J. Membrane Biol. 130, 183–190 (1992)

The Journal of
Membrane Biology

Opposite Effects of Angiotensin II and the Protein Kinase C Activator OAG on Cardiac Na^+ Channels

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Summary. Elementary Na^+ currents were recorded at 19°C in cell-attached and inside-out patch-clamp experiments to study the influence of the vasoactive peptide angiotensin II (A II) and of the diacylglycerol analogue OAG (1-oleoyl-2-acetyl-sn-glycerol) on open probability and gating properties of single cardiac Na^+ channels from cultured neonatal rat cardiocytes. Treating the cardiocytes with A II caused Na^+ channel activation: reconstructed peak I_{Na} increased to 137 ± 17.5% of control at 3 μmol/liters and to 176 ± 42% at 30 μmol/liter. This NP_o increase developed without major changes in open state and burst activity, even at 30 μmol/liter. OAG (6 μmol/liter) did not mimic this A II action. By contrast, OAG treatment of the cardiocytes had the opposite effect on NP_o and diminished reconstructed peak I_{Na} to 67 ± 4.9% of the control. The putative protein kinase C inhibitor staurosporine (0.2 μmol/liter) abolished this I_{Na} depression and led to a normalization of NP_o. OAG had the same effect on isolated Na^+ channels. Exposure of the cytoplasmic surface of inside-out patches to 1 μmol/liter OAG reversibly depressed, in the simultaneous presence of 50 μmol/liter Mg-ATP, the reconstructed peak I_{Na} to 40 ± 9.7% of the control but left i_{unit}, τ_{open} and burst activity unaffected. No NP_o depression was obtained in the absence of Mg-ATP indicating that Mg-ATP may serve as phosphate donor. Obviously, after phosphorylation by protein kinase C, cardiac Na^+ channels attain a reduced open probability but appear to preserve their kinetic properties. It is also concluded that activation of protein kinase C is not the mechanism underlying the A II induced channel activation.

Key Words Na^+ channel properties · protein kinase C · angiotensin II · OAG · phosphorylation

Introduction

Modulation of Na^+ channel activity represents an important principle for altering excitability and impulse conduction in heart muscle and other excitable tissues, where Na^+ channels play the major role in the generation of action potentials. The superfamily of Na^+ channels in excitable membranes constitute the prototype of a voltage-gated ionic channel and, from a very orthodox but classical point of view, are considered to be exclusively controlled by the membrane potential. However, there is increasing evidence that Na^+ channels share with many other ionic channels the sensitivity to influences other than voltage, such as hormones and metabolites. In heart muscle, for example, β_1-adrenergic stimulation is accompanied by an I_{Na} depression (for review *see* Catterall, 1988), mediated by the second messenger cAMP and finally caused by an activation of protein kinase A, which is generally believed to result in Na^+ channel phosphorylation. This may underlie the reduction in open probability while gating itself remains largely preserved. Since the activated G_s-protein can directly interfere with cardiac Na^+ channels thereby reducing their open probability (Schubert et al., 1989), control of cardiac excitability by catecholamines can be achieved extremely rapidly. Less clear is the importance of the cytosolic environment. Cardiac Na^+ channels sensitively react with gating changes to some metabolites of the glycolytic pathway (Kohlhardt, Fichtner & Fröbe, 1989), lysophosphatidylcholine (Burnashev et al., 1989), 6-aminopurines (Herzig & Kohlhardt, 1991) or internal Mg^{++} variations (Benz & Kohlhardt, 1991). Particularly internal Mg^{++} variations may functionally couple Na^+ channels to the cellular metabolic state thereby modulating cardiac excitability, since the loss of cellular ATP is well established to be accompanied by a rise in cytosolic Mg^{++}.

The vasoactive peptide angiotensin II (A II) recently described in neonatal and adult cardiocytes of different species to be capable of increasing I_{Na} (Moorman et al., 1989; Nilius, Tytgat & Albitz, 1989) is another example for an endogenous modulator of cardiac Na^+ channels. A II was tentatively supposed to exert this action by an activation of protein kinase

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C (Moorman et al., 1989). A possible theoretical implication is that cardiac Na^+ channels may be dualistically controlled by phosphorylation, protein kinase C and protein kinase A inducing opposite functional effects upon channel protein phosphorylation.

The present patch-clamp experiments in neonatal rat cardiocytes dealt with the sensitivity of cardiac Na^+ channels to A II, compared the A II action with the influence of OAG, a diacylglycerolanalogue capable of activating protein kinase C directly, and, thus, focused not only on the mode of action of A II but also on the significance of protein kinase C activation on Na^+ channel activity. To compare the actions of A II and OAG, Na^+ channel activity was analyzed in the cell-attached mode. This offers the advantage that Na^+ channel activity can be analyzed without methodological complications as they may be inherent in whole-cell recordings of I_{Na} and, furthermore, without dialyzing the cell interior.

Materials and Methods

Elementary Na^+ currents were recorded in cell-attached and inside-out patches from cultured neonatal rat cardiocytes with a L-M/EPC 5 amplifier by employing the standard patch clamp technique (Hamill et al., 1981). The cultivating procedure and the handling of the short-time (18–24 hr) cultured cardiocytes were essentially the same as described in detail earlier (Kohlhardt, Fröbe & Herzig, 1986). Briefly, the cardiocytes were kept in an isotonic K^+ solution during the whole experiment in order to avoid spontaneous activity with unpredictable changes of the membrane potential. Under these conditions, the resting potential was close to 0 mV (±5 mV). During cell-attached experiments, the cardiocyte under examination was periodically monitored to detect an eventual development of cellular granulation. In this case, the experiment was discarded since cellular granulation is usually accompanied by a run-down of Na^+ channel activity. After patch formation, an initial equilibration period of 10 min was allowed to see whether stable Na^+ channel activity occurred. Cell-attached and inside-out experiments began with a period of 15–20 min to collect a sufficiently large ensemble of sweeps under control conditions. Inside-out experiments are particularly complicated by unpredictable, spontaneously occurring changes in Na^+ channel activity (Kohlhardt, 1991). Therefore, the reversibility of experimentally-induced NP_o changes was tested during a washout phase.

Na^+ channel activity was triggered by rectangular membrane depolarizations from a holding potential (−110 mV to −130 mV) to a test potential (mostly −60 mV) with a rate of 0.67 Hz. The step depolarizations lasted 70 msec. The patch-clamp recordings were filtered at 1 kHz, on-line digitized with a sampling rate of 5 kHz, and stored on floppy discs. The dead time was 0.2 msec.

By subtracting leakage and residual capacity currents, the patch-clamp recordings were idealized. Open times of and gap times between non-overlapping single events were analyzed by setting a threshold at 50% current amplitude (Colquhoun and Sigworth, 1983). Probability density functions yielded τ_{open} and, by neglecting the first bin of 0.4 msec, 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 best fit of the probability density functions resulted from the least square method. The reopening analysis was based on a counting of sequential openings but was essentially complicated by the presence of multiple functioning Na^+ channels in each patch. Thus, sequential openings, even in activity sweeps without superpositions (the activity sweep type taken for analysis), will not a priori indicate the repetitive activity of one and the same Na^+ channel. Counting sequential openings, therefore, will yield only a rough estimate of reopening.

Groups, each consisting of 40 sweeps and periodically collected from the continuously stepped patch, were ensemble-averaged to reconstruct the macroscopic I_{Na}. Since $I_{Na} = i\,N\,P_o$ (i means unitary current, N is the number of channels and P_o means open probability), I_{Na} provides a measure of NP_o if i_{unit} remains unchanged; peak I_{Na} refers to the moment where NP_o attains its maximum during membrane depolarization and was taken as an index of the latter.

Sweeps without detectable channel openings are referred to as null sweeps. Whenever possible, the data are given as mean ± SEM.

Solutions (composition in mmol/liter)

(A) Isotonic K^+ solution: KCl 140; $MgCl_2$ 5; Na-pyruvate 2.5; glucose 20; $CaCl_2$ 0.05 or 0 (in the latter case, buffered with EGTA 2); HEPES 10; pH 7.4. This solution faced the cell surface in the cell-attached experiments and the cytosolic membrane surface in the inside-out experiments.

(B) Pipette solution: NaCl 150; $MgCl_2$ 1; $CaCl_2$ 0.03; HEPES 10; pH 7.4.

Temperature (controlled by a Peltier element device): 19 ± 0.5°C.

Compounds

All compounds were freshly dissolved before use in solution A. OAG (1-oleoyl-2-acetyl-sn-glycerol) and staurosporine were dissolved in dimethylsulfoxide (DMSO); appropriate amounts of the stock solutions were added to solution A to give a final DMSO concentration of 2.4 to 4.8×10^{-7} mol/liter. In the OAG and staurosporine experiments, solution A was supplemented with the same DMSO concentration under control and washout conditions.

A II (Hypertensin) was a gift of Ciba-Geigy Ltd. (Basel, Switzerland). All other compounds except staurosporine (Boehringer Mannheim, Germany) were purchased from Sigma Chemical (Munich, Germany).

Results

A first series of cell-attached experiments dealt with the influence of A II. From both of the cell populations in culture, spherocytes and rod-shaped cardiocytes, the latter type was selected to study the A II action in an advanced developmental cellular stage. A near-threshold test potential (mostly −60 mV) was

intentionally chosen since Na^+ inactivation proceeds rather slowly in this voltage range thus facilitating burst activity of Na^+ channels and, consequently, improving the yield of single channel events. Regardless of the absence or presence of 50 μmol/liter Ca^{++}, the exposure of cardiocytes to 3 μmol/liter A II increased Na^+ channel activity within 60–90 sec. This became evident by an increase of both the fraction of activity sweeps and the fraction of sweeps with superpositions, at the expense of the fraction of null sweeps (Fig. 1*A*). Consequently, in the experiment depicted in Fig. 1, reconstructed peak I_{Na} rose to 139% of the control. Open-state kinetics (Fig. 1*B*) and unitary current size remained unaffected. Reopening proved almost insensitive (Fig. 1*C*). Although an ultralong opening sequence not fitting the monoexponential frequency distribution of sequential openings occurred in the A II experiment depicted in Fig. 1 (*see* Fig. 1*C*), this particular activity mode cannot a priori be considered to be provoked by A II since Na^+ channels may also occasionally switch into this activity mode under normal conditions (Patlak & Ortiz, 1985; Kohlhardt et al., 1988).

In contrast to recent observations of Moorman et al. (1989) in neonatal cardiocytes and of Nilius et al. (1989) in adult myocytes, a significant increase of NP_o required in the present experiments A II concentrations higher than 0.3 μmol/liter. Reconstructed peak I_{Na} rose, at −60 mV, to 137 ± 17.5% (n = 8) at 3 μmol/liter and to 176 ± 42% (n = 5) at 30 μmol/liter. At 30 μmol/liter, τ_{open} tended to increase slightly and was 116 ± 3.1% (n= 5) of the control value. The mean number of sequential openings remained almost unaffected in this concentration range. It was, therefore, not surprising that I_{Na} decay kinetics, too, varied only insignificantly: τ_{decay} was 107 ± 3.7% (n = 8) at 3 μmol/liter and 118 ± 21.5% (n = 5) at 30 μmol/liter.

A II can stimulate the phosphoinositide hydrolysis thereby enhancing the tissue level of diacylglycerol (Griendling et al., 1986; Allen et al., 1988), the physiological activator of protein kinase C. Nevertheless, OAG was found not to mimic the A II effect on cardiac Na^+ channels (Fig. 2). After superfusing the cardiocytes with 6 μmol/liter OAG, reconstructed peak I_{Na} declined to 67 ± 4.9% (n = 8) of the control value. This NP_o decline developed without changes in either the open-state kinetics or the burst activity. τ_{open} was 101 ± 9.4% (n = 8) and the mean number of sequential openings 93 ± 4% (n = 8) of the control value. In the continued presence of OAG, three cardiocytes were subsequently exposed to the putative protein kinase C inhibitor, staurosporine (0.2 μmol/liter). Staurosporine abolished within 2–3 min the OAG action and caused NP_o to return to the control value as observed in the OAG-free solution.

Isolated cardiac Na^+ channels in cell-free conditions only responded to the presence of cytoplasmic OAG under conditions which allow a phosphorylation reaction. After excision, the inside-out patches were initially equilibrated for 15 min to clean the cytoplasmic membrane surface from possible contamination with cellular metabolites and to ensure that stable Na^+ channel activity was achieved. A first series of experiments revealed that, in the absence of Mg-ATP, a cytoplasmic treatment with OAG had no modulating effect on cardiac Na^+ channels: reconstructed peak I_{Na} varied insignificantly over a 5% range (n = 3). NP_o declined significantly, however, when OAG (1 μmol/liter) was administered in the simultaneous presence of Mg-ATP (50 μmol/liter). This response is most likely due to a channel phosphorylation brought about by activated protein kinase C and suggests that this enzyme is membrane-bound and spatially close to the Na^+ channels. Mg-ATP was present throughout in these experiments (i.e., before and after OAG treatment) since ATP itself can modulate the activity of cardiac Na^+ channels (Herzig & Kohlhardt, 1991). Under these conditions, OAG led to a decrease of the fraction of activity sweeps while the fraction of null sweeps rose (Fig. 3*A*). Elementary Na^+ channel properties including i_{unit}, open-state kinetics and burst activity remained unaffected (*see* Fig. 3 and Table). The inhibitory OAG effect developed within a few tenths of seconds and, thus, faster than in the cell-attached experiments where OAG has to pass through the membrane to reach its internal surface. I_{Na} declined without changes of the I_{Na} decay kinetics (*see* Fig. 3*C*), the latter remained a first-order reaction and could be best fitted by a single exponential. OAG washout (*see* Fig. 3*C*) caused I_{Na} to return to the initial control value within 1–2 min. In a total of four inside-out experiments, reconstructed peak I_{Na} declined to 40 ± 9.7% in the presence of 1 μmol/liter OAG (50 μmol/liter Mg-ATP) and was 95 ± 3.1% of the control after OAG washout.

Fluctuating levels of phosphoinositide metabolites at the cytoplasmic membrane surface may occur upon hydrolysis of phosphatidyl-4,5-bisphosphate. The latter represents a membrane-associated storage form of the two second messengers, diacylglycerol and inositoltrisphosphate. Interestingly, the common structural group of phosphoinositide metabolites, inositol, was found in another series of inside-out experiments to be capable of interfering with cardiac Na^+ channels (*see* Fig. 4): after treating the cytosolic surface with 10 μmol/liter inositol, reconstructed peak I_{Na} declined to 52 + 8.1% (n = 3)

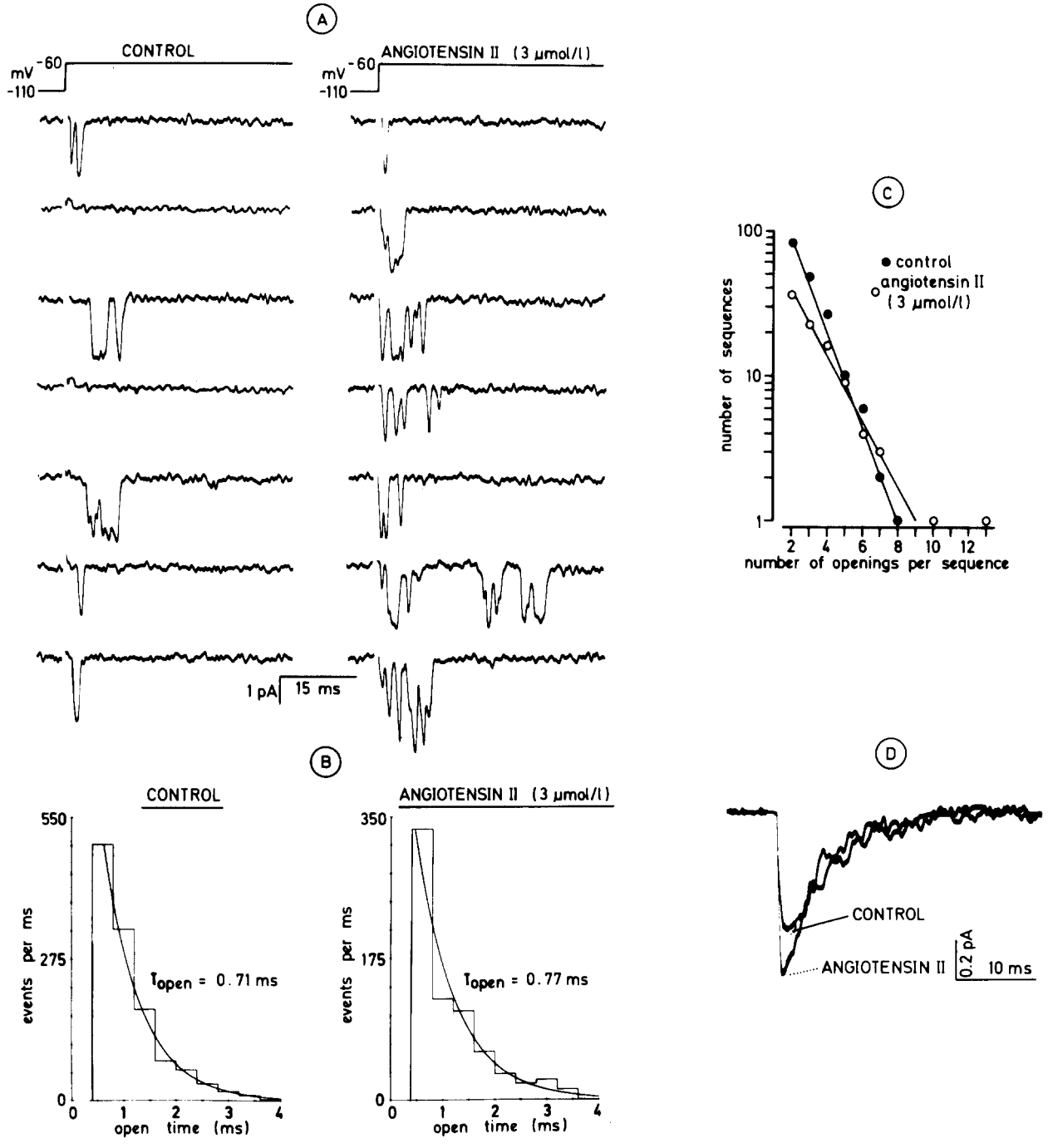

Fig. 1. The response of single cardiac Na^+ channels to angiotensin II. (*A*) Consecutive recordings of elementary Na^+ currents before (left) and after (right) angiotensin II treatment (3 µmol/liter) of the cardiocyte. (*B*) Open time probability density functions before (left) and after (right) angiotensin II treatment. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 1200 \exp(-t/0.00071)$ and by $N(t) = 615 \exp(-t/0.00077)$, respectively. (*C*) Frequency distributions of the number of openings per opening sequence before (filled circles) and after (open circles) angiotensin II treatment. The lines are fitted by eye. (*D*) Reconstructed macroscopic I_{Na} before and after angiotensin II treatment. Ensemble averages of 160 sweeps. Patch 638CA (cell-attached recording mode); −60 mV.

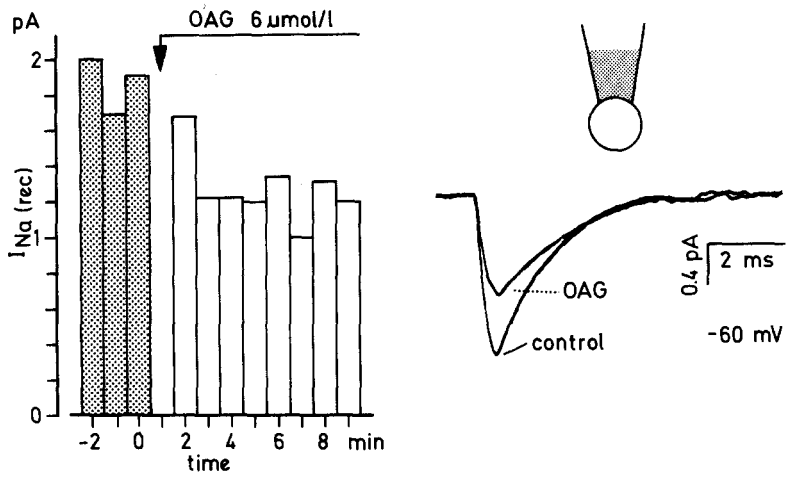

Fig. 2. The influence of OAG (6 μmol/liter) on reconstructed peak I_{Na} in the cell-attached mode. (Left) NP_0 profile before and after treating the cardiocyte with OAG. (Right) Superimposed reconstructed macroscopic Na^+ currents before and after OAG treatment of the cardiocyte. Patch 693CA (cell-attached recording mode); −60 mV.

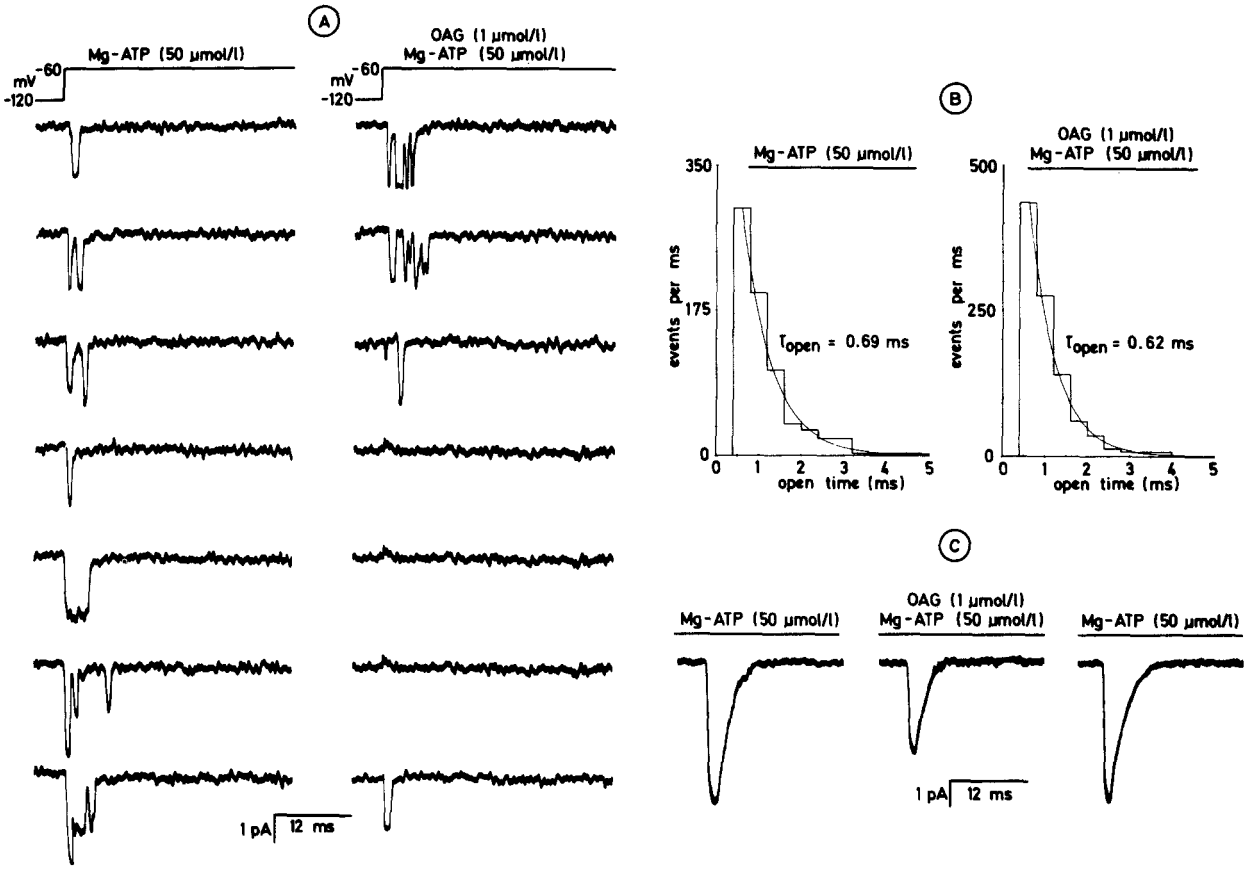

Fig. 3. The response of single cardiac Na^+ channels in cell-free conditions to OAG (1 μmol/liter) and the simultaneous presence of Mg-ATP (50 μmol/liter). (*A*) Consecutive recordings of elementary Na^+ currents before (left) and after (right) the cytosolic OAG administration. (*B*) Open-time probability density functions before (left) and after (right) cytosolic OAG administration. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 725 \exp(-t/0.00069)$ and by $N(t) = 1211 \exp(-t/0.00062)$, respectively. (*C*) Macroscopic Na^+ currents each reconstructed from an ensemble average of 120 sweeps before cytosolic OAG treatment (left), with OAG (middle), and 3 min after OAG washout. Patch 683IO (inside-out recording mode); −60 mV.

Table 1. The effect of OAG on cardiac Na^+ channels in cell-free conditions[a]

	CONTROL (50 μmol/liter Mg-ATP)				OAG (1 μmol/liter) (50 μmol/liter Mg-ATP)			
exp	τ_{open}	reopening	τ_{decay}	I_{Na}	τ_{open}	reopening	τ_{decay}	I_{Na}
682IO	0.85 msec	3.12	2.8 msec	1.7 pA	0.75 msec	3.66	4.6 msec	0.7 pA
683IO	0.69 msec	2.67	3.5 msec	1.4 pA	0.62 msec	2.54	2.7 msec	0.8 pA
684IO	1.07 msec	2.91	3.2 msec	1.0 pA	1.11 msec	3.00	3.3 msec	0.5 pA
685IO	0.90 msec	3.04	3.3 msec	1.9 pA	0.76 msec	2.47	3.0 msec	0.3 pA
mean	0.88	2.94	3.2		0.81	2.92	3.4	
	±0.08 msec	±0.1	±0.15 msec		±0.10 msec	±0.27	±0.42 msec	

[a] (1 μmol/liter in the presence of 50 μmol/liter Mg-ATP).
Reopening is expressed as the mean number of openings per opening sequence (for definition, see Materials and Methods) and τ_{decay} refers to I_{Na} decay kinetics. Membrane potential −60 mV.

of the control within a few tenths of seconds. This inhibition was not accompanied by changes of i_{unit}, open-state kinetics or burst activity and was found to disappear upon inositol washout.

Discussion

The present patch clamp experiments with cultured neonatal cardiocytes confirm that A II can modulate the activity of cardiac Na^+ channels. In the absence of accompanying changes of elementary channel properties, A II led to an increase in NP_o, an effect that could not be mimicked by the activator of protein kinase C, OAG. Obviously, the vasoactive peptide hormone A II exerts its influence on cardiac Na^+ channels by a mechanism other than through stimulation of protein kinase C, at least in cultured neonatal cardiocytes.

High-affinity A II receptors have been described in the sarcolemma of several cardiac tissues including neonatal rat heart, adult bovine and rabbit myocardium (Wright et al., 1983; Baker et al., 1984; Rogers, 1984; Rogers, Gaa & Allen, 1986). A II receptor stimulation is followed by a cascade of molecular events which finally causes an activation of protein kinase C (Baker & Singer, 1988). Although the biochemical responsiveness of the cultured cardiocytes to A II had not been tested in the present experiments, Griendling et al. (1986) provided evidence that, in cultured vascular smooth cells, a phosphoinositide hydrolysis caused by A II leads to a rise of the cytosolic diacylglycerol level, the key reaction involved in protein kinase C activation. It was surprising, therefore, that treatment of the cardiocytes with OAG had the opposite effect when compared with A II, and produced a depression of NP_o of cardiac Na^+ channels. In this context, it is important to discuss this discrepancy on the basis of A II receptor subtypes (Whitebread et al., 1989) of which only one, the AT_1 subtype, appears to be coupled to phosphoinositide turnover and, finally, protein kinase C stimulation. The other subtype, AT_2, has been proposed recently by Bottari et al. (1992) to be coupled to activate a protein tyrosine phosphatase. Assuming the existence and functional significance of AT_2 receptors in the neonatal rat cardiocyte, A II and OAG may well lead to different cellular responses, based on protein dephosphorylation or phosphorylation, respectively. It seems unlikely that the special cell type or the specific cultivating and experimental conditions of the present study underlie the unexpected opposite effects of A II and OAG since A II was reported in adult guinea pig myocytes to exert the same biophysical response, namely an increase in NP_o (Nilius et al., 1989).

Besides 6-aminopurines including ATP (Herzig & Kohlhardt, 1991), inositol was identified as another metabolite capable of modulating NP_o of cardiac Na^+ channels, but its functional significance, particularly with respect to the A II action, awaits further clarification. In other words, the mechanism underlying the NP_o increase after A II stimulation remains to be elucidated.

It is likewise an open question whether A II acts as a physiological activator of cardiac Na^+ channels and plays a role in regulating cardiac excitability. The normal A II plasma concentration is known to be close to 1 nmol/liter but submicromolar (Moorman et al., 1989; Nilius et al., 1989) or, in the present study, even micromolar A II concentrations are needed to augment NP_o.

Consistent with the responsiveness of brain Na^+ channels (Sigel & Baur, 1988; Numann, Catterall & Scheuer, 1991), a stimulation of protein kinase C by OAG had an inhibitory influence on cardiac Na^+

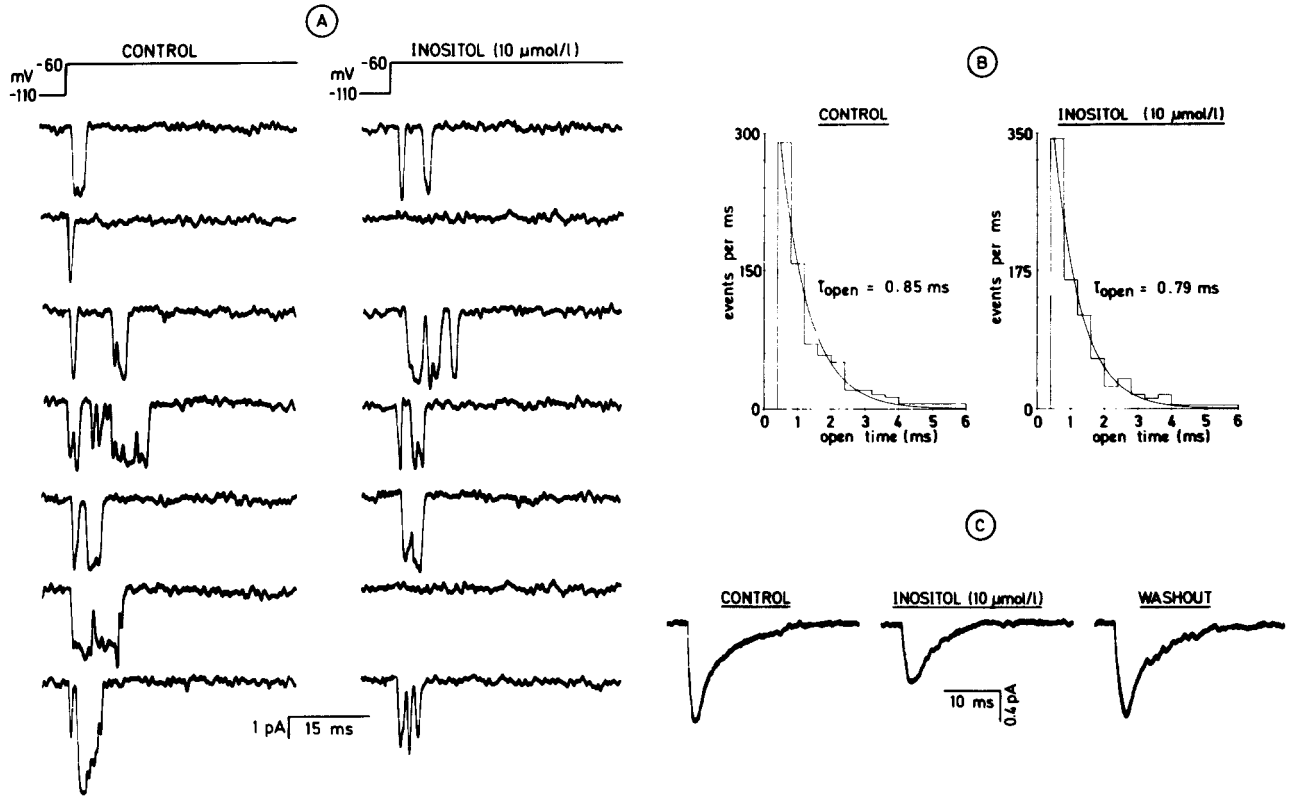

Fig. 4. The response of single cardiac Na^+ channels in cell-free conditions to the cytoplasmic presence of inositol (10 μmol/liter). (*A*) Consecutive recordings of elementary Na^+ currents before (left) and after (right) the cytosolic inositol administration. (*B*) Open-time probability density functions before (left) and after (right) cytosolic inositol administration. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 515 \exp(-t/0.00085)$ and by $N(t) = 660 \exp(-t/0.00079)$, respectively. (*C*) Macroscopic Na^+ currents each reconstructed from an ensemble average of 120 sweeps before cytosolic inositol treatment (left), with inositol (middle), and 3 min after inositol washout. Patch 671IO (inside-out recording mode); −60 mV.

channels and reduced NP_o while gating remained unaffected. Evidence has been accumulated that the pore-forming 260 kD α-subunit of Na^+ channels possesses two distinct phosphorylation sites, one of them located at the cytosolic channel surface in the connecting loop between segment six of domain I and segment one of domain II (for review *see* Catterall, 1988), being a target for phosphorylation by protein kinase A. A second phosphorylation site has been very recently localized on the cytosolic loop between domains III and IV (West et al., 1991) where protein kinase C is acting. West et al. (1991) obtained, with micromolar OAG concentrations, a reduction of peak I_{Na} together with a significant slowing of Na^+ inactivation while Sigel and Baur (1988) reported, likewise in brain Na^+ channels, an I_{Na} depression without concomitant changes of Na^+ inactivation. The latter pattern of response is consistent with the reaction of cardiac Na^+ channels to OAG as observed in the present experiments in the cell-attached and cell-free recording mode.

Phosphorylation by protein kinase C was found to occur at a single amino acid, serine 1506, which is located in the highly conserved region of the cytosolic linker between domains III and IV (West et al., 1991). This linker is of outstanding significance for Na^+ channel gating; it has been identified in mutagenesis experiments (Stühmer et al., 1989) and by using a site-directed antibody (Vassilev et al,. 1989) to play a crucial role for Na^+ inactivation. Although showing preserved gating properties, mutant Na^+ channels devoid of serine 1506 proved to be completely insensitive to protein kinase C and responded neither with a decrease in open probability nor with a delayed Na^+ inactivation (West et al., 1991). No obvious explanation can be offered as to why cardiac Na^+ channels retain their normal burst activity in the presence of the protein kinase C activator, OAG, while NP_o was actually reduced.

Na^+ channel phosphorylation by protein kinase A has a very similar functional consequence. Stimulation of β_1-adrenoceptors was shown in whole-cell clamped cardiocytes to depress I_{Na} (Ono, Kiyouse

& Arita, 1989) while I_{Na} decay kinetics remained unaffected. Further work is required to elucidate the relative significance of the aforementioned phosphorylation pathways in regulating NP_o or cardiac Na^+ channels and, thereby, the Na^+-dependent excitability in heart muscle via protein kinases A and C.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (Ko 778/2-3), Bonn.

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Received 24 February 1992; revised 14 May 1992