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Progress in Biophysics and Molecular Biology 90 (2006) 249–269

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Progress in
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Review

Actions of emigrated neutrophils on Na^+ and K^+ currents in rat ventricular myocytes

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Available online 9 August 2005

Abstract

Interactions between neutrophils and the ventricular myocardium can contribute to tissue injury, contractile dysfunction and generation of arrhythmias in acute cardiac inflammation. Many of the molecular events responsible for neutrophil adhesion to ventricular myocytes are well defined; in contrast, the resulting electrophysiological effects and changes in excitation–contraction coupling have not been studied in detail. In the present experiments, rat ventricular myocytes were superfused with either circulating or emigrated neutrophils and whole-cell currents and action potential waveforms were recorded using the nystatin-perforated patch method. Almost immediately after adhering to ventricular myocytes, emigrated neutrophils caused a depolarization of the resting membrane potential and a marked prolongation of myocyte action potential. Voltage clamp experiments demonstrated that following neutrophil adhesion, there was (i) a slowing of the inactivation of a TTX-sensitive Na^+ current, and (ii) a decrease in an inwardly rectifying K^+ current.

One cytotoxic effect of neutrophils appears to be initiated by enhanced Na^+ entry into the myocytes. Thus, manoeuvres that precluded activation of Na^+ channels, for example holding the membrane potential at -80 mV, significantly increased the time to cell death or prevented contracture entirely. A mathematical model for the action potential of rat ventricular myocytes has been modified and then utilized to integrate these findings. These simulations demonstrate the marked effects of (50-fold) slowing of the inactivation of 2–4% of the available Na^+ channels on action potential duration and the corresponding intracellular Ca^{2+} transient. In ongoing studies using this combination of

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approaches, are providing significant new insights into some of the fundamental processes that modulate myocyte damage in acute inflammation.

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Keywords: Neutrophil; Myocyte; Na⁺ current; Inflammatory response

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1. Introduction

Inflammation within the ischaemic myocardium is a complex process. Numerous immunological and cell signalling mechanisms contribute to tissue damage. Alone or in combination, these can result in reduced myocardial function. Recently, attention has focussed on the deleterious effects of macrophages in inflamed myocardium (Entman et al., 1992; Mehta and Li, 1999). Within hours of initiation of an inflammatory response there is an infiltration of myocardial tissues by neutrophils as assessed by both histological changes (Binah, 1994; Bohle et al., 1993; Birdsall et al., 1997) and biochemical markers (Chen et al., 1995; Lefer et al., 1996; Cuevas et al., 1997; Sato et al., 1997). Adhesion of emigrated neutrophils to ventricular myocytes compromises their function and it may result in cell death (Wilson et al., 1993; Lefer et al., 1993; Mayers et al., 1996; Semb et al., 1989).

Neutrophil-mediated target cell responses involve a cascade of distinct events. After tissue injury the production of cytokines and chemoattractants results in the activation of neutrophils and enhanced expression of adhesion molecules (Entman et al., 1992). This is followed by extravation of neutrophils from the circulation to the site(s) of injury. These emigrated neutrophils adhere strongly to myocytes and ultimately cause myocyte dysfunction (Entman et al., 1990; Smith et al., 1991; Poon et al., 1999). Although the process of leukocyte recruitment is now quite well understood (cf. Poon et al., 1999); the neutrophil–myocyte interactions have been studied in less detail. In their important, original studies, Entman and colleagues (1990, 1992) demonstrated that neutrophils can adhere to myocytes via CD11/CD18 or B₂-integrins. Recently, we have identified an additional type of adhesion molecule, α_4 -integrin, and shown that it is also an important mediator of neutrophil adhesion to myocytes (Reinhardt et al., 1997). Following adhesion to myocytes, the activated neutrophils release a number of cytotoxic compounds, each of

which can cause myocyte dysfunction (Rossen et al., 1985; Engler, 1989; Lucchesi et al., 1989) sometimes including arrhythmias (Dhein et al., 1995).

Previous studies have demonstrated that application of a number of the substances known to be released by neutrophils can quickly result in dramatically altered mechanical and electrophysiological responses in cardiac myocytes (Pallandi et al., 1987; Barrington et al., 1988). Hoffman et al (1997) reported that circulating neutrophils, when bound to canine myocytes, produced electrophysiological effects including delayed repolarization, early afterdepolarizations (EAD) and, ultimately, marked depolarization of the resting potential. These results provided important insights into the way in which neutrophils may generate rhythm disturbances, and also suggested that neutrophils may be important contributors to reperfusion-induced arrhythmias. These findings also raised questions concerning the underlying ionic mechanism(s) i.e. whether neutrophil-induced changes in transmembrane ionic currents could be identified. These questions and the known functional differences between activated and emigrated neutrophils (Zimmerli et al., 1986; Yee et al., 1994; Poon et al., 1999) provided the background to and the motivation for these neutrophil/myocyte studies.

The present experiments were designed to record the electrophysiological changes induced following adherence of either circulating or emigrated neutrophils to rat ventricular myocytes, and to identify the corresponding changes in ionic current(s). Our results show that shortly after adherence of emigrated neutrophils, rapid and marked electrophysiological changes (increased action potential duration, depolarization of resting membrane potential) occur. These changes may quickly result in myocyte death. Corresponding voltage clamp measurements demonstrate that neutrophil adherence leads to alterations in the (i) TTX-sensitive Na^+ current and (ii) inwardly rectifying K^+ current, I_{K1} . When the voltage clamp method was used to prevent neutrophil-mediated membrane depolarization and/or activation of Na^+ current, myocyte damage was reduced and the myocyte death increased dramatically. We suggest, therefore, that the primary cytotoxic effects of neutrophils on cardiac myocytes appears to arise from neutrophil-induced changes of TTX-sensitive Na^+ channels. These electrophysiological findings have been incorporated into a modified version of our mathematical model for the rat ventricular action potential (Pandit et al., 2001). In this way, our results have been integrated compared with previous literature, and evaluated in terms of a number of different working hypotheses for the underlying electrophysiological and electrochemical changes.

2. Materials and methods

2.1. Ventricular cell isolation

The procedure for the isolation of adult rat ventricular myocytes has been described previously by Ward and Giles (1997). Briefly, animals were killed by decapitation and the heart was rapidly removed and mounted on a canula for retrograde perfusion at a rate of 10 ml min^{-1} using a standard Langendorff apparatus. Hearts were initially perfused for 5 min with Tyrode's solution containing 1 mM CaCl_2 . This was followed by 5 min perfusion with nominally Ca^{2+} -free Tyrode's solution. After this, the heart was perfused for 7 min with Tyrode's solution to which collagenase (0.02 mg/ml ; Yakult Co Ltd, Japan) and protease (0.004 mg/ml ; type XIV; Sigma Chemical Co, St

Louis, MO) had been added. The entire right ventricle was then removed from the heart and minced in 10 ml of Tyrode's solution containing collagenase (0.5 mg/ml), protease (0.1 mg/ml), bovine serum albumin (BSA; 5 mg/ml; Sigma) and CaCl_2 (50 μM). After the tissue was gently agitated for 10 to 20 min at 34 °C in a shaking water bath, aliquots of supernatant were drawn off (at 3 min intervals) and placed into 4 ml of Tyrode's solution containing CaCl_2 (50 μM). The ventricular myocytes were stored in this solution until use.

2.2. Neutrophil isolation

To obtain emigrated neutrophils, rats were injected intraperitoneally with 10 ml of 1% oyster glycogen in a phosphate buffered saline (PBS) (cf. Poon et al., 1999). After 4 h, these animals were killed, and the peritoneal fluid was collected. After centrifugation, the emigrated neutrophils were resuspended in Tyrode's solution at approximately 8×10^6 cells/ml. Circulating rat neutrophils were isolated from citrate-anticoagulated whole blood collected by cardiac puncture. Red blood cells were removed by dextran sedimentation followed by hypotonic lysis. Neutrophils were further purified by centrifugation through a Histopaque gradient and then resuspended in Tyrode's solution at approximately 8×10^6 cells/ml. These neutrophils were activated by treating them with 20 μM *N*-formyl-Met-Leu-Phe (fMLP) immediately before they were added to the superfusion chamber which contained the myocytes.

2.3. Solutions

The control Tyrode's solution used for the isolation of myocytes contained (mM): NaCl 140, KCl 5.4, MgCl_2 1, Na_2HPO_4 1, HEPES 5, glucose 10, CaCl_2 1, pH was adjusted to 7.4 with NaOH. This solution was continuously gassed with 100% O_2 . The internal solution used to fill the microelectrodes contained (mM): KCl 20, K-aspartate 110, NaCl 10, MgCl_2 1, CaCl_2 1, EGTA 10, HEPES 10 and K_2ATP 5. The pH was adjusted to 7.2 with 1 M KOH. Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co (St. Louis, USA).

2.4. Electrophysiological methods

Membrane currents and action potentials were recorded using standard voltage-clamp and current-clamp methods. Borosilicate glass shanks were pulled to form patch pipettes on a microprocessor-controlled, multiple stage puller (model P87, Sutter Instruments, Novato, CA). When filled with internal solution, pipette resistance ranged from 1.5 to 3 M Ω . Tip potentials were zeroed immediately prior to gigaseal formation. All measured membrane potentials were corrected by -10 mV to compensate for junction potentials between the external and internal solutions. Currents and action potentials were recorded using customized software (Cellsoft, D. Bergman, University of Calgary) with a List EPC-7 amplifier (List Electronics, Darmstadt, Germany). Whole-cell currents and membrane potentials were filtered at 3 kHz and sampled at 5–10 kHz with a 12-bit A–D converter (DT2821; Data Translation, Marlborough, MA) and were stored on a microcomputer for later analysis.

Most electrophysiological experiments were done using the nystatin perforated patch technique to prevent intracellular dialysis of the myocytes as described by Korn and Horn (1989). Nystatin,

dissolved in DMSO, was added to the pipette solution to a final concentration of 0.2 mg/ml immediately prior to each attempt to form a gigohm seal. From a holding potential of -80 mV, 30 ms test pulses to -75 mV were applied to the cell at 1 Hz to monitor the extent of perforation. Following stabilization of capacitive transients (~ 7 min), an additional 5 min time period was allowed prior to recording the capacitive transients, which were used for calculations of cell capacitance and series resistance. The total cell capacitance, C_m , measured as the integral of the displacement current resulting from the 5 mV depolarizing steps, averaged 112 ± 13 pF ($n = 43$). Uncompensated series resistance measured 12.2 ± 3.4 M Ω ($n = 43$). Recordings in which the series resistance exceeded 20 M Ω were excluded from analysis. Action potentials in ventricular myocytes were elicited by a brief (5 ms), 600 pA current injections applied at 0.2 Hz. Whole-cell currents were elicited by applying either rectangular or ramp waveforms, as indicated. Following the required baseline recordings, activated neutrophils were added to the superfusion chamber, using a custom built rapid solution changer. In all experiments, myocytes were superfused with neutrophils for only 1 min and then the superfusion solution was returned to Tyrode's solution. These conditions were chosen to provide the suitable 'time window' needed to resolve the neutrophil-induced time-dependent changes in action potentials and underlying ionic currents. More aggressive neutrophil treatment resulted in virtually immediate myocyte death. Electrophysiological parameters were continuously monitored throughout each experiment. Action potential and membrane current recordings were analysed using Cellsoft. All experiments were carried out at room temperature (21 – 22 °C).

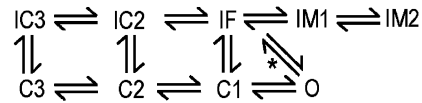
In one part of this study the cell-attached patch recording technique was used to record the single channel transitions which underlie I_{Na} . Fire-polished electrodes were coated with Sylgard 184 (Dow Corning) and filled with Ca^{2+} -free Tyrode's solution to which 100 μ M $BaCl_2$ was added to block I_{K1} . Following tight seal formation (> 20 G Ω) I_{Na} was elicited by holding the patch 50 mV negative to the resting membrane potential and delivering 50 ms depolarizing steps to 10 mV positive to the membrane potential. Current traces in which channel activity was absent were used to correct for capacitance transient and leakage current by subtraction. Ensemble currents were obtained by averaging at least 50 consecutive sweeps. Statistical analysis was done using Student's *t*-test.

2.5. Mathematical model of the rat ventricular myocyte

All simulations of changes in inactivation of the Na^+ current, I_{Na} , are based on our previous model of the rat ventricular myocyte (Pandit et al., 2001). However, for the purposes of this study I_{Na} was formulated as a Markovian model of single channel transitions, rather than the Hodgkin–Huxley formulation used in the original Pandit et al. model. The Markovian formulation is based on the model used previously by Clancy and Rudy (1999), and Bondarenko et al., (2004). All rate constants have magnitudes and membrane potential dependence identical to those in Bondarenko et al. (2004). A second important set of changes are in the formulations we have used for the L-type Ca^{2+} current and for Ca^{2+} -induced Ca^{2+} release. Those were taken from Shiferaw et al. (2003).

In our model of the Na^+ channel it is assumed that the channels can exist in a number of closed (Cx), and closed inactivated (ICx) states, as well as a single open state (O), a 'fast' inactivated state (IF), and two 'slow' 'intermediate' inactivated states (IM1, IM2). Transitions are allowed between

Cx and ICx states. The ‘slow’ inactivated states (IM1, IM2) are virtually ‘absorbing’ at depolarized potentials. The state diagram for this model is shown below.



The effects of activated neutrophils on Na^+ currents in rat ventricular myocytes was modelled by assuming that in a small percentage of the channel population (2–4%), the rate constant for the ‘O to IF’ transition (indicated by * in the diagram above) was reduced by a factor of 50-fold. This modification produced Na^+ currents having a very slow component of inactivation which was completely absent in the modelled ‘control’ I_{Na} . Baseline or control I_{Na} records inactivated completely within approximately 10 ms at -20 mV.

3. Results

In all experiments, emigrated neutrophils adhered strongly to freshly isolated quiescent ventricular myocytes. Under our experimental conditions, on average, 4.2 ± 0.4 ($n = 39$) emigrated neutrophils adhered to each ventricular myocyte. In contrast, when the same density of circulating neutrophils were added to the superfusion solution, only 1.5 ± 1.0 ($n = 4$) neutrophils/myocyte adhered. Fig. 1 shows representative microscopic fields of myocytes in the presence of either (A) circulating or (B) emigrated neutrophils. Close examination revealed that the emigrated neutrophils were affixed to both the myocyte and the underlying glass coverslip. Emigrated neutrophils were strongly activated as assessed by the large population of mobile, non-spherical cells.

To define the electrophysiological consequences of neutrophil adherence to ventricular myocytes, action potentials were recorded continuously at 0.2 Hz before and after addition of emigrated neutrophils to the superfusate (Figs. 1C and 1D). Typical patterns of electrophysiological changes occurred within 3–7 min. These changes include: (i) an initial small depolarization of the resting membrane potential ($n = 5/19$); (ii) prolongation of action potential duration ($n = 9/19$) as illustrated in Fig. 1C; or (iii) both depolarization and prolongation ($n = 5/19$) as shown in Fig. 1D. Under experimental conditions in which the superfusion solution was maintained at $31\text{--}32^\circ\text{C}$, the changes in action potential configuration were rapid. On average, myocytes survived for 370 ± 44 s ($n = 19$; see Table 1) following adhesion of emigrated neutrophils. When the same protocols were performed at room temperature, $20\text{--}22^\circ\text{C}$, the time to myocyte death was prolonged to 605 ± 48 s ($n = 4$; see Table 1), even though there was no significant difference in the number of neutrophils (4.4 ± 0.6 ($n = 19$) and 3.6 ± 0.3 ($n = 4$), respectively) which had adhered to each myocyte. Infact, there was no correlation between the number of adhered neutrophils and time to myocyte death. As we have reported previously (Poon et al., 1999), a single emigrated neutrophil was able cause myocyte death.

Voltage clamp measurements were done to identify the ionic current changes responsible for these neutrophil-induced alterations in the resting potential and/or action potential waveform. All of these measurements were made at room temperature, to take advantage of

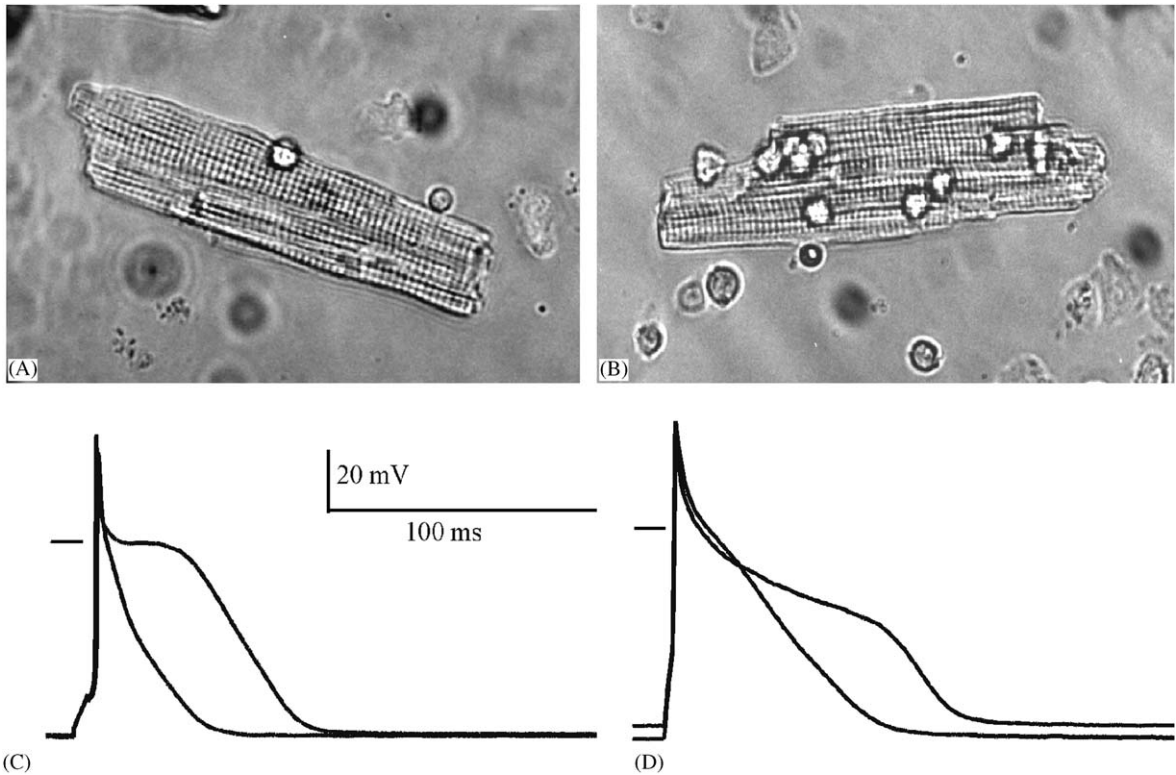


Fig. 1. Photomicrographs which illustrate circulating (A) and emigrated (B) neutrophils adhering to rat ventricular myocytes. Circulating neutrophils were exposed to 20 μ M fMLP prior to being added to the recording chamber (see Methods). Panels C and D show representative action potentials recorded following adhesion of emigrated neutrophils. The marked action potential prolongation (C) and a small depolarization of the resting potential (D) was also present in 60–70% of the myocytes studied.

Table 1
Myocyte longevity following adhesion of neutrophils

Group	Experimental manoeuvre	Time to death (s)	<i>n</i>
1	Action potential recordings—circulating neutrophils (22 °C)	1680 \pm 120	4
2	Action potential recordings—emigrated neutrophils (31 °C)	370 \pm 44	19
3	Action potential recordings—emigrated neutrophils (22 °C)	605 \pm 48*	4
4	Ramp protocol—without tetrodotoxin (22 °C)	792 \pm 101*	5
5	Ramp protocol—with tetrodotoxin (22 °C)	1240 \pm 86* ^{#,‡}	6
6	Hyperpolarizing pulses from a holding potential—80 mV (22 °C)	1533 \pm 103* [‡]	4

Cell death is defined as the time at which electrophysiological recordings became unstable and visible morphological changes (contracture) occurred in the ventricular myocyte. In experiments in which myocytes survived, the recordings were terminated at 1800 s.

*Indicates data significantly different ($P < 0.05$) from group 2.

[#]Indicates data significantly different ($P < 0.05$) from group 4.

[‡]Indicates data which is not significantly different ($P < 0.05$) from group 1.

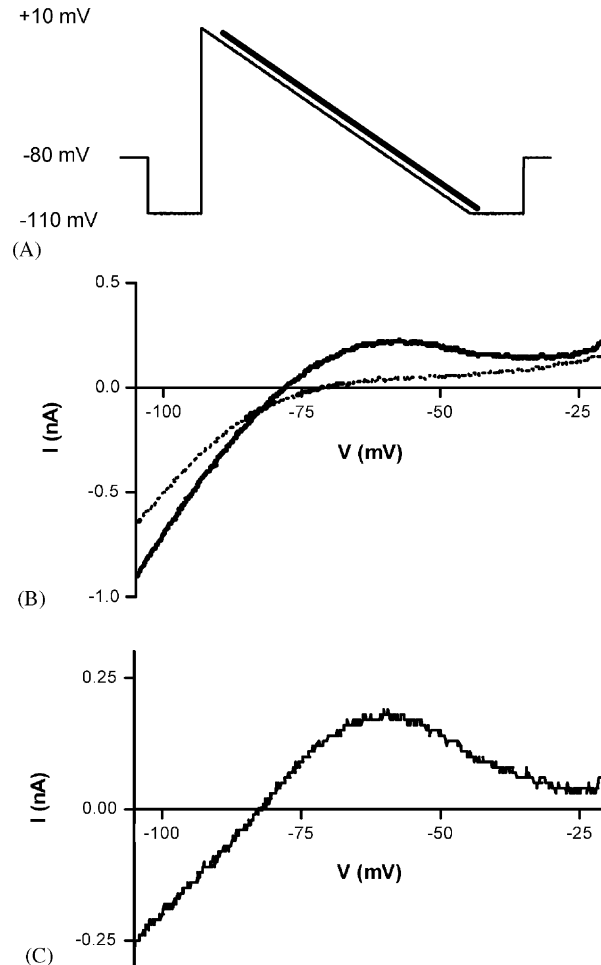


Fig. 2. Effects of emigrated neutrophils on whole-cell currents recorded from rat ventricular myocytes using the nystatin-perforated patch technique at room temperature (21–22 °C) in the presence of 20 μ M tetrodotoxin. The ramp voltage-clamp protocol is illustrated in Panel A. Panel B illustrates typical whole-cell currents obtained within the voltage range indicated by the heavy line in Panel A. These two sets of currents were recorded: (i) immediately following neutrophil adhesion and (ii) just prior to cell death. The difference current shown in Panel C illustrates the neutrophil-induced current change 15 min after neutrophil adhesion. These and all other voltage clamp records in this paper were obtained at room temperature (21–22 °C).

the increased window of time between onset of action potential shape changes and cell death. Membrane potential was controlled using the ramp voltage clamp waveform illustrated in Fig. 2A. In these experiments the interval between neutrophil adherence and myocyte death was 792 ± 101 s ($n = 5$; see Table 1). In the presence of strongly activated, adherent neutrophils, there was an increase in inward current at all membrane potentials negative to approximately -30 mV (data not shown). Thus, at -50 mV, where control cells exhibit a net outward K^+ current, adherence of neutrophils caused a marked decrease from 116 ± 30 to 28 ± 46 pA ($n = 5$). This current change was more pronounced negative to E_K at -100 mV where

currents of -676 ± 151 and -944 ± 115 pA ($n = 5$) were measured before and following neutrophil adhesion, respectively.

To determine whether a time- and voltage-dependent Na^+ influx may contribute to the neutrophil-induced inward current, identical protocols were carried out in the presence of $20 \mu\text{M}$ tetrodotoxin (TTX). Under these conditions, the pattern of neutrophil binding and adherence was unchanged. Fig. 2B, which consists of typical current–voltage relationships shows that in the presence of TTX the adhering of neutrophils caused a significantly different pattern of current changes. Under these conditions there was a decrease in the inward current negative to -85 mV. At -100 mV this current change was -637 ± 113 to -467 ± 91 pA ($n = 6$) when measured prior to, and following, neutrophil adhesion, respectively. This figure also shows that after addition of TTX a neutrophil-induced reduction in the outward current occurred between -70 and -30 mV (from 85 ± 38 to -27 ± 60 pA ($n = 6$)), together with a decrease of the negative slope of the current–voltage relationship.

Based on the observation that in most experiments a small depolarization of the resting potential occurred soon after neutrophil adhesion, a more detailed examination of the inward rectifier K^+ current (I_{K1}) was undertaken. In the presence of 10 – $30 \mu\text{M}$ TTX, whole-cell currents were elicited by 500 ms rectangular depolarizations to membrane potentials between -120 and -10 mV, from a holding potential of -80 mV. This protocol was repeated in the presence of $100 \mu\text{M}$ Ba^{2+} to determine the Ba^{2+} -sensitive K^+ current, I_{K1} . Following neutrophil adherence, a clamp pulse to -110 mV was applied repetitively to monitor changes in I_{K1} . After this current had decreased and stabilized, the Ba^{2+} -sensitive component was again assessed over a broad range of membrane potentials. Representative I – V relationships for the Ba^{2+} -sensitive currents determined prior to (Fig. 3A), and following (Fig. 3B) neutrophil adherence are shown. Note that neutrophil adherence caused significant decreases in both the inward (at -120 and -110 mV) and outward (-70 to -50 mV) components of this current, I_{K1} .

A second important observation made in these experiments in which TTX was included in the superfusate was that there was a significant increase in the time to myocyte death following neutrophil adherence; this parameter averaged 1240 ± 86 s ($n = 6$; see Table 1) in TTX, compared to 792 ± 101 s ($n = 5$; see Table 1) under control conditions. This finding suggested that the increase of inward Na^+ current observed in the absence of TTX is an essential factor for initiating the neutrophil-induced pathophysiological changes, which ultimately causes myocyte death. This working hypothesis was examined in experiments in which myocytes were voltage clamped at a holding potential of -80 mV, to prevent activation of I_{Na} . 500 ms rectangular hyperpolarizations to -110 mV were applied at a frequency of 0.1 Hz to record the background K^+ current, I_{K1} . As expected, following neutrophil adhesion, there was a progressive decrease in I_{K1} . Myocytes in this experimental group survived on average for 1533 ± 103 s ($n = 4$; see Table 1); very similar to the results obtained in the presence of TTX.

To gain further insight into the molecular mechanism(s) and the types of ion channels in the myocyte that can be altered by neutrophil adherence, the Ca^{2+} -independent transient outward K^+ current, I_{to} , which generates the initial rapid repolarization of the action potential was studied (cf. Clark et al., 1996; Campbell et al., 1995; Giles et al., 1996). I_{to} was activated by 500 ms depolarizing steps to 50 mV, from a holding potential of -80 mV. This was followed by a 500 ms hyperpolarizing step to -110 mV to monitor I_{K1} under these same conditions. TTX ($20 \mu\text{M}$) was present to block I_{Na} . These results (data not shown) revealed that even 15 min after neutrophil

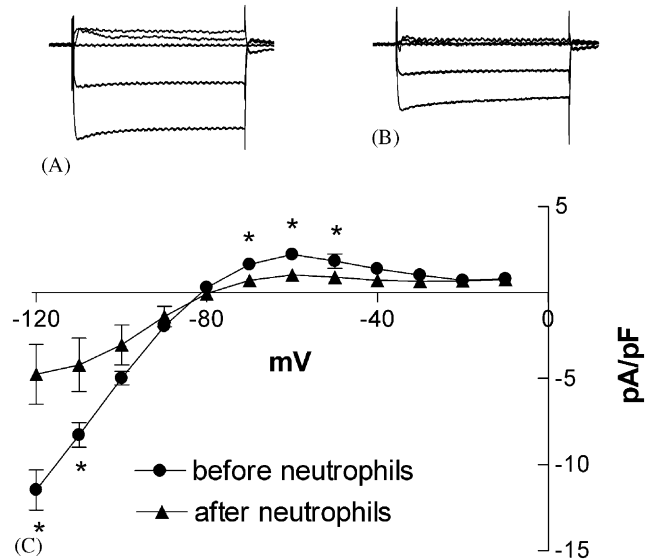


Fig. 3. Effects of emigrated neutrophils on the inward rectifying K^+ current, I_{K1} . Representative tracings of the Ba^{2+} -sensitive currents (at membrane potentials of -120 , -100 , -80 , -60 and -40 mV) recorded prior to (A) and following neutrophil adhesion (B) are illustrated. The current–voltage relationships for these Ba^{2+} -sensitive difference currents is shown in C. Values represent the mean \pm standard error of the mean for $n = 4$ myocytes. (*) indicates membrane potentials at which neutrophil-adherent data are significantly different ($p < 0.05$) from pre-adherent values.

adhesion, I_{to} remained unchanged, while I_{K1} decreased, demonstrating that neutrophil-induced effects had developed. Thus, the effects of neutrophils on ventricular myocyte ion channels appear to be selective for certain ion channel targets.

The next set of experiments addressed the mechanism(s) by which neutrophil adherence leads to alterations in Na^+ current in ventricular myocytes. The cell-attached patch technique was applied, so that single channel events could be recorded. Representative traces of I_{Na} before, and following, neutrophil adherence are illustrated in Figs. 4A and 4B, respectively. For comparison, ensemble records, obtained by summing at least 50 consecutive sweeps, were normalized to the maximum peak amplitude and then superimposed (Fig. 4C). Peak inward currents were not significantly different between the experimental groups with values of 25 ± 6 ($n = 6$) and 28 ± 13 pA ($n = 6$) for neutrophil-free and neutrophil-adherent myocytes, respectively. The I_{Na} current observed following neutrophil adhesion is consistent with a slowing of the rate of fast inactivation of the macroscopic current by the adhering neutrophils.

The remaining component of this study consisted of attempting to summarize and integrate these experimental findings using mathematical simulation(s). Our mathematical model of the electrophysiological properties of rat ventricular myocytes (Pandit et al., 2001) was used as a starting point. Our initial goal was to examine the extent to which changes in individual kinetic parameters or maximal conductances of selected ion currents underlying the action potential may contribute to the observed electrophysiological changes. The kinetic parameters which regulate I_{Na} were altered one at a time. The resulting action potential duration, $[Ca^{2+}]_i$ transients, and $[Na^+]_i$ levels are shown in Figs. 5–8.

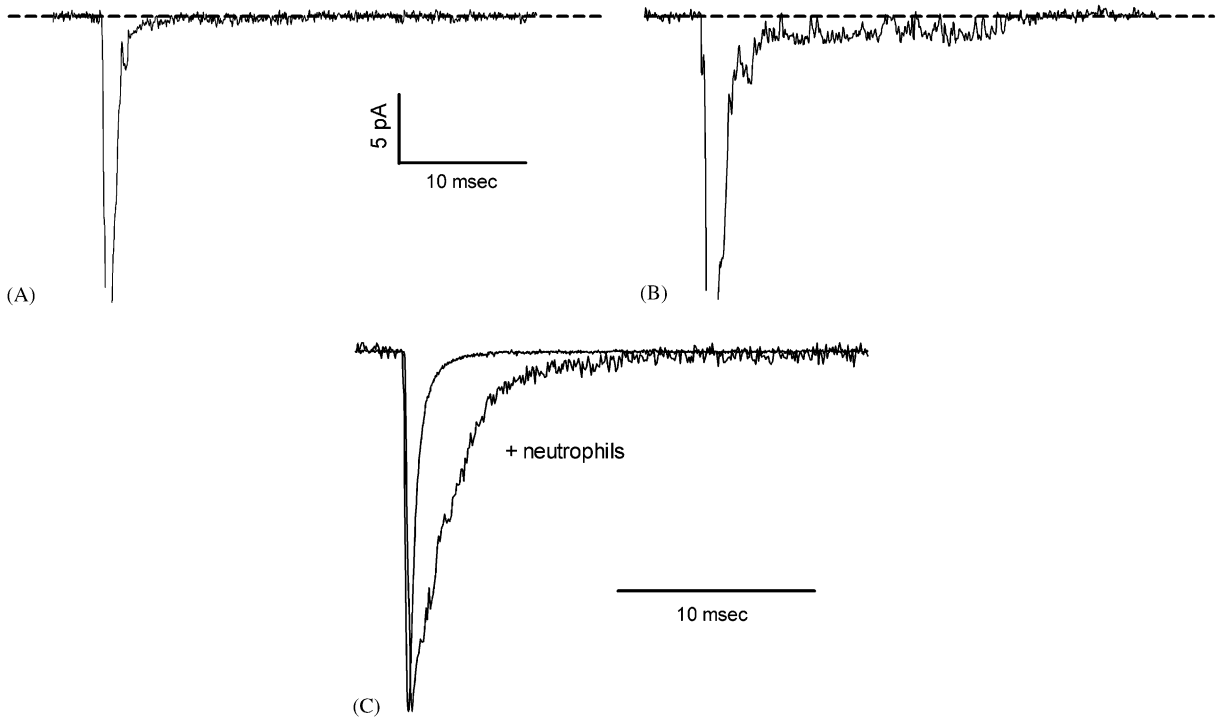


Fig. 4. Representative cell-attached recordings of I_{Na} in control conditions (A) and 15 min after (B) an emigrated neutrophil had adhered to the myocyte. Ensemble recordings, normalized to peak current amplitude, are illustrated in C. Each ensemble average was obtained from at least 50 sweeps. The two ensemble averaged records in Panel C have been normalized for the purpose of illustrating changes in the time-course of inactivation. Prior to the averaging procedure leak and capacitive currents were removed from each record by subtraction of data from a sweep in which no unitary current changes were detected.

Our working hypothesis was that when emigrated neutrophils release a number of reactive oxygen species, e.g. H_2O_2 , an important initial effect is a marked slowing of the inactivation of I_{Na} , and that the resulting small, but significant ‘late inward current’ can prolong the action potential duration (cf. Ward and Giles, 1997). These changes in the repolarization waveform can alter the time-course of the time- and voltage-dependent Ca^{2+} current and the extent and time-course of Ca^{2+} release from the sarcoplasmic reticulum (SR), thus altering Ca^{2+} homeostasis and myocyte contractions (cf. (Bouchard et al., 1993)). To address these possibilities, and in an attempt to relate our calculations to other well established approaches to this type of analysis, our model of the rat ventricular action potential (Pandit et al., 2001) was modified in two significant ways:

- (1) I_{Na} was reformulated in terms of a Markovian model of the single channel transitions governing this conductance. This formulation was taken from published work originally developed by the Winslow Group (Iyer et al., 2004) and by Clancy and Rudy (1999).
- (2) The mathematical expressions for the L-type Ca^{2+} current, and for Ca^{2+} -induced Ca^{2+} release from the SR were reformulated according to the mathematical formalisms in an important recent paper by Shiferaw et al. (2002).

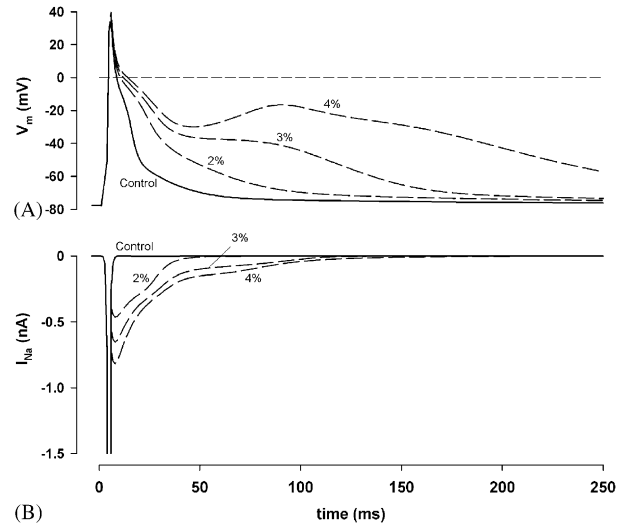


Fig. 5. (A) Mathematical simulation of rat ventricular action potentials (0.2 Hz) assuming a ‘control’ population of Na^+ channels (solid line), and then with Na^+ channel populations in which 2%, 3% and 4% of the channels had a ‘modified’ ‘O to IF’ transition rate (50-fold slower than control). (B) Na^+ currents generated during each of the action potentials which are shown in (A). Note that the peak amplitude of the large initial inward Na^+ current during the upstroke of the action potentials has been truncated to more clearly illustrate its inactivation phase.

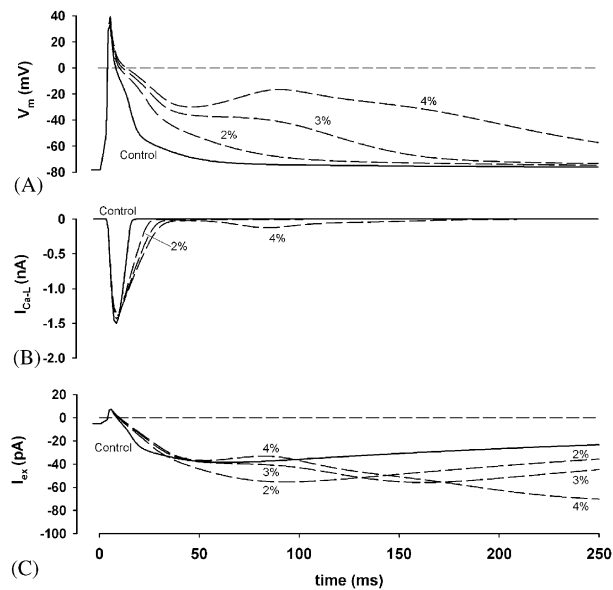


Fig. 6. Comparison of L-type Ca^{2+} current (I_{Ca-L}) and Na^+ - Ca^{2+} exchanger current (I_{ex}) during control action potentials and action potentials with ‘modified’ Na^+ channels. (A) Control action potentials (solid line) and action potentials generated after 2%, 3% and 4% of the Na^+ channel population has been changed i.e., ‘modified’ ‘O to IF’ transition rate (dashed lines) as in A. (B) I_{Ca-L} corresponding to the action potentials shown in (A). (C) I_{ex} corresponding to the action potentials shown in (A).

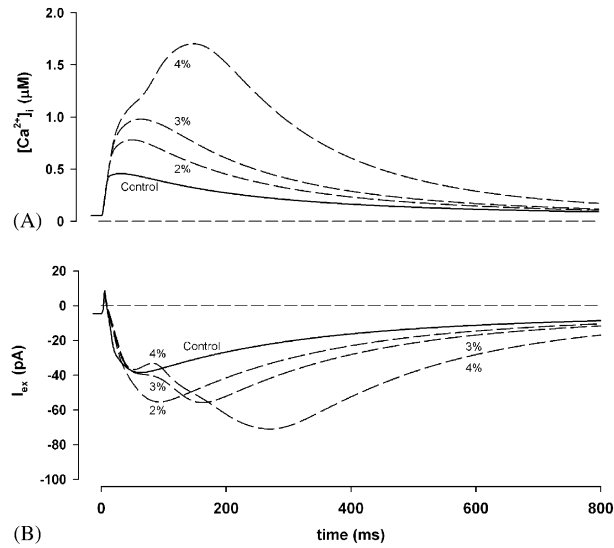


Fig. 7. (A) Simulation of intracellular $[Ca^{2+}]_i$ during action potentials with unmodified Na^+ channels (control; solid line), and during action potentials with 2%, 3% and 4% of the population of Na^+ channels with a ‘modified’ ‘O to IF’ transition rate (compare with Fig. 2). (B) Na^+ – Ca^{2+} exchanger current (I_{ex}) generated during control action potentials, and action potentials with 2%, 3% and 4% ‘modified’ Na^+ channels.

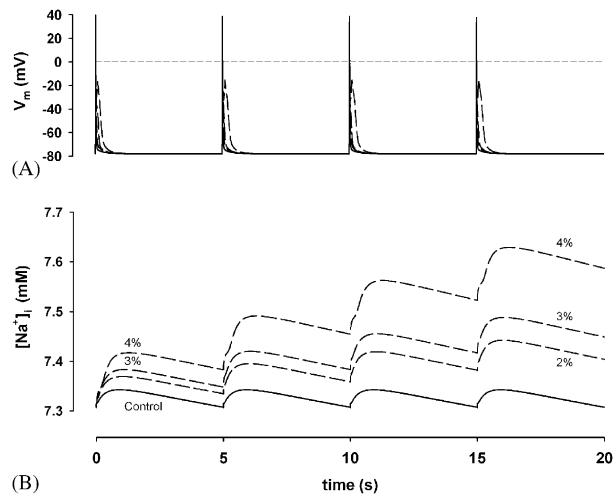


Fig. 8. Comparison of (A) action potentials and (B) intracellular $[Na^+]_i$ under control Na^+ channels (solid line) conditions, and Na^+ channel populations in which 2%, 3% and 4% of the channels had a ‘modified’ ‘O to IF’ transition rate (see Fig. 5). This simulation shows the first four action potentials (0.2 Hz), starting from a ‘resting’ condition.

The simulations in Figs. 5–8 illustrate the effects of slowing the ‘O to IF’ transition rate of a small fraction (1–5%) of the Na^+ channels in this model of the rat ventricular myocyte (see Section 2 for details). Effects on action potential waveform and Na^+ currents are illustrated in

Fig. 5 which compares the action potential waveforms (Fig. 5A) and Na^+ currents (Fig. 5B) in the simulated conditions where 2%, 3%, or 4% of the Na^+ channels were ‘modified’. Specifically, the ‘O to IF’ transition rate was slowed by a factor of 50. This single change produced greatly prolonged action potentials having distinct plateau regions (Fig. 5A). The duration of the plateau increased, and the plateau became more depolarized with a progressively larger fraction of modified Na^+ channels. In this simulation, modification of 4% of the Na^+ channels produced an action potential with an ‘EAD’. When 5% of the Na^+ channels were assumed to have this type of modified inactivation, the simulated action potentials failed to repolarize (not shown). In addition, if 100% of the Na^+ channels were modified with a 10-fold reduction in the O to IF transition rate, (which produced a Na^+ current similar in time course to the neutrophil-treated ensemble-averaged current shown in Fig. 4B), no significant prolongation of the simulated action potential occurred. From these computations, we have concluded that it is essential to include a small, but very slowly inactivating component of Na^+ current in the model, to reproduce action potential prolongation similar to that of the recorded action potentials shown in Fig. 1. Although such a current component is not apparent in the ensemble-averaged current shown in Fig. 4B, its absence can be accounted for as follows. If modification of inactivation of as few as 2–4% of the channels is adequate to produce the large action potential broadening shown in Fig. 5, a slow current component would likely be too small to detect or record reliably in ensemble-averaged currents obtained from relatively small ensemble averages, approx ~ 50 records as was the case in this experiment.

Fig. 6 compares these same action potential waveforms with those of the L-type Ca^{2+} current, $I_{\text{Ca-L}}$, and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger current, I_{ex} , during the action potentials. The time course of both $I_{\text{Ca-L}}$ and I_{ex} were prolonged during action potentials in which a very small percentage of the Na^+ channels were assumed to exhibit this type of modified inactivation. This prolongation was progressively greater as the percentage of modified channels increased. Note that the 4% case, $I_{\text{Ca-L}}$ had a small, secondary inward current component corresponding to the time course of the EAD.

Fig. 7 compares $[\text{Ca}^{2+}]_i$ transients (Fig. 7A) and I_{ex} (Fig. 7B) during action potentials computed assuming either control conditions or the same modified Na^+ channel conditions described in Figs. 5 and 6. Note that slowing of the inactivation of a small fraction of the Na^+ current produced very large increases in the magnitude and the duration of the intracellular Ca^{2+} transients. The time course of I_{ex} approximately paralleled that of the $[\text{Ca}^{2+}]_i$ transients. However, the combination of $[\text{Ca}^{2+}]_i$ and membrane potential during the action potentials determined the precise time course of I_{ex} . That is, although the waveform of the action potential produced by ‘modified’ Na^+ channels sets the voltage trajectory for the plateau and repolarization, the intrinsic voltage dependence of the I_{ex} can result in marked changes in electrogenic ion transporter.

Fig. 8 illustrates the simulated changes in $[\text{Na}^+]_i$ during control conditions and after the action potential waveforms have been prolonged by slowing inactivation of I_{Na} . This model output shows the first two action potentials following a prolonged ‘rest’ period. Note that $[\text{Na}^+]_i$ rose slightly during the first control action potential, but then declined to the rest level prior to the start of the next action potential (5 s) later. In contrast, under conditions where the action potentials had been prolonged due to modified inactivation of 2%, 3% or 4% of the Na^+ channels, this increase in $[\text{Na}^+]_i$ during the first action potential was significantly larger; and the $[\text{Na}^+]_i$ level did

not return to its rest level before the next action potential was initiated. Hence $[\text{Na}^+]_i$ showed progressive and increased ‘accumulation’ with successive action potentials. Extending this simulation to explore the effects of applying a longer train of action potentials on $[\text{Na}^+]_i$ would provide information on the steady-state increase in $[\text{Na}^+]_i$ that this marked action potential prolongation can cause. This would be one way of providing additional insights into the electrophysiological and electrochemical mechanisms for neutrophil-induced electrical and contractile activity. The effects of the initial, small neutrophil-induced depolarization of the myocyte on $[\text{Na}^+]_i$ was very small (not shown).

4. Discussion

Recent studies have demonstrated the presence of neutrophils in inflamed myocardium, either as a consequence of primary inflammation or following reperfusion of previously ischaemic myocardium (Lefer et al., 1993; Wilson et al., 1993; Sato et al., 1997; Mehta and Li, 1999). Neutrophil-mediated events can give rise to tissue injury and may contribute to the generation of life threatening arrhythmias (Dhein et al., 1995). Previously, we have reported that neutrophils could adhere in a selective, robust fashion to rat ventricular myocytes; and have also shown that following their transmigration, a novel α integrin-dependent process is activated (Reinhardt et al., 1997). Shortly after even a single neutrophil adheres the targeted myocyte loses its structural integrity (round up) and generates abnormal spontaneous contractions (Poon et al., 1999). These and other findings provided indirect evidence that activated neutrophils exert significant deleterious effects on cardiac myocytes.

The present study was done to examine this sequence of events, and to reveal underlying mechanisms by identifying the electrophysiological consequences of activated neutrophil adhesion to rat ventricular myocytes. Intracellular recordings from isolated myocytes demonstrated that following adhesion of emigrated neutrophils, the resting membrane potential depolarized, the action potential waveform lengthened, and myocyte death quickly followed. These changes in action potential waveform are due to (i) a change in time-course of inactivation of TTX-sensitive Na^+ current, and (ii) a neutrophil-dependent decrease in the inward rectifier K^+ current. Voltage clamp experiments show that a very important initial event is the neutrophil-mediated increase in Na^+ influx as a result of slowing of the time-course of inactivation of I_{Na} . The decrease in I_{K1} is usually, but not always observed. This response may be a consequence of myoplasmic acidification (see below).

Hoffman et al. (1997) have reported previously that neutrophils isolated from the circulation and activated with zymosan-containing serum adhered to canine myocytes. This resulted in a dramatic prolongation of action potential duration. In contrast, under our experimental conditions using rat ventricular myocytes, circulating neutrophils had no consistent effect on action potential waveforms (data not shown), even though they did adhere to the myocytes. It is quite likely that the chemical agent used by Hoffman et al (1997) to activate neutrophils was more potent than the one we chose for our experiments. Thus the neutrophils in their study may have been more noxious. This variability is one reason that we have used emigrated neutrophils which have previously been reported to respond to a stimulus with much more robust proteolytic

activity. Under our conditions emigrated, but not circulating, neutrophils consistently resulted in rapid myocyte damage/death of myocytes from rat ventricles.

Definitive identification of the cellular mechanism(s) by which neutrophils alter myocyte electrophysiology is challenging due to the complexity of neutrophil pathophysiology and signalling. Neutrophils are known to release a large number of potentially harmful substances following their adhesion to a target cell (Engler, 1989; Lucchesi et al., 1989; Jordan et al., 1999). This release is directed into a restricted extracellular space and then into the myoplasm of the host cell. The release of compounds (such as oxidants and proteases) can lead to damage of ventricular myocytes. Much of the research on these types of neutrophil–myocyte interactions has focused on the role of reactive oxygen species. Entman et al (1992) reported that following adhesion; neutrophil undergo an intracellular oxidative burst, and this is followed quickly by myocyte contracture and death. Since extracellular free radical scavengers were ineffective in preventing these neutrophil effects, it was suggested that these oxidants were secreted directly into the myocyte. A later study by King et al (1997) demonstrated that myeloperoxidase-derived oxidants were also formed when circulating neutrophils were utilized. Chemically stimulated, circulating neutrophils produce oxidants but are unlikely to degranulate and release proteases. In contrast, emigrated neutrophils have a greater tendency to degranulate and therefore a proteolytic reaction may also contribute to the myocyte dysfunction.

Our results suggest that the cytotoxic effect of neutrophils on myocytes is initiated by an increase of Na^+ influx through TTX sensitive Na^+ channels. In a previous study (Ward and Giles, 1997), we examined the electrophysiological effects of the reactive oxygen species, hydrogen peroxide (H_2O_2) on rat ventricular myocytes. These results showed that H_2O_2 caused a pronounced TTX-sensitive increase in action potential duration. Corresponding voltage clamp experiments demonstrated that H_2O_2 can prolong action potential duration by slowing the rate of Na^+ channel inactivation. This Na^+ -channel mediated effect appears to be mediated by a protein kinase C-dependent mechanism. H_2O_2 is known to be produced by activated neutrophils (Jordan et al., 1999); hence this mediator was a major focus of our working hypothesis for the ionic mechanism which causes the prolonged action potential durations observed in this study.

The neutrophil-induced reduction in the inwardly rectifying background K^+ current is expected to have both direct and indirect electrophysiological effects. Inhibition of I_{K1} may result in a depolarization of resting membrane potential and can prolong action potential duration (Shimoni et al., 1992; Nichols and Lopatin, 1997). Both of these electrophysiological events can contribute to rhythm disturbances (Orchard and Cingolani, 1994). It is unlikely that the effect of neutrophils on the inwardly rectifying K^+ current is caused by oxidants, as I_{K1} has previously been shown to be relatively insensitive to oxidation (Cerbai et al., 1991). However, it is well known that I_{K1} is sensitive to block by intracellular protons (Ito et al., 1992; Orchard and Cingolani, 1994;), i.e. acidification of the myoplasm. Hackam et al (1996) demonstrated that following emigration, neutrophils exhibit altered pH_i regulation. After being stimulated with fMLP, the cytosol of the neutrophil becomes strongly acidic as a result of increased metabolic activity (Demaurex et al., 1996). Following adherence to a ventricular myocyte this ‘proton load’ may be introduced into the myocyte myoplasm, thereby causing a reduction of I_{K1} .

Following neutrophil attack additional acidification of the myocyte may also be caused by the augmented Na^+ influx due to neutrophil-induced slowing of inactivation of Na^+ channels. An increase of intracellular Na^+ would inhibit the activity of the Na^+/H^+ exchanger, resulting in

augmented intracellular H^+ (Fliegel and Wang, 1997; Karmazyn et al., 1999). This could occur (at a reduced rate) even in the presence of TTX; since in our experiments the concentrations of TTX used do not fully block I_{Na} (Satin et al., 1992). Sufficient Na^+ may have entered the myocyte to cause an intracellular acidification, but not cell death. Another plausible alternative is that the reduced electrochemical gradient for Na^+ would result in diminished Na^+/Ca^{2+} exchange activity (Bouchard et al., 1993, 1995, 2004; Blaustein and Lederer, 1999). The increase in intracellular Ca^{2+} levels could activate, modulate or enhance a number of biochemical reactions, alter excitation–contraction coupling, and ultimately result in cell contracture and death. Additional work, in which spectrophotometric measurements of pH_i , $[Na^+]_i$ and $[Ca^{2+}]_i$ are made in conjunction with cell shortening or electrophysiological parameters are needed to further address these complex and interdependent phenomena.

To attempt to integrate our experimental findings, and to begin to relate some of them to previous papers on excitation–contraction coupling in rat ventricular myocytes, a modified mathematical model of some of the important electrophysiological and electrochemical phenomena was developed. These computations focussed on simulating the effects of the small irreversible changes in the rate of inactivation of I_{Na} caused by emigrated neutrophils. I_{Na} appears to be one important target following neutrophil attack (Fig. 4). As indicated, based on an extensive literature and our previous work, we have assumed that the electrophysiological changes are a consequence of free radical production by the neutrophil with the most likely species being hydrogen peroxide (Ward and Giles, 1997).

It is apparent from Figs. 5 and 6 that even a relatively small number of Na^+ channels which inactivate slowly, or fail to inactivate can result in a very substantial prolongation of the action potential duration (Fig. 5). This is a consequence of the fact that the plateau region of the cardiac action potential has a relatively high resistance. Our computations suggest that this I_{Na} -induced prolongation of APD can secondarily, but significantly, alter the waveform of the L-type Ca^{2+} current. The prolonged action potential and augmented Ca^{2+} influx can result in corresponding changes in the activity of the Na^+/Ca^{2+} exchanger. As shown in Table 1, myocyte death occurred approximately 3–5 min after neutrophil attack (in experiments done at room temperature). Additional experimental and computational work is needed to more fully assess the electrochemical changes in the myocyte which are responsible for this. However, myocyte damage does not appear to be due to acidification of the myoplasm following attachment of emigrated neutrophils. Thus, in our model when an *in silico* structure having a volume equivalent to a neutrophil and an intracellular pH of 4.0 was assumed to ‘inject’ this hydrogen ion load into the myocyte, no significant change in intracellular pH of the ventricular cell was predicted. This was also the case when the number of neutrophils was increased from 1 to 6; or when the adherent neutrophils were assumed to ‘burst’ and hence inject a series of some 6–8 acidic challenges into the subjacent myocyte (results not shown).

The contracture of the myocyte immediately following neutrophil attack could be caused by the prolongation of the action potential per se. This results in an increased Ca^{2+} load in the SR for two reasons: (i) the L-type Ca^{2+} current is prolonged, and (ii) the duration of the diastolic interval is significantly decreased, thus resulting in reduced effectiveness of the Na^+/Ca^{2+} exchanger to extrude Ca^{2+} from the cell. However, as shown in Fig. 8, at relatively long times after neutrophil attack or under circumstances when the action potential firing rate of the myocyte is substantial, an activity-induced increase in intracellular Na^+ may also develop. This increase in $[Na^+]_i$ would

be expected to reduce the ability of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to extrude Ca^{2+} . In the extreme case, it may result in the $\text{Na}^+/\text{Ca}^{2+}$ exchanger running backwards thus providing a significant additional source of extra Ca^{2+} influx. Under this circumstance, it is well-known that isolated myocytes from mammalian ventricle quickly develop irreversible contractures.

The computations shown in Fig. 8 are instructive. However, it will be important to extend them so that the experimental conditions on which Table 1 is based are modelled in a more comprehensive fashion. In this way the maximal change in $[\text{Na}^+]_i$ can be estimated; and the possibility that myocyte injury results in part from Ca^{2+} influx due to the $\text{Na}^+/\text{Ca}^{2+}$ exchanger operating in ‘reverse mode’ (Baczko et al., 2003, 2004) can be addressed.

Additional experimental and computational work is ongoing. It focuses on more detailed assessment of this sequence of events by carrying out computations in which the neutrophil-induced Na^+ influx enters only a relatively small fraction of the total intracellular volume within the myocyte. A second condition which needs to be studied in detail is inhibition of glycolytic metabolism and hence, reduced efficacy of the Na^+/K^+ pump. These and other plausible working hypothesis for explaining the complex pathophysiological processes involved in neutrophil–myocyte interactions in the setting of acute inflammation should be further explored using genetically modified mouse experimental models. We and others have shown that the general experimental approaches outlined in this study can be applied to adult mice. Thus, they can be used in conjunction with tissue-specific modification of the relevant receptors, and enzyme subtypes in these two cells. A comprehensive mathematical model of the action potential for the mouse ventricular myocyte has been published recently (Bondarenko et al. 2004).

Acknowledgments

The study was supported by the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Canada. WRG held an Alberta Heritage Foundation for Medical Research (AHFMR) Medical Scientist Award and was also supported (2004–2005) by the Departments of Bioengineering and Medicine at the University of California San Diego. CAW was supported by Heart and Stroke Foundation of Canada and AHFMR Postdoctoral Fellowships. The financial assistance of the Research Chair of the Heart and Stroke Foundation of Alberta and the N.W.T. to W.R.G. is also gratefully acknowledged.

Editor’s note

Please see also related communications in this volume by Ten Tusscher et al. (2005) and Sarai et al. (2005). For further information and downloadable content please see <http://www.physio.me.org.nz/publications/PBMB-2005-89/Ward/>

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