A Novel, Voltage-Dependent Nonselective Cation Current Activated by Insulin in Guinea Pig Isolated Ventricular Myocytes

Yin Hua Zhang, Jules C. Hancox

Abstract—Insulin regulates cardiac metabolism and function by targeting metabolic proteins or voltage-gated ion channels. This study provides evidence for a novel, voltage-dependent, nonselective cation channel (NSCC) in the heart. Under voltage clamp at 37°C and with major known conductances blocked, insulin (1 nmol/L to 1 μmol/L) activated an outwardly rectifying current ($I_{\text{insulin}}$) in guinea pig ventricular myocytes. $I_{\text{insulin}}$ could be carried by Cs$^+$, K$^+$, Li$^+$, and Na$^+$ ions but not by NMDG$^+$. It was inhibited by the NSCC blockers gadolinium and SKF96365 but not flufenamic acid. $I_{\text{insulin}}$ was largely blocked by the insulin receptor tyrosine kinase inhibitor HNMPA-(AM)$_3$ and by the phospholipase C inhibitor U73122 but not by its inactive analogue U73433. Staurosporine, a potent blocker of protein kinase C, did not prevent the activation of $I_{\text{insulin}}$. Application of analogue of diacylglycerol, 1-oleoyl-2-acetyl-sn-glycerol, mimicked the effect of insulin. This activated an outwardly rectifying NSCC that could be carried by Cs$^+$, K$^+$, Li$^+$, or Na$^+$ and that was blocked by gadolinium but not by flufenamic acid or staurosporine. We conclude that the intracellular pathway leading to activation of this novel cardiac NSCC involves phospholipase C, is protein kinase C–independent, and may depend on direct channel activation by diacylglycerol. (Circ Res. 2003;92:●●●●●●.)

Key Words: cardiac myocytes n diacylglycerol n insulin n nonselective cation current

Insulin is an essential metabolic hormone$^{12}$ that influences cardiac metabolism, the inotropic state of the heart,$^{3–5}$ cardiac protection,$^{6}$ hypertrophy,$^{7}$ and cardiomyopathy in patients with diabetes mellitus.$^{8}$ Insulin exerts its biological effects through the insulin receptor, an intrinsic tyrosine kinase, and its downstream signal transduction pathways.$^{1,2,6}$ Recently it has been reported that agents that increase tyrosine phosphorylation can activate nonselective cation channels (NSCCs) in smooth muscle cells.$^9$ However, functional expression of tyrosine kinase–activated NSCC in cardiac myocytes has not yet been reported.

In the present study we provide evidence that insulin can activate a novel NSCC in isolated ventricular myocytes. This NSCC is distinct from other NSCCs in the heart,$^{10–12}$ such as background NSCC and stretch-activated NSCC,$^{10}$ which are voltage-independent. In contrast, the insulin-activated NSCC ($I_{\text{insulin}}$) exhibits outward rectification. The ion selectivity of this current and the signal transduction mechanism involved in its activation are also described.

Materials and Methods

Ventricular myocytes from male guinea pigs (400 to 600 g) were isolated as described previously.$^{13}$ Whole-cell patch-clamp measurements were made at 37°C. Detailed methodological information is supplied in the online data supplement, available at http://www.circresaha.org.

Results and Discussion

Both application of insulin (1 μmol/L) progressively increased both outward and inward current components over 20 to 180 seconds, after which the response magnitude gradually decreased to a steady-state level with time (Figures 1A and 1B, n=15). $I_{\text{insulin}}$ did not show instantaneous activation on step depolarization to +80 mV but showed time dependence in its development (Figure 1A). This time dependence of current activation is reminiscent of that recently reported for a ligand-gated vaniloid receptor channel.$^{14}$ Plots of the peak outward and inward current densities against time (Figure 1B) show that the effect of insulin on outward current was more prominent than on inward current. With symmetrical pipette and external Cs$^+$, the current-voltage ($I$-$V$) relationship for $I_{\text{insulin}}$ showed prominent outward rectification and a reversal potential ($E_{\text{rev}}$) close to 0 mV (Figure 1A, inset, $E_{\text{rev}}$, $-0.13±1.71$ mV, n=9). Given that major known ionic current components had been blocked (see the online data supplement), the identity of $I_{\text{insulin}}$ was investigated additionally. Its cation selectivity was determined by altering the dominant pipette cation without altering the nature of the dominant permeant anion (chloride). $I_{\text{insulin}}$ could be observed when pipette Cs$^+$ was replaced by K$^+$, Li$^+$, or Na$^+$ ($P$<0.1 ANOVA, with Bonferroni post-hoc test) but not when NMDG$^+$ was substituted for Cs$^+$ ($P$<0.01). The peak outward densities of $I_{\text{insulin}}$ for the various cations are shown in
Collectively, these data suggest that insulin was an outwardly rectifying NSCC. Insulin magnitude was concentration-dependent over the insulin concentration range of 1 nmol/L to 1 μmol/L (Figure 1D).

Voltage-dependent NSCCs in the tissues other than the heart exhibit differential sensitivities to pharmacological NSCC blockers. For example, the vascular α1-adrenoceptor–activated cation channel (of which the transient receptor potential homologue TRPC6 is an essential component) is blocked by gadolinium (Gd) and SKF96365 but enhanced by flufenamic acid (FFA), whereas TRPC3 and TRPC7 can be inhibited by FFA. Therefore, we studied the sensitivities of insulin to these 3 blockers. As summarized in Figure 1E, Gd (100 μmol/L) significantly attenuated insulin and so did SKF96365 (10 μmol/L). However, FFA (100 μmol/L) did not affect insulin. Collectively, the observations described in Figures 1A through 1E indicate that insulin activated a novel voltage-dependent NSCC that differs from NSCCs reported to date from the heart.

Activation of the insulin receptor (an intrinsic tyrosine kinase) induces the activation of phospholipase Cγ (PLCγ) to hydrolyze phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate and diacylglycerol (DAG) and inositol 1,4,5-trisphosphate and DAG then act as second-messenger molecules to mobilize intracellular calcium and activate protein kinase C (PKC), respectively. The involvement of these signal-transduction pathways in the activation of insulin was studied. Pretreatment of cells with hydroxy-2-naphthalenyl-methyl phosphonic acid tris-acetoxy-methyl ester [HNMPA-(AM)3; 1 mmol/L for >15 minutes] followed by the application of insulin with HNMPA-(AM)3 abolished the activation of insulin (Figure 1F). A specific PLC inhibitor, U73122 (10 μmol/L), significantly inhibited activation of insulin, whereas an inactive analogue of U73122, U73433 (10

Figure 1. Insulin activates a voltage-dependent NSCC. A, Representative current traces showing the time-dependent activation of insulin. Inset shows the current in symmetrical Cs+ conditions. B, Current density of peak outward and inward Iinisulin (these typically occurred at between ~65 and ~78 mV and over a plateau range between ~69 and ~114 mV, respectively). C, Current densities (pA/pF) for maximal outward Iinisulin with different internal monovalent cations: Cs+, n=21; Li+, n=8; K+, n=10; Na+, n=7; and NMDG+, n=4. D, Concentration-dependent activation of maximal outward Iinisulin. E, Effects of NSCC blockers on maximal outward Iinisulin (Gd, n=5, *P<0.01 against control; SKF96365, n=10, *P<0.01; and flufenamic acid, n=6, P>0.1). F, Maximal outward Iinisulin densities with HNMPA-(AM)3 (n=8, *P<0.01 against control; U73122, n=10, *P<0.01; U73433, n=6, P>0.1; and staurosporine, n=8, P>0.1).
m/L), did not affect the response, suggesting that a PLC-dependent pathway is involved in the activation of insulin. A potent inhibitor of PKC, staurosporine (100 nmol/L), did not prevent the activation of insulin (Figure 1F). These data suggest that mobilization of PKC is not obligatory for the activation of insulin (additional supporting results are described in the online data supplement).

Recent evidence suggests that DAG can itself act as an intracellular messenger and directly activate voltage-dependent NSCC. The potential role for DAG in activating insulin was investigated by using a membrane-permanent analogue of DAG, 1-oleoyl-2-sn-acetyl-glycerol (OAG). OAG (50 μmol/L) increased both outward and inward current components in a time-dependent manner (Figures 2A and 2B). With symmetrical Cs⁺, the OAG-activated current (I_{OAG}) showed an outwardly rectifying I-V relationship (E_{rev} was +1.34±0.54 mV; n=6; Figure 2A inset). Similar to I_{insulin}, I_{OAG} could still be activated when internal Cs⁺ was replaced by Na⁺, Li⁺, or K⁺ but not when a NMDG⁺-rich pipette solution was used (Figure 2C; P<0.01, ANOVA with Bonferroni post-hoc test). I_{OAG} was significantly inhibited by 100 μmol/L Gd but unaltered by flufenamic acid (100 μmol/L; Figure 2D). In addition, staurosporine (100 nmol/L) did not prevent the activation of I_{OAG} (Figure 2D). These data indicate that OAG activated a voltage-dependent NSCC similar to insulin and suggest that the insulin-activated NSCC could be mediated directly through an action of DAG without requiring mobilization of PKC.

Ligand-gated, voltage-dependent NSCCs have been reported in several tissue types (eg, smooth muscle cells, Figure 2).

Figure 2. An analogue of diacylglycerol, OAG activates a NSCC similar to insulin. A, Representative records showing the time-dependent activation of voltage-dependent current by OAG (50 μmol/L). Inset shows the I-V relationship of OAG-activated current with symmetrical Cs⁺. B, Current density of peak outward and inward I_{OAG} with time. C, Current densities (pA/pF) for maximal outward I_{OAG} with different internal monovalent cations: Cs⁺, n=10; Li⁺, n=5; K⁺, n=5; Na⁺, n=6; and NMDG⁺, n=6). D, Similar to I_{insulin}, I_{OAG} was not sensitive to FFA (P>0.1) but largely inhibited by Gd application (n=5, *P<0.01). E, I_{OAG} was not sensitive to staurosporine (100 nmol/L, n=4, P>0.05).
endothelial cells and sensory neurons in which NSCCs may be important in depolarizing the membrane potential or inducing calcium entry. Our data provide clear evidence for the existence of a hitherto unreported, voltage-dependent, and ligand-activated NSCC in the heart. Moreover, both insulin and OAG induced shortening of the AP duration in the presence of staurosporine (Figures 3A through 3C; insulin and OAG shortened APD90 by 26.5 ± 4.2%, OAG, 33.4 ± 1.8%, respectively). The changes of action potential profile by insulin and OAG in the presence of PKC inhibition suggest that insulin has the potential to modulate cardiac electrophysiology (see the online data supplement for additional discussion of this issue). Additional investigation of the regulation and potential roles of this novel NSCC is now warranted to understand its contributions to normal and pathological cardiac electrophysiology.

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References


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Materials & Methods.

*Myocyte isolation* Male guinea-pigs (400-600 g) were humanely killed using a UK Home Office-approved ‘Schedule 1’ procedure, their hearts removed and single ventricular myocytes isolated as described previously.\(^1\) Isolated cells were kept in high-K\(^+\), low Cl\(^-\) storage medium (Kraft-Brühe, KB medium) at 4°C\(^1\).

*Voltage-clamp technique* Whole-cell patch-clamp experiments were performed using an Axopatch 200A amplifier (Axon instruments, USA). Patch pipettes (Corning 7052 glass, AM Systems) were pulled using a Flaming/Brown P87 puller and fire-polished to a final resistance of \(~2\) MΩ (Narishige MF 83 microforge, Japan). A descending voltage ramp from +80 mV to −120 mV (dV/dt, 0.4 V s\(^{-1}\); holding potential of −80 mV) was employed to measure whole cell membrane current. Protocols were generated and data recorded on-line with p-Clamp 6.0 software via an analogue-to-digital converter (Digidata 1200B, Axon instrument, USA).

*Solutions* Once in the recording chamber, cells were bathed in a standard Tyrode’s solution containing (in mM): 140 NaCl, 5 HEPES, 10 glucose, 4 KCl, 2.5 CaCl\(_2\), 1 MgCl\(_2\) (pH adjusted to 7.45 with NaOH). The pipette dialysate contained (in mM): 113 Cs, 20 NaCl, 4 Mg-ATP, 0.5 Na-GTP, 10 HEPES, 5 glucose, 10 BAPTA, 20 tetraethylammonium chloride, 1 CaCl\(_2\), pH 7.2 (titrated with CsOH). Na-GTP was included because G-proteins are important in signal transduction mechanisms downstream of the insulin receptor.\(^2\)\(^-\)\(^4\) The combination of 10 BAPTA and 1 CaCl\(_2\) gave a free pipette Ca concentration of 20 nM (calculated with the Maxchelator program, WEBMAX v 2.10, Stanford, USA). For experiments in which the dominant internal cation was different from Cs, 113 mM Cs-aspartate was replaced with equimolar K-, Li-, NMDG-, or Na-aspartate.
When recording $I_{\text{insulin}}$, the extracellular solution was switched to a K$^+$-free Tyrode's solution containing 10 μM strophanthinidin (to inhibit Na-K pump current), 10 μM nifedipine (to eliminate Ca current) and 1 mM BaCl$_2$ (to block residual K and background conductances), 2 mM NiCl$_2$ (to block Na-Ca exchange current) and 100 μM glibenclamide (to block cAMP-activated Cl$^-$ current). This solution was used for all experiments except those in which symmetrical Cs was used; for those experiments, external NaCl was replaced with CsCl. Experimental solutions were externally applied using a temperature-controlled (37°C), rapid application device.

**Chemicals.** Apart from the collagenase used for cell isolation (Worthington, supplied via Lorne Laboratory Ltd, UK), all chemicals used in this study were obtained from Sigma or CalBiochem. Insulin was dissolved in distilled water (Milli-Q) to make a 0.35 mM stock solution. For our experiments, OAG was dissolved in DMSO to make a 50mM stock solution and added to the pipette solution to a final concentration of 50 μM. Gadolinium, flufenamic acid and SKF96365 were dissolved in the distilled water to produce stock solutions. For experiments in which the sensitivity of the novel non-selective cation conductance to these agents was examined, gadolinium was acutely applied after OAG or insulin, whilst cells were pre-incubated with flufenamic acid and SKF96365 for 1-2 minutes before applying insulin or OAG. It should be noted that we observed a rapid breakdown of the gigaseal in the combined presence of OAG and SKF96365 and therefore it was not possible to determine the effects of SKF96365 on $I_{\text{OAG}}$. Hydroxy-2-naphthalenylmethyl phosphonic acid tris-acetoxymethyl ester (HNMPA-(AM)$_3$) staurosporine, chelerythine, U-73122, U-73433, 1-oleoyl-2-acetyl-sn-glycerol (OAG) were dissolved in DMSO to make stock solutions. The stock solutions were made so that the final concentrations of agents in experimental solutions were at least 1/500 of their appropriate stock solutions. We used maximally
effective concentrations of experimental agents in order to avoid failing to observe the involvement of particular pathways in mediating actions of insulin.

*Data analysis.* Data are expressed as mean ± S.E.M. Comparison of magnitudes of \( I_{\text{insulin}} \) or \( I_{\text{OAG}} \) (Figures 1D and 2C) between the range of different pipette cations was made using ANOVA and a Bonferroni post-hoc test for pair-wise comparison. A Student’s t-test was employed to determine significance of the reduction in magnitude of \( I_{\text{insulin}} \) or \( I_{\text{OAG}} \) by NSCC blocking agents and of receptor and intracellular-messenger antagonists. \( P \) values of less than 0.05 were taken as significant.

**Results and Discussion**

*Identification of \( I_{\text{insulin}} \) and \( I_{\text{OAG}} \) as NSCCs.*

The experimental solutions used in this study can be expected to have inhibited most conductances that could overlap a NSCC. Moreover, although chloride ions were present and so could in theory be available to carry a ligand-activated current, a number of features of the data shown in Figures 1-2 indicate that \( I_{\text{insulin}} \) and \( I_{\text{OAG}} \) are NSCCs. First, the results in Figures 1C and 2C with cation replacement (\( \text{Cs}^+, \text{Na}^+, \text{K}^+, \text{Li}^- \) and \( \text{NMDG}^+ \)) show that pipette \( \text{NMDG}^+ \) largely abolished both \( I_{\text{insulin}} \) and \( I_{\text{OAG}} \), despite the fact that \( \text{Cl}^- \) was present internally and externally to a similar level as with the other internal cations. Without any change to transmembrane \( \text{Cl}^- \) gradient, the loss of current correlated with the change to the dominant internal cation. Second, the concentration of glibenclamide used in the present study (100\( \mu \text{M} \)) is almost 10 fold of the \( I_{\text{Cl}_{50}} \) for glibenclamide inhibition of the cAMP-activated \( \text{Cl}^- \) current.\(^5\) Third, the volume-activated \( \text{Cl}^- \) current (\( I_{\text{Cl}_{\text{vol}}} \)) that is known to be regulated by tyrosine kinase signaling pathways is also sensitive to glibenclamide (at 100 \( \mu \text{M} \), glibenclamide has been reported to inhibit \( I_{\text{Cl}_{\text{vol}}} \) at positive potentials by \( \sim 80\% \)). Fourth, flufenamic
acid (FFA) can block at least some Cl⁻ conductances in some tissues (e.g.⁶), but did not inhibit $I_{\text{insulin}}$ or $I_{\text{OAG}}$. Fifth, the sensitivity of $I_{\text{insulin}}$ to Gd$^{3+}$ and to SKF96365 is consistent with its identity as a receptor-activated NSCC. Taken collectively, these points provide compelling evidence that insulin activated a NSCC rather than a Cl⁻ conductance under our conditions and that application of OAG also led to activation of a NSCC.

*Activation of NSCC by a pathway ultimately leading to direct channel activation by DAG.*

As shown in Figure 1F of the main text of this Report, HNMPA-(AM)₃ abolished the activation of $I_{\text{insulin}}$, demonstrating the involvement of insulin-receptor tyrosine kinase in the activation of $I_{\text{insulin}}$. Use of a specific PLC inhibitor, U73122, but not of an inactive analogue, U73433 significantly inhibited activation of $I_{\text{insulin}}$ which implicated a PLC-dependent pathway in the activation of $I_{\text{insulin}}$. It should also be noted that under our recording conditions, bulk Ca$^{2+}$ was highly buffered with BAPTA therefore an obligatory role for IP₃-dependent Ca$^{2+}$-release in the activation of $I_{\text{insulin}}$ was unlikely. Moreover, the data obtained with staurosporine (Figure 1F), suggested that activation of PKC was not necessary for activation of $I_{\text{insulin}}$ to occur. Two further pieces of evidence support this. First, we also found that $I_{\text{insulin}}$ could also be observed with another inhibitor of PKC, chelerythrine (data not shown). Second, in a recent study from our laboratory performed under similar conditions, application of phorbol 12-myristate 13-acetate (PMA), a potent PKC activator, did not induce outwardly rectifying current similar to $I_{\text{insulin}}$⁷. These observations, together with the fact that OAG activated an outwardly rectifying current that was carried by the same cations as $I_{\text{insulin}}$ and that was blocked by Gd$^{3+}$ but not FFA, suggest that insulin activated a NSCC by a pathway that involved activation of PLC, subsequent DAG production and then direct channel activation by DAG.
$I_{\text{insulin}}$ and $I_{\text{OAG}}$ showed similarities to one-another in their outward rectification, cation selectivity and sensitivities to FFA and Gd$^{3+}$, suggesting that the two currents were carried by a similar NSCC. In addition, as shown in Figure 1B, the activation of $I_{\text{insulin}}$ showed a biphasic time-dependence, with current activating to a peak over $\sim 150$ s and then declining to a lower level over the following 200 s. The mechanism underlying the decline in $I_{\text{insulin}}$ following its activation was not investigated in this study. However, our data indicate that observation of $I_{\text{insulin}}$ was consequent upon activation of the insulin tyrosine-kinase receptor and of its downstream internal second messenger cascade(s). Therefore, the decline of $I_{\text{insulin}}$ after its activation can be postulated to result from mechanisms such as receptor desensitization, or time-dependent interactions between different intracellular proteins/pathways linked to the insulin receptor. Whilst it is clear that direct application of the membrane-permeant DAG analogue OAD elicited a NSCC, independent of surface-receptor activation, we were unable to ascertain whether or not, following its activation, $I_{\text{OAG}}$ showed a similar decline in amplitude to $I_{\text{insulin}}$. This is because, under our experimental conditions, it proved difficult to maintain stable recordings with prolonged exposure of myocytes to externally applied OAG; this limited reliable recording periods to $\sim 180$ s (Figure 2B). Further investigation is required to identify the precise mechanism underlying the biphasic nature of the activation $I_{\text{insulin}}$.

Physiological relevance of the novel NSCC.

The normal physiological concentration range of insulin lies in the nM range. Since $I_{\text{insulin}}$ was activated in a concentration-dependent manner by insulin concentrations between 1nM and 1 µM, some activation of the current might occur physiologically. This is unlikely to be the only effect exhibited by insulin, though, since it has been reported to alter cardiac sarcolemmal ionic currents by conventional signalling pathways involving protein kinase activation. For example, in experiments conducted at room temperature, L-type calcium current has been observed to be increased by
The cardiac Na-Ca exchanger has also been reported to be regulated by insulin (e.g.\textsuperscript{11}) and insulin can also influence cardiac K channels (e.g.\textsuperscript{12}). The action potential experiments in Figure 3 were therefore specifically designed to address the question as to whether insulin (and OAG) could alter action potential configuration when protein kinase pathways involving serine or threonine phosphorylation were inhibited by the presence of staurosporine – maximising the likelihood of observing the consequences of direct NSCC activation by DAG/OAG. The resultant action potential shortening observed with insulin and OAG must, however, be interpreted within the context that under normal conditions, insulin might be anticipated to modulate other currents in addition to activating the novel NSCC reported here. The overall net effect of insulin might be anticipated to depend on the balance of actions on depolarising and repolarising conductances, which \textit{in vitro} may conceivably vary between different cells. Consistent with this notion, in three of five cells from which action potentials were recorded in the absence of staurosporine insulin produced moderate lengthening of $APD_{90}$, whilst in two of five cells $APD_{90}$ was shortened by insulin. When data from the five cells were pooled the mean alteration of $APD_{90}$ by insulin (prolongation by $17.74 \pm 15.58 \%$) was statistically insignificant ($p>0.3$). The contribution of the insulin-/OAG-activated NSCC to the overall electrophysiological effects of insulin is at present not easy to isolate, however, due to the lack of a selective and rapidly-acting blocker of the current that could be externally applied during action potential recording.

Although the findings of the present study are relevant to the cardiac actions of insulin, they also have a wider, and arguably more fundamental, significance. First, the NSCC described here appears not to be restricted to the guinea-pig heart because in preliminary experiments on rat atrial and ventricular myocytes we have observed a similar insulin-activated current. Thus channels mediating this NSCC are present in
different species and in different regions of the heart. Second, whilst insulin is the
agonist that has allowed us to identify and study this novel NSCC, the observation that
any agonist might activate a NSCC in the heart via a pathway involving a direct action
of DAG is important. From this, two questions arise that now require investigation.
The first is whether or not other hormones than insulin that work via a receptor-linked
tyrosine kinase (or indeed via other receptor linked pathways) are able to activate a
similar outwardly rectifying NSCC in mammalian heart cells. The second intriguing
question posed by our data that must be addressed relates to the underlying molecular
identity of this novel NSCC.
References for the online supplement


