

Atrionatriuretic Peptide and Calcium-Conducting Sodium Channels

L. A. Sorbera and M. Morad (1) propose that atrionatriuretic peptide (ANP) regulates the cardiac Na^+ channel "by an alteration of its ionic selectivity." They conclude that "permeation through the Na^+ channel may occur somewhat similarly to that described for the Ca^{2+} channel" and that "alteration in the ionic selectivity of a channel by a hormone may serve as a rapid regulatory mechanism." We have been unable to substantiate their finding that ANP increases Na^+ channel conductance to Ca^{2+} , but present evidence that ANP exerts its effect by shifting the voltage-dependent availability of the Na^+ channel.

We measured ionic current responses to step depolarizations from a holding potential at which full Na^+ channel availability was assured (Fig. 1A) (2). Under these conditions exposing the cell to 100 nM ANP produced a small reduction in current (Fig. 1B). The peak current-voltage (I - V) relations are shown in Fig. 2. Although 100 nM ANP had little effect on peak current when availability was maximal, it shifted the half-point of voltage-dependent availability 4.5 mV in the negative direction (Fig. 3). This effect was rapidly reversible after the peptide was washed out. In three cells, ANP shifted availability -4.8 ± 0.3 mV (SEM), and peak conductance was reduced to $94 \pm 2\%$ (SEM) of control. When we replaced extracellular Na^+ with *N*-methyl-D-glucamine (retaining 2 mM Ca^{2+}) and the cell was exposed to 100 nM ANP ($n = 3$) we did not observe inward currents (in contrast to the report of Sorbera and Morad). Only outward currents (at positive potentials) were recorded (Fig. 4), as expected because

the channel is approximately 3% permeable to Cs^+ (3). Therefore, we were unable to confirm the finding of Sorbera and Morad (1) that ANP increased Ca^{2+} permeability of the Na^+ channel in the absence of extracellular Na^+ .

Many of the discrepancies between our data and those of Sorbera and Morad can be explained by the change in voltage-dependent availability of the Na^+ channel induced by ANP. The Na^+ channel availability depended on holding potential (4), and dropped e -fold in approximately 5 mV (Fig. 3). Sorbera and Morad do not report an availability half-point, but it is likely that it was in the range of -90 to -80 mV in control solutions (4). Thus, their holding potential may have been on the steep portion of the availability relation. Figure 5 illustrates the variable reduction in Na^+ current (I_{Na}) in response to ANP when channel availability was altered by changing holding potential (5). Furthermore, cells with different availability half-points would cause the magnitude of the ANP effect to appear variable.

Sorbera and Morad report that the current in Na^+ -free solution in the presence of ANP disappeared when extracellular Ca^{2+} was removed [figure 3 in (1)] and that, upon exposure to ANP, the reduction in current in Na^+ -containing, Ca^{2+} -free solutions was much smaller than in Ca^{2+} -containing solutions. These observations could also be explained by holding potential and shifts in voltage-dependent availability. Divalent cations bind to and screen surface charge sites on or near Na^+ channels and shift voltage-dependent channel availability (6). A reduc-

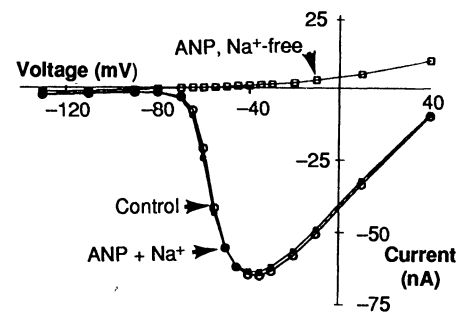


Fig. 2. Peak I - V relation for control ($\circ$), ANP + Na^+ ($\blacksquare$), and ANP, Na^+ -free ($\square$). Peak currents were calculated from capacity, but not leak-corrected currents.

tion in current would be expected when extracellular Ca^{2+} was reduced to $20 \mu\text{M}$. This reduction would explain why removal of Ca^{2+} appeared to eliminate current in the presence of ANP [figure 3 in (1)]; had the membrane been hyperpolarized, the current would have reappeared. Sorbera and Morad report ANP had little effect in Na^+ -containing, Ca^{2+} -free solutions, but their data were obtained in the presence of 10 mM Mg^{2+} [figure 2 in (1)]. High Mg^{2+} concentrations would shift availability to more positive potentials and bring the holding potential of -80 mV closer to the top of the availability

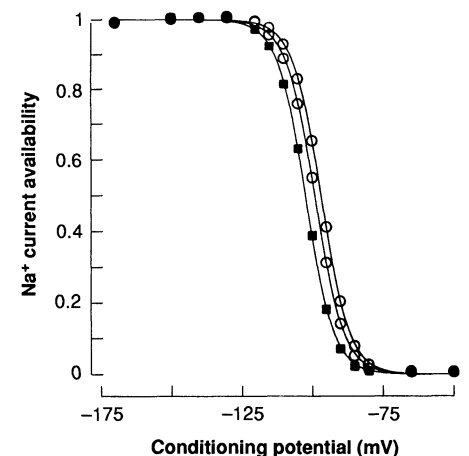


Fig. 3. Effect of 100 nM ANP on voltage-dependent availability. Control ($\circ$) and in the presence of ANP ($\blacksquare$). Solutions and recording as in Fig. 1. Cells were held for 500 ms at the voltage indicated on the abscissa and depolarized to 0 mV for 25 ms so that availability could be assessed. One and one-half seconds were allowed for recovery at -150 mV between conditioning pulses. Currents are shown normalized to the fully available value. Peak current in 100 nM ANP was 93% of the control value (-42.8 nA). The availability curve measured after washing returned toward the control curve but was not superimposed on it. A hyperpolarizing shift in kinetics is a characteristic of the preparation and was not affected by the ANP. In three cells it was $0.5 \text{ mV min}^{-1} \pm 0.06 \text{ mV min}^{-1}$ (SEM). The ordinate shows maximum availability normalized to one.

Fig. 1. Effect of ANP on the Na^+ current of canine Purkinje myocytes. Cells were isolated according to the procedure of Sheets *et al.* (2) and voltage-clamped with a large bore, double-barreled glass pipette (2). Intracellular solution: 150 mM CsF, 10 mM Hepes, pH 7.2. All currents are shown without leak or capacity correction. Cell B1.03, 56 pF, background resistance 56 megohms, 18°C , primary charging time constant $20 \mu\text{s}$, depolarizations 1 s^{-1} . (A) Superimposed raw currents recorded at 100 kHz, analog filtered (8-pole Bessel) at 10 kHz, for 50-ms depolarizations from -150 mV to -70 , -65 , -60 , -55 , -50 , -40 , -30 , -10 , $+10$, and $+40$ mV. Extracellular solution: 120 mM CsCl, 30 mM NaCl, 2 mM CaCl_2 , 10 mM Hepes, pH 7.2. (B) Superimposed raw currents, as in (A), except with the addition of 100 nM ANP (α -dog/human 1-28, Peninsula Labs, San Diego, California). ANP was dissolved in acetic acid; final solution was titrated to pH 7.2 with CsOH.

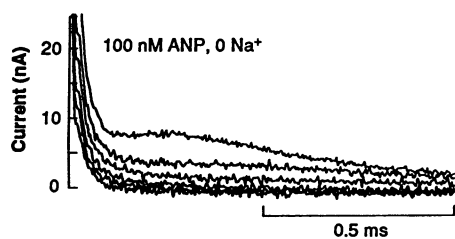


Fig. 4. Superimposed raw currents recorded at 300 kHz for 10-ms depolarizations from -150 mV to -70 , -55 , -40 , -20 , 0 , $+30$ mV. Extracellular solution: 150 mM *N*-methyl-D-glucamine, 2 mM CaCl_2 , 100 nM ANP, 10 mM Hepes, pH 7.2 , titrated with tris base. Note the expanded time base and increase in gain.

relation. Under conditions of full or near full availability, ANP has little effect on the I - V relation (Fig. 5). A similar effect explains the increase in current after the addition of 5 mM Ni^{2+} [figure 4B in (1)].

The presence of current in ANP, Na^+ -free solutions that can be blocked by tetrodotoxin (TTX) in experiments of Sorbera and Morad remains unexplained. Although it is impossible to exclude differences between our intracellular solutions and theirs as the cause, they provide evidence that substitution of Cl^- with aspartate, addition of cyclic adenosine $3',5'$ -monophosphate (cyclic AMP) or addition of guanosine $5'$ - O -(3-

thiotriphosphate) ($\text{GTP}\gamma\text{S}$) or guanosine $5'$ - O -(2-thiodiphosphate) ($\text{GTP}\beta\text{S}$) did not affect their results. We suggest that a contaminant might have been present in their ANP solutions because we were unable to reproduce their result, and their proposed selectivity change is unlikely on the basis of their own data. They report that peak current in 137 mM Na^+ was 5.5 nA and was 3.8 nA when 2 mM Ca^{2+} accounted for the conductance; peak current requires roughly 50 times greater conductance per millimolar for Ca^{2+} than for Na^+ . Therefore, Sorbera and Morad should have found that changes in extracellular Ca^{2+} (taking into account shifts in voltage availability) had dramatic effects on the size of their macroscopic current. Alternatively, the selectivity change may be peculiar to guinea pig and rat ventricular myocytes or to rat ANP. If this is the case, then the selectivity change does not involve an endogenous ANP binding site, as we could not substantiate the effect with dog-human peptide acting on canine cells.

Sorbera and Morad appear to have been hampered by a slow voltage clamp and by difficulty with voltage control. Currents through Na^+ channels at 18°C achieve a peak value earlier than 500 μs at positive potentials (Figs. 1 and 5). In the traces published by Sorbera and Morad, the capacity transient persisted longer than 500 μs , making assessment of peak currents and reversal difficult. Our data support the conclusion that the action of ANP on cardiac Na^+ current shifts voltage-dependent availability rather than alters selectivity of the channel. While this effect is not so dramatic as the action proposed by Sorbera and Morad, it could act as a regulatory mechanism in ANP-secreting cells.

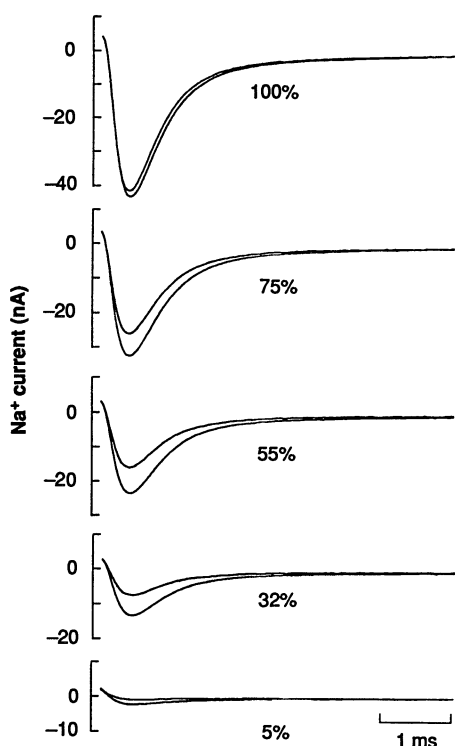


Fig. 5. Capacity-corrected currents in depolarizations to 0 mV from various holding potentials in control solution and with 100 nM ANP. In all cases the smaller current was measured in the presence of ANP. Labels indicate availability as assessed under the control condition.

observed." In these instances currents in ANP would be even smaller.

6. S. Weidmann, *J. Physiol. (London)* **129**, 568 (1955); B. Frankenhaeuser and A. L. Hodgkin, *ibid.* **137**, 218 (1957); B. Hille, A. M. Woodhull, B. I. Shapiro, *Philos. Trans. R. Soc. London Ser. B* **270**, 301 (1975).
7. We thank S. Krueger for technical assistance and E. Page for supplying the ANP.

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Response: The suppressive effect of rat atrionatriuretic peptide (ANP) on Na^+ current has been found consistently in our laboratory in rat, guinea pig, and frog ventricular cells as well as in rat atrial cells (1). It is a large effect that cannot easily be explained by poor voltage clamp control of the cell. In a solution deficient in Na^+ (10 mM), Na^+ current was strongly enhanced by ANP, and its reversal potential was shifted to more positive potentials (Fig. 1). The removal of Ca^{2+} negated the ANP-induced enhancement of Na^+ current and the change in reversal potential. The enhancement of Na^+ current in low Na^+ and normal Ca^{2+} was observed not only at positive potentials, where it may be difficult to separate the rapidly inactivating ionic current from the capacitive spikes, but also at negative potentials (-50 to -10 mV), where the current activates and inactivates relatively slowly. Sheets and Hanck do not report an effect resembling ours.

The experimental techniques used by Sheets and Hanck were quite different from those that we used. We used rat ANP and the whole-cell voltage clamp technique to measure ionic currents in enzymatically dissociated ventricular and atrial cells from rodents and frog (1). Sheets and Hanck used a ruptured cell voltage clamp technique and applied dog-human α ANP to single Purkinje cells from dogs. In the ruptured cell voltage clamp technique, about half of a cell is sucked into a large glass pipette, then its membrane is ruptured mechanically to establish a pathway with low access resistance to the intact part of the cell. While this technique yields superior frequency response and effective internal dialysis, it also washes away cellular constituents of high molecular weight that are essential to the regulation of ionic channels. These constituents are retained when 1 - to 2 -megohm pipettes are used to penetrate an intact cell (2).

In our experiments, extracellular Na^+ was varied between its normal physiological concentration (137 mM) and 10 mM or less. Sheets and Hanck used extracellular Na^+ at 30 mM or less. We used an internal solution (typically 110 mM CsCl, 0 to 10 mM NaCl, 5 mM MgATP, 20 mM Hepes, 14 mM EGTA, 10 μM cAMP, pH 7.2) that was

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5. ANP shifted channel availability by 5 mV in Fig. 5. Sorbera and Morad report, "in some cells, however, a 10 mV shift toward more negative potentials was

more complex and probably supported regulatory processes better than the solution used by Sheets and Hanck (150 mM CsF, 10 mM Hepes, pH 7.2). Sheets and Hanck used a high intracellular concentration of F^- . This ion blocks Ca^{2+} permeation in neurons (3), and blocks G protein modulation of ionic channels (4). Finally, we used a concentration clamp technique that increased the concentration of ANP around the myocyte within a few milliseconds whereas Sheets and Hanck applied ANP by diluting it in the bath.

We discount the possibility of accidental contamination in our laboratory because we used several batches of ANP prepared over the course of a year and a half. The presence of a contaminant that could suppress Na^+ current at high extracellular Na^+ concentrations, but enhance it at low extracellular Na^+ concentrations was unlikely. We established that biologically active ANP was present in the experimental solutions and that the tissue in question responded to ANP: There was a dose-dependent suppression of the Ca^{2+} current consistent with the binding constant of ANP receptors (5) and in agreement with previous reports (6). It is possible that the solutions used by Sheets and Hanck were contaminated or did not contain biologically active ANP. For instance, in the absence of a chelator like EGTA, Al^{3+} is a common contaminant (7) that together with F^- blocks G protein regulation by mimicking the effect of $GTP\gamma S$ (4, 7). Furthermore, it seems that Sheets and Hanck did not establish that their ANP was biologically active or at least that it suppressed Ca^{2+} current.

Sheets and Hanck explain the suppressive effect of ANP on Na^+ current by a potential shift in the steady-state availability curve. However, their measured shifts in inactivation of Na^+ current were small (-4.8 ± 0.3 mV, $n = 3$) and are not supported by dose-response measurements or biological assay of the potency of their ANP solution. Much larger shifts of this type occur spontaneously (8). We occasionally found that ANP induced a small negative shift (<10 mV) in the steady-state inactivation curve of Na^+ current (1), but only in addition to a strong suppression of the maximally available current at -120 to -150 mV. Such shifts cannot explain the dramatic increase in inward current when ANP is added to an external solution containing 2 mM Ca^{2+} and 10 mM Na^+ (Fig. 2). Hyperpolarization of the prepulse potential to -150 mV, in control solution, did not remove inactivation so that an inward current comparable in magnitude to the current induced by ANP was revealed (Fig. 2, upper panel). In fact, identical steady-state availability curves were obtained in the presence and absence

Fig. 1. The enhancement of Na^+ current by ANP in a frog ventricular myocyte is accompanied by a shift in its reversal potential from the equilibrium potential of Na^+ (0 mV) to a positive value. The voltage dependence of the peak inward currents (top panel) is measured from traces (lower panels) recorded in the absence ($\circ$) and presence ($\bullet$) of 100 nM ANP. External solution: 93 mM CsCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, with CsOH. Internal solution: 77.5 mM CsCl, 10 mM NaCl, 5 mM $MgATP$, 10 mM Hepes, 10 mM EGTA, pH 7.2, with CsOH.

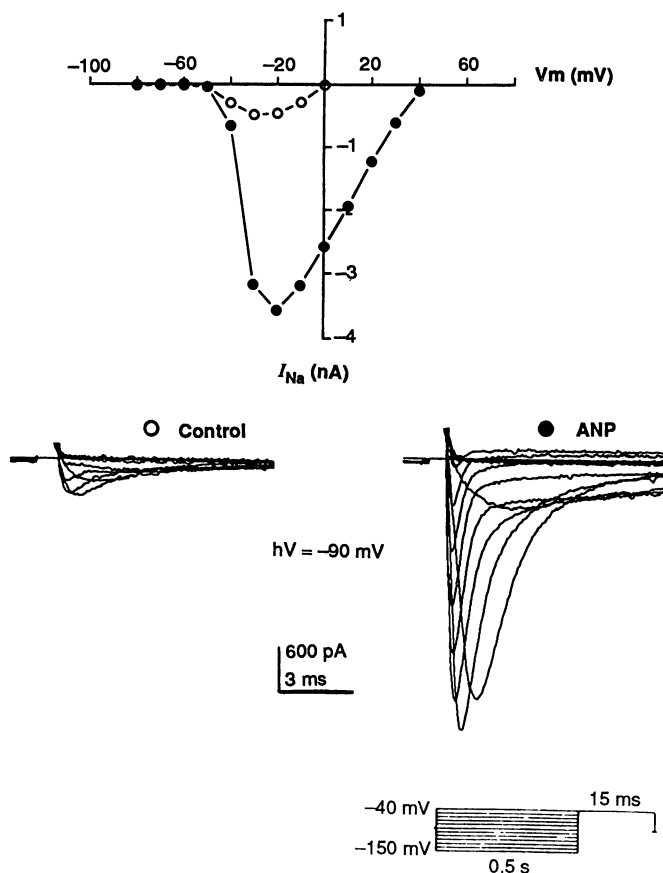
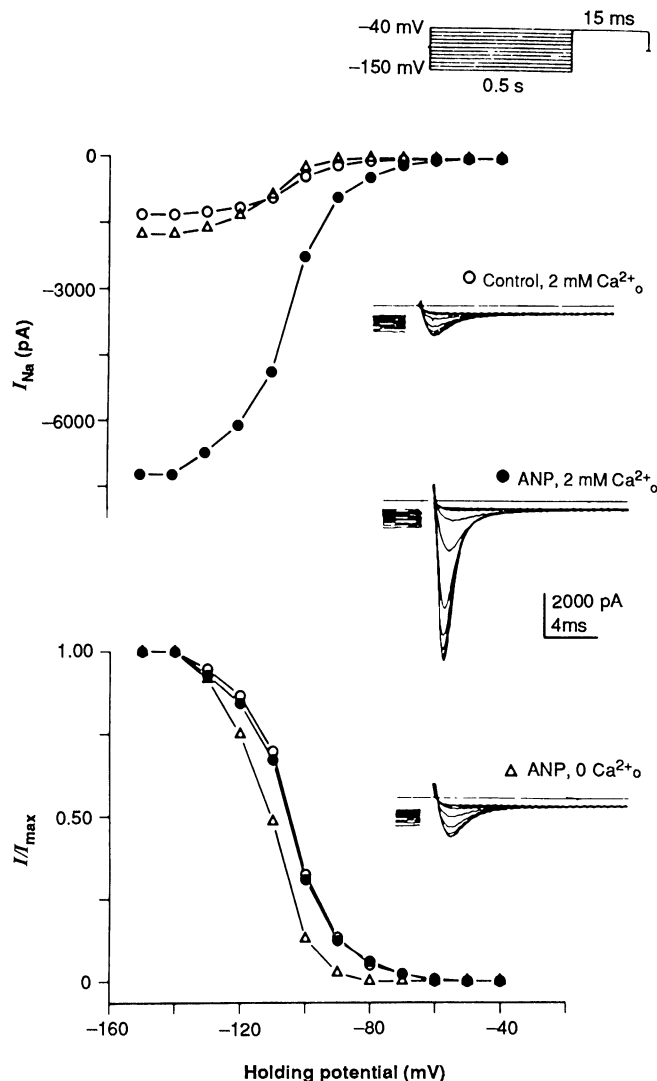


Fig. 2. The ANP-induced enhancement of inward current, and its dependence on extracellular Ca^{2+} , are independent of holding potential and cannot be explained by shifts in the inactivation curve. The top panel shows peak inward current at -40 mV measured after a 0.5-s conditioning pulse to lower negative potentials. The Ca^{2+} requirements of the ANP-induced currents were observed independently of holding potential ($\circ$, control; $\bullet$, addition of ANP; Δ , removal of Ca^{2+} in the presence of ANP). The normalized currents are plotted as inactivation curves in the lower panel and show only minor potential shifts when Ca^{2+} is removed. Insets show sample records. Rat ventricular myocyte bathing in solution containing 127 mM CsCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes, 0 or 2 mM $CaCl_2$, 10 mM glucose, titrated to pH 7.4, with CsOH, and dialyzed with 115 mM CsCl, 5 mM NaCl, 5 mM $MgATP$, 20 mM Hepes, 14 mM EGTA, titrated to pH 7.2, with CsOH.



of ANP (Fig. 2, lower panel). Sheets and Hanck suggest that the subsequent removal of extracellular Ca^{2+} shifts the availability curve of Na^+ current in a way that can account for the suppression of the ANP-induced current. We do not see how a shift in the availability curve to more negative potentials could result in enhancement of Na^+ current. The removal of Ca^{2+} indeed causes a small negative shift in the inactivation curve (Fig. 2, lower panel), but in the wrong direction to account for an enhancing effect of ANP at extremely low negative potentials (Fig. 2, upper panel). We conclude that the shifts in the inactivation curve of Na^+ current are much too small in normal extracellular Na^+ , and in the wrong direction in low extracellular Na^+ , to explain the ANP effects and their dependence on extracellular Ca^{2+} .

The voltage clamp technique used by Sheets and Hanck clearly has an excellent frequency response (8). Nevertheless, their measurements of the kinetics, voltage dependency, and steady-state properties of Na^+ currents were quite similar to ours. With our technique, we cannot measure shifts in inactivation curves with an accuracy of 0.3 mV, but this accuracy is not necessary for our conclusions.

Sheets and Hanck calculate the selectivity of Na^+ and Ca^{2+} per millimolar and argue that an ANP-induced permeation of Ca^{2+} through the Na^+ channel in low Na^+ solution [figure 3 in (1)] is implausible, as we did not find a similar ANP-induced increase in inward current in solutions with normal extracellular $[\text{Na}^+]$. We think that the "independence principle" should not be used for the formulation of experimental solutions or the interpretation of results. There are numerous effects that can be explained only by ionic interactions. These effects include the titration of surface charge by polyvalent cations, saturation phenomena, single-file diffusion to anomalous mole fraction effects (9), and changes in ionic selectivity evoked by binding of ions to regulatory sites in or near a channel. Consider, for example, the intracellular Ca^{2+} -dependent inactivation of the Ca^{2+} channel and the permeation of Na^+ through this channel when the Ca^{2+} concentration is very low ($<1 \mu\text{M}$) (10) or when the H^+ concentration is abruptly increased (11). Such findings demonstrate that, not only the gating parameters, but also the ionic selectivity of a channel can depend, in a complex fashion, on the presence or absence of ions and other metabolites. Considering this background and our

experimental evidence, we have proposed an ANP-induced permeation of Ca^{2+} through the Na^+ channel in low Na^+ solution.

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