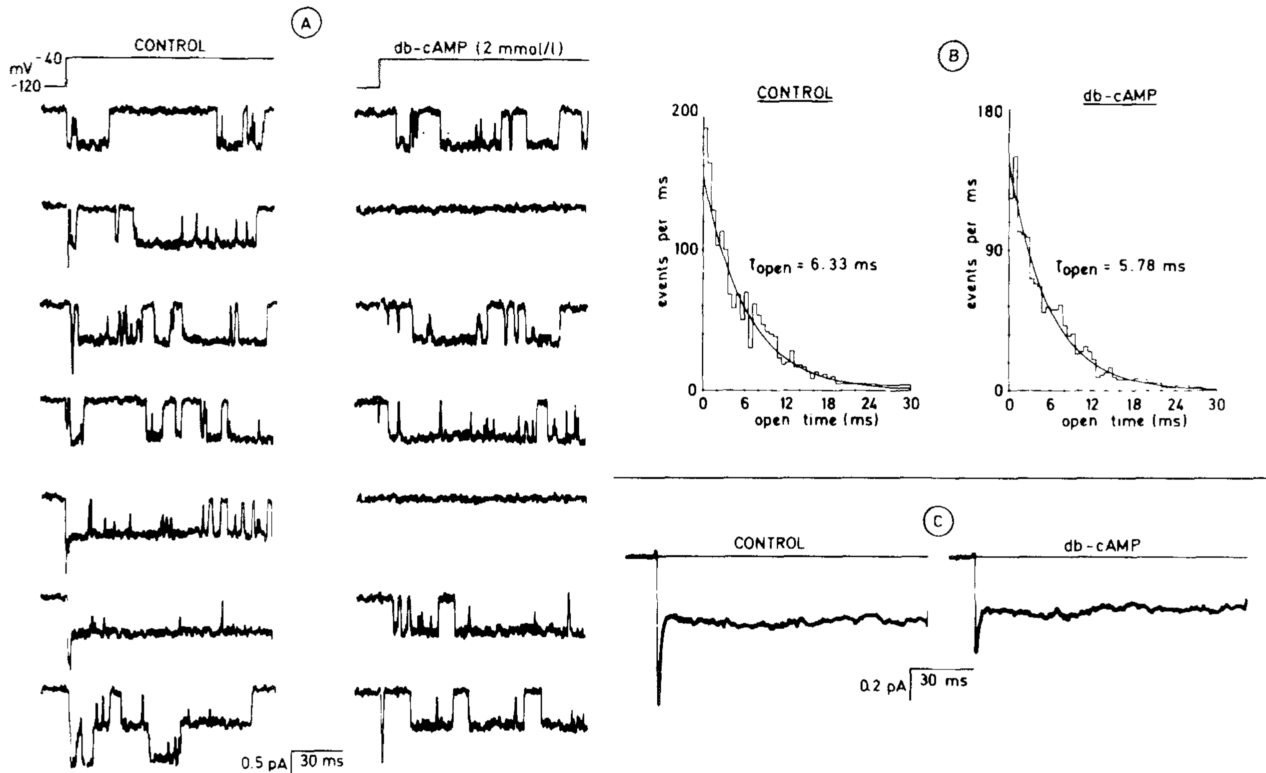

Fig. 2. The influence of db-cAMP on DPI-modified Na^+ channels under cell-attached conditions. (*A*) Consecutive recordings of elementary Na^+ currents in the absence (left) and presence (right) of 2×10^{-3} mol/liter db-cAMP. (*B*) Open-time probability density functions based on 1250 events under control conditions and on 980 events with db-cAMP. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 153 \exp(-t/0.00633)$ and by $N(t) = 146 \exp(-t/0.00578)$, respectively. (*C*) Macroscopic Na^+ currents reconstructed from 275 records before and of 253 records after db-cAMP treatment. Patch 330CA; −40 mV

reopening (Fig. 1*C* and *D*). As judged from the frequency event distribution of the number of sequential openings (Fig. 1*C*) and with the precautions mentioned in Materials and Methods, the number of sequential openings during membrane depolarization decreased. Concomitantly, the fraction of activity sweeps showing sequences declined, in the experiment illustrated in Fig. 1, from 56 to 37% (*see* Fig. 1*D*) which is another manifestation of reduced channel reopening.

The db-cAMP action is reminiscent in one aspect of the effect of several class 1 antiarrhythmics since Na^+ channel blockade evoked by these drugs likewise occurs as an all-or-none event (Kohlhardt & Fichtner, 1988; Grant et al., 1989). The protection of the open state against blocking antiarrhythmic molecules is, however, readily lost in kinetically modified Na^+ channels when removal of inactivation prolongs the conductive state (Kohlhardt et al., 1989*b*). Cell-attached experiments with DPI-modified Na^+ channels excluded the possibility that db-cAMP treatment might be followed by a similar flicker blockade. Figure 2 demonstrates the well-established DPI effect characterized by long-lasting, burst-like Na^+ channel activity with a several-fold prolonged open state giving rise to a noninactivating, sustained I_{Na} component (Kohlhardt et al., 1986). Exposing the cardiocytes to 2×10^{-3} mol/liter, db-cAMP reduced peak I_{Na} to 64 ± 5% and sustained I_{Na} to 59 ± 21% ($n = 2$) of the initial control value. The absence of any flicker blockade is proven by the unchanged values for τ_{open}. Moreover, burst duration remained unaffected, too.

Apart from β-adrenergic stimulation, the cellular cAMP level can be also elevated by the membrane-permeable compound forskolin. This plant diterpene activates directly the adenylate cyclase (Seamon & Daly, 1986) and may mimick the catecholamine action. In a total of four cell-attached experiments with the β-adrenergic isoproternol (1×10^{-7} mol/liter), no changes in Na^+ channel acitivity occurred. This is in contrast to results in whole-cell clamped adult cardiocytes (Ono et al., 1989) and also to earlier V_{max} measurements in papillary muscles (Windisch & Tritthart, 1982; Arita et al., 1983; Gillis & Kohlhardt, 1988) but may be primarily related to

the smaller isoproterenol concentration used in the present experiments. Forskolin (1×10^{-6} mol/liter), on the other hand, was found to be effective and reduced reconstructed peak I_{Na} strongly to 28 ± 10% ($n = 3$) of the initial control value. Again, this depression was not accompanied by changes of unitary current size or open-state kinetics. To ensure that the reduced Na^+ channel activity was not caused by a local anesthetic side effect of forskolin (Castle, 1989), the influence of dideoxyforskolin was tested. Treating a cardiocyte with this biologically inactive forskolin analog (1×10^{-6} mol/liter) failed to exert an inhibitory action since I_{Na} changed insignificantly to 98 ± 4% ($n = 2$) of the initial control value.

Although H_2 receptor stimulation can also effectively increase cAMP synthesis, it was surprising to see that histamine treatment of the cardiocytes evoked an effect on Na^+ channels which was opposite to that of db-cAMP. 2.5×10^{-6} mol/liter increased peak I_{Na} to 148 ± 12% ($n = 3$) which took 5–10 min for full development. Elementary channel properties including unitary current size, reopening and open-state kinetics remained unaffected (*see* Fig. 3).

Isolated cardiac Na^+ channels sensitively respond to the cytoplasmic presence of cAMP under conditions which exclude a phosphorylation reaction. This was found in inside-out patches whose cytoplasmic membrane surface faced an environment with a quasi-physiological ionic milieu but lacking the phosphate donor for protein phosphorylation, ATP. After excision, the inside-out patches were initially equilibrated for 15 min in this medium in order to clean the cytoplasmic membrane surface from a possible contamination with cellular metabolites including ATP. This equilibration period was also useful to stabilize the Na^+ channel activity in the cell-free recording mode. NP_o was monitored periodically and mostly attained a steady state within 5 min after patch excision. Otherwise, the patch was discarded. Figure 4*A* and *B* show that db-cAMP alone is capable of depressing Na^+ channel activity. In response to the cytoplasmic administration of 1×10^{-3} mol/liter, reconstructed peak I_{Na} declined within 2 min to 47 ± 7% ($n = 3$) of the initial control value. Again, elementary channel properties remained unaffected. cAMP tested at the same concentration exerted the same effect. A quite similar NP_o decrease developed after exposing the cytoplasmic channel surface to another adenine nucleotide, ATP (1×10^{-3} mol/liter) (*see* Fig. 4*B* and *D*). As seen in a washout experiment, the inhibitory ATP effect is reversible, at least partially. That the inhibitory action of both adenine nucleotides does not reside in their phosphate groups can be concluded from the results of three inside-out patches treated cytoplasmatically with adenosine (1×10^{-4} mol/liter) where peak I_{Na} declined to 40 ± 5% of the control value. The cytoplasmic presence of adenine (*see* Fig. 4*C*) and of the structurally closely related hypoxanthine produced essentially the same effect: at 1×10^{-5} mol/liter, peak I_{Na} declined to 40 ± 5% ($n = 2$) and 44 ± 6% ($n = 2$), respectively. The reversibility of this inhibitory effect was tested in the adenine experiments: NP_o returned within a few minutes to almost the initial control value after removing the purine base from the cytoplasmic membrane surface.

Discussion

The present patch-clamp experiments, first of all, identified a novel family of Na^+ channel inhibitors comprising adenosine, ATP and cAMP and show that their effectiveness essentially resides on the adenine group of the molecule. Analogous to other inhibitory agents, like, for example, local anesthetics, they could find a channel-associated binding site at the cytoplasmic channel surface or in a less superficial region of the channel and, by interacting with this hypothetical site, might finally hinder the channel to open. Alternatively, this interaction could decrease NP_o by a hyperpolarizing shift of steady-state inactivation. Influencing the Na^+ channel by an accumulation in the surrounding lipid phase of the membrane seems less likely in view of the strong hydrophilicity of the adenine derivatives.

Metabolites like adenine nucleotides are not unique in affecting cardiac Na^+ channels. As likewise found in cell-free conditions, several intermediate products of glycolysis including glyceroaldehyde phosphate and 2,3-diphosphoglycerate may also exert an influence thereby activating Na^+ channels and causing them to enhanced burst-like activity (Kohlhardt et al., 1989*a*). Apparently the same effect appears when lysophosphatidylcholine is present at the cytoplasmic surface of inside-out patches (Burnashev et al., 1989) which represents a degradation product of the membrane constituent phosphatidylcholine.

Of particular interest could be the ATP sensitivity. ATP is of importance for several membrane-associated functions and, by influencing the activity of ATP-dependent K^+ channels, controls, particularly in heart muscle, action potential duration and refractoriness. Weiss and Lamp (1989) presented circumstantial evidence that it is preferentially the glycolytically formed ATP which maintains the normal cellular electrophysiological function. Interestingly, as also shown by these authors in dialyzed whole-cell clamped cardiocytes, the equilibration of

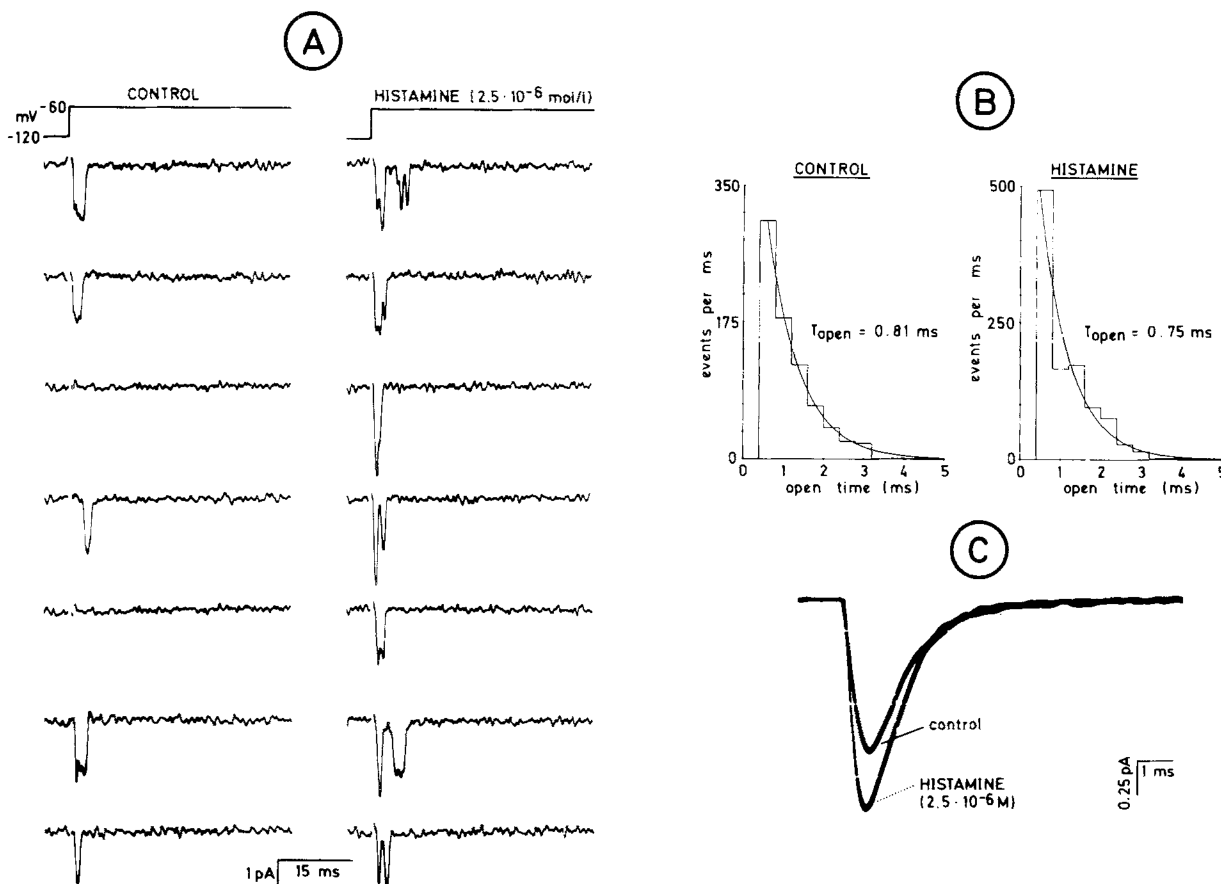

Fig. 3. The response of cardiac Na^+ channels to histamine treatment of a cardiocyte. (*A*) Consecutive recordings of elementary Na^+ currents before and after 2.5×10^{-6} mol/liter histamine. (*B*) Open-time probability density functions from 367 events under control conditions and from 497 events with histamine. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 635\exp(-t/0.00081)$ and by $N(t) = 927\exp(-t/0.00099)$, respectively. (*C*) Superimposed reconstructed macroscopic Na^+ currents before and after histamine treatment. Patch 527CA; −60 mV

the cell interior with an ATP concentration as high as 15 mmol/liter cannot prevent the activation of the ATP-dependent K^+ current after glycolytic inhibition. Because of its capability to interfere with Na^+ channels, ATP could directly control cardiac excitability. However, it cannot be excluded that this susceptibility to adenine nucleotides is related to patch excision and the exposure of the cytoplasmic channel surface to an artificial environment. Assuming that Na^+ channels *in situ* have a similar sensitivity, the influence of ATP would be dominant. Compared with the ATP concentration which is in the range of 5 mmol/liter, the intracellular cAMP concentration is about 1000 times smaller and will barely exceed an upper limit of about 10–15 μmol/liter after β_1-adrenergic stimulation. Fluctuations in cAMP are, therefore, ineffective to change the total intracellular adenine nucleotide concentration at the cytoplasmic membrane surface significantly.

The functional significance of the ATP sensitivity is difficult to judge for still another reason since the susceptibility to adenine nucleotides could be a property of channels expressed in neonatal cardiac cells. Embryonic precursors of adult myotubes, for example, preferentially express Na^+ channels being highly resistant to tetrodotoxin, whereas maturation is associated with the occurrence of an increasingly higher proportion of highly tetrodotoxin-sensitive Na^+ channels (Weiss & Horn, 1986). Neonatal rat heart Na^+ channels are not yet proven to share exactly the same properties with their adult relatives. Thus, it cannot be excluded that the sensitivity to adenine nucleotides changes during cardiac development.

The α-subunit of rat heart Na^+ channels was recently reported to be a good substrate for phosphorylation by cAMP-dependent protein kinase (Gordon et al., 1988). I_{Na} depression seen with db-cAMP in the cell-attached mode may well be due to a net phosphate gain of the α-subunit. Phosphoryla-

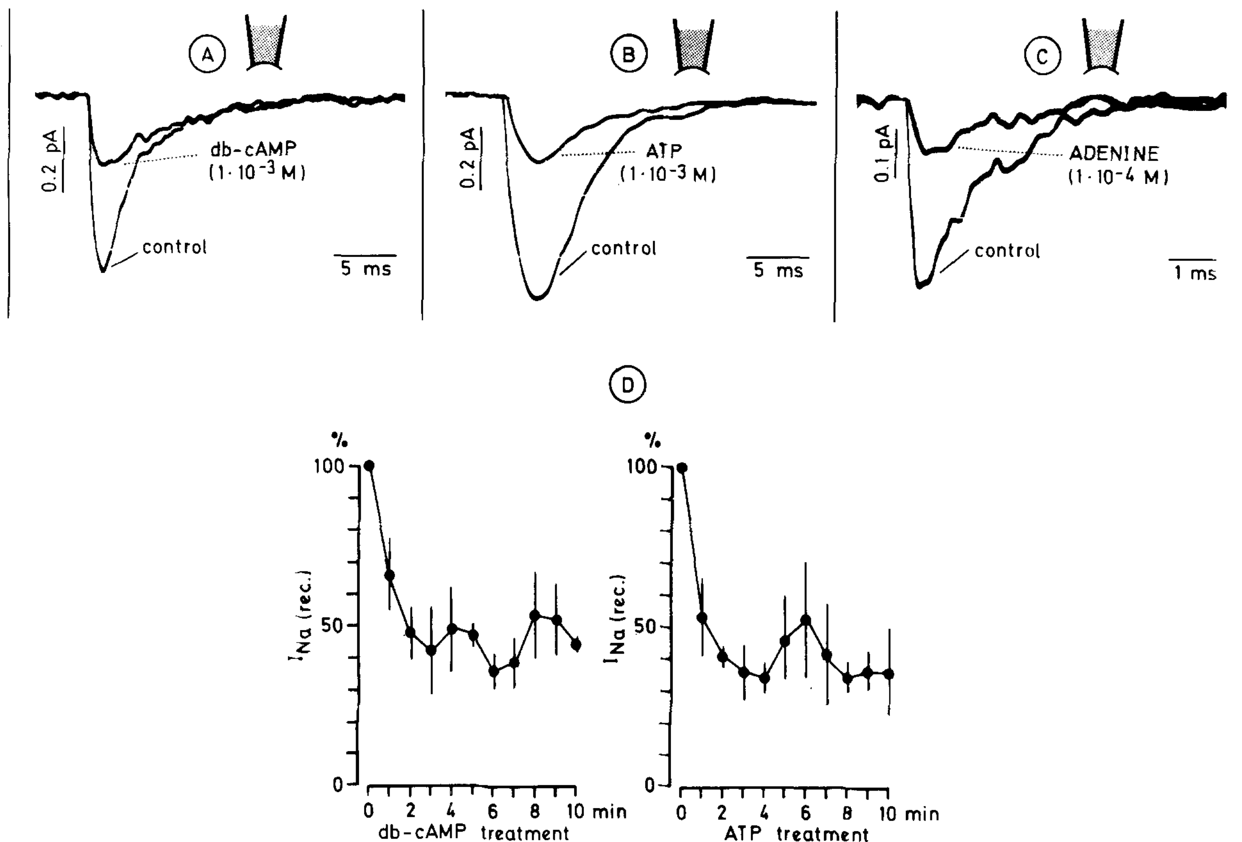

Fig. 4. Sensitivity of Na+ channels in the inside-out mode to the cytoplasmic presence of db-cAMP, ATP and adenine. (*A–C*) Superimposed, reconstructed macroscopic Na^+ currents before and after treating the inside-out patches with db-cAMP (patch 548IO; −60 mV), ATP (patch 550IO; −60 mV) and adenine (patch 570IO; −55 mV). (*D*) Time course of I_{Na} depression. Mean relative changes (±SEM) after cytoplasmic treatment of the inside-out patches with 1×10^{-3} mol/liter db-cAMP (left; $n = 3$) and with 1×10^{-3} mol/liter ATP (right; $n = 3$). 100% refers to the control I_{Na}. The control I_{Na} was obtained from an ensemble average of 200 records during a 5-min period directly preceding the administration of the adenine nucleotides. The patches were kept at holding potentials between −110 and −120 mV

tion would not be followed by major change in elementary channel properties except for a decrease in burst-like activity which points to a more rapidly developing inactivation during membrane depolarization. Of particular interest is the resistance of the open state in DPI-modified, noninactivating Na^+ channels since they are kinetically reminiscent of L-type Ca^{2+} channels. Phosphorylation of the latter not only increases their open probability but also prolongs the conductive state (Reuter, 1983).

Cardiac Na^+ channels are rather resistant to β_1-adrenergic stimulation. As inferred from earlier indirect evidence in papillary muscles with $\dot{V}_{max}$ as an indicator for I_{Na} (Windisch & Tritthart, 1982; Arita et al., 1983) and from recent I_{Na} recordings in whole-cell clamped myocytes (Ono et al., 1989), micromolar agonist concentrations are needed for a depression amounting to only 16% with 1×10^{-6} mol/liter norepinephrine (Ono et al., 1989). Gillis and Kohlhardt (1988) compared the ED_{50} of isoproterenol to produce a positive inotropic effect with the ED_{50} of this compound to depress $\dot{V}_{max}$ in Na^+-dependent action potentials and concluded that, in the physiologically relevant submicromolar range, Na^+-dependent excitability is only weakly sensitive to β_1-adrenergic agonists, if at all. Accordingly, stimulation of neonatal cardiocytes in the present cell-attached experiments with 1×10^{-7} mol/liter isoproterenol left NP_o unchanged. It seems to be important to note that these cells are basically sensitive to β_1-adrenergic agonists and respond to isoproterenol with a measurable elevation in cellular cAMP even under hypoxic conditions (Thandroyen et al., 1989).

The generally accepted concept is, therefore, not invalidated that elevation of cytoplasmic cAMP after physiological stimulation of β_1-adrenoceptors with endogenous agonists has negligibly small significance for excitability and impulse conduction in

normal heart muscle. In partially depolarized myocardium, however, an enhanced effectiveness was consistently found (Windisch & Tritthart, 1982; Arita et al., 1983; Hisatome et al., 1985; Gillis & Kohlhardt, 1988; Ono et al., 1989). Local anesthetics exert a similar voltage-dependent action in that their inhibitory efficacy becomes accentuated upon membrane depolarization.

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