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Sustained Depolarization and ADP-Ribose Activate a Common Ionic Current in Rat Peritoneal Macrophages

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Phagocytosis is associated with large changes in the membrane potential of macrophages, but the functional significance of this is unknown. Whole cell recordings were made from rat peritoneal macrophages. Sustained (>30 s) depolarization of the cells progressively activated a conductance that remained high (several nanoSeimens) for several tens of seconds. This current: 1) was linearly dependent on potential between −100 and +50 mV; 2) reversed close to 0 mV in a physiological external solution; 3) could also be carried in part by N-methyl-D-glucamine (P_{NMDG}/P_{Na} 0.7), chloride (P_{Cl}/P_{Na} 0.5), or calcium (P_{Ca}/P_{Na} 1.3); and 4) was blocked by intracellular ATP (5 mM) or ADP (10 mM) and by extracellular lanthanum (half-maximal concentration 1 mM). A current with all the same properties was recorded in cells when the intracellular solution contained ADP-ribose (10–300 μM) or β-NAD (1 mM) (but not any other nucleotide analogs tested). The results suggest that prolonged depolarization leads to an increased intracellular level of ADP-ribose, which in turn activates this nonselective conductance(s).


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3 Abbreviations used in this paper: βNAD, β-NAD free acid; βNADH, β-NAD reduced salt; βNADP, β-NADP reduced salt; βNADPH, β-NADPH reduced salt; NMDG, (N-methyl-D-glucamine); SBFI, 1,3-benzenedicarboxylic acid, 4,4'-[[1,4,10-trioxa-7,13-diazacyclopentadecane-7,13-diylbis(5-methoxy-6,2-benzofuranylidyl)]bis-, tetra-ammonium salt; SKF96365, (1-b-[3-(4-methoxyphenylpropoxy)-4-methoxophenethyl]I4-umidazole hydrochloride.

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growth medium (Dulbecco’s) supplemented with 10% heat-inactivated FCS and 2 mM d-glutamine. Cells were grown to confluence and plated on coverslips, and recordings were made 2–48 h later.

Whole cell recording

Ion currents were studied using the whole cell configuration of the patch-clamp technique, as described by Hamill et al. (17). Glass electrodes (Harvard Apparatus, Edenbridge, U.K.) ranged from 6 to 8 MΩ in resistance and were fire polished. The indifferent electrode was an Ag-AgCl electrode connected to the bath through a short 3 M KCl agar bridge. All the experiments were performed at room temperature using an EPC9 patch-clamp system (HEKA, Lambrecht, Germany). The cells were kept in an extracellular solution containing (mM): NaCl, 147; KCl, 2; MgCl2, 1; CaCl2, 2; HEPES, 10; and glucose, 23 (pH brought to 7.3 with HCl; NaOH). The NMDG solution contained (mM): NMDG, base 154; HEPES, 10; ADP, ADP-glucose, ADP-ribose, cyclic ADP-ribose, AMP, ATP, EGTA, lanthanum chloride, t-rutinoside, t-riboside 5-phosphate, NMDG, xanthine, and xanthine oxidase were purchased from Sigma-Aldrich. EC50 is the ADP-ribose concentration evoking half of the maximal current, IC50 is the ADP-ribose concentration evoking half of the maximal current, and n is the Hill coefficient.

Chemicals

ADP, ADP-glucose, ADP-ribose, cyclic ADP-ribose, AMP, ATP, EGTA, lanthanum chloride, t-rutinoside, t-riboside 5-phosphate, NMDG, xanthine, and xanthine oxidase were purchased from Sigma-Aldrich. EC50 is the ADP-ribose concentration evoking half of the maximal current, IC50 is the ADP-ribose concentration evoking half of the maximal current, and n is the Hill coefficient.

Results

Macrophages had a capacitance of 11.4 ± 0.4 pF (n = 110). The cell diameter was 9.1 ± 0.2 μm (n = 30). Step depolarizations from a holding potential of −80 mV (10 mV increments to +40 mV, 500 ms duration) revealed no time-dependent currents when a sodium chloride-based internal solution was used. When a potassium chloride-based internal solution was used (150 mM KCl, 10 mM HEPES, 10 mM EGTA), the resting potential of the cells was −30.9 ± 0.7 mV (n = 11).

Current activated by depolarization

Cells held at −80 mV showed no change in holding current or conductance during 5–10 min of whole cell recording. However, if the cell was held at +10 mV for several tens of seconds and then returned to −80 mV, a large inward current was observed (Fig. 1A). This current quickly ( < 5 s) reached its peak amplitude (several hundred pA) and then declined during the next few minutes (Fig. 1A; in 10 cells the amplitude after 2 min was 19 ± 4% of the peak amplitude). The duration of the depolarization required to evoke the subsequent inward current was not studied in detail; however, a depolarization to +10 mV for only 10 s was not effective (n = 5). After an interval of several minutes, a second depolarization again elicited a large inward current with similar properties (n = 10).

The inward current was associated with a large increase in cell capacitance. The current-voltage relation of the cell remained essentially linear, and reversed close to 0 mV (−3.1 ± 0.24 mV, n = 18) in normal external solution (Fig. 1, B and C) (Table I). The time course of the conductance increase was followed by holding the cell at +10 mV, but repeatedly applying brief ramp voltage commands (−130 to +50 mV in 500 ms; 5 s intervals). Fig. 1C shows the time course of the resulting conductance increase. The current (at −80 mV) grew more slowly and was much smaller with a sustained holding potential of −40 mV (Fig. 1D), and it was larger with depolarization to +40 mV.

In low chloride extracellular solution (40 mM NaCl), the current at −80 mV was reduced to 51 ± 16% (n = 3) of its control value; the residual current reversed polarity at −13.1 ± 1.6 mV (n = 4) (Fig. 2A); from equation 1 this corresponds to PCr/PNa of 0.5 (Table I). When extracellular sodium was substituted by NMDG, the current evoked by depolarization was reduced to 79 ± 2% (n = 6) of its control value (measured at −80 mV). The residual current reversed polarity at −5.4 ± 0.45 mV (n = 7) (Table I; Fig. 2A), which indicates that NMDG is significantly permeable (PNa/NMDG = 0.74; from equation 2). When extracellular glutamate replaced chloride, the current at −80 mV was reduced to 91 ± 4% (n = 4), and when extracellular calcium replaced sodium, the inhibition was to 48 ± 6% (n = 6) of control (Fig. 2B). By measuring the reversal potential of the residual current (Table I), it was evident

Measurements of permeability

For the sodium concentration at which I = 0.5 ± I max, and n is the Hill coefficient. Concentration-current curves were fitted with I max = PCr-PNa × [(ICr × IEc)/ (ICr + IEc)], where I is the current at a given lanthanum concentration expressed as percentage of the current in the absence of lanthanum (I max). ICr is the lanthanum concentration at which I = 0.5 × I max, and n is the Hill coefficient. Concentration-current curves were fitted with I max = 100 [(ADP-ribose) [I/(ADP-ribose)] + EC50]), where I is the current activated by a given concentration of ADP-ribose and expressed as percentage of the maximal currents (I max). IC50 is the ADP-ribose concentration evoking half of the maximal current, and n is the Hill coefficient.

Data analysis

The results shown in the figures are means ± SEM. Curve fits of the pooled data were performed using Kaleidagraph (Synergy Software, Reading, PA) and Excel (Microsoft, Redmond, WA) software. The lanthanum inhibition curve was fitted with I max = 100 [(ICr × IEc)/ (ICr + IEc)], where I is the current at a given lanthanum concentration expressed as percentage of the current in the absence of lanthanum (I max). ICr is the lanthanum concentration at which I = 0.5 × I max, and n is the Hill coefficient. Concentration-current curves were fitted with I max = 100 [(ADP-ribose) [I/(ADP-ribose)] + EC50]), where I is the current activated by a given concentration of ADP-ribose and expressed as percentage of the maximal currents (I max). IC50 is the ADP-ribose concentration evoking half of the maximal current, and n is the Hill coefficient.
that the conductance activated by prolonged depolarization is significantly permeable to chloride and gluconate, NMDG, and calcium ions. In each of these ion substitutions, the current-voltage relation remained essentially linear (Fig. 2).

We conducted control measurements of the permeability of P2X receptors expressed in HEK293 cells, using the same intracellular and extracellular solutions. We used the agonist ATP (30 μM) to activate the inward current in cells expressing P2X 2/3 subunits, which was typically ~1 nA in amplitude (20). We observed that the agonist-induced current reversed polarity at 0.8 ± 0.5 mV (n = 23) in control extracellular solution, −33 ± 1.4 mV (n = 4) in 40 mM NaCl, and −86.5 ± 0.4 mV (n = 4) in NMDG solution. These reversal potentials correspond (from equations 1 and 2) to relative permeabilities of PCL/PNa = 0.02 and PNMDG/PNa = 0.03 (see Ref. 21).

The current induced by depolarization was substantially blocked by lanthanum (10 μM) (Fig. 1); the IC50 for this action was ~1 μM (Fig. 3A). The current was also significantly inhibited (76 ± 10%, n = 4) by SKF96365 (100 μM); at 10 μM SKF96365 inhibited the current in some (75 ± 8%, n = 3) but not other (n = 4) cells. SKF96365 has been widely used to block nonselective cation channels and store-operated calcium entry in nonexcitable cells since its introduction by Merritt et al. (22). The current evoked by depolarization was also greatly reduced by including in the pipette either ATP (5 mM, inhibited by 89 ± 3% (n = 4) or ADP (10 mM, inhibited by 97 ± 4%, n = 5) (Fig. 3B).

**Current activated by ADP-ribose**
An inward current progressively developed over tens of seconds in cells held at −80 mV, when the recording electrode contained

<table>
<thead>
<tr>
<th>Depolarization</th>
<th>Remaining Current (%)</th>
<th>Erev (mV)</th>
<th>PCL/PNa</th>
<th>Depolarization</th>
<th>Remaining Current (%)</th>
<th>Erev (mV)</th>
<th>PCL/PNa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51 ± 16 (3)</td>
<td>−3.1 ± 0.24 (18)</td>
<td>0.47</td>
<td>Control</td>
<td>69 ± 2 (4)</td>
<td>−2.7 ± 0.65 (11)</td>
<td>0.45</td>
</tr>
<tr>
<td>Low chloride</td>
<td>79 ± 2 (6)</td>
<td>−13.1 ± 1.6 (4)</td>
<td>0.74</td>
<td>Low chloride</td>
<td>75 ± 2 (4)</td>
<td>−13.5 ± 1.2 (4)</td>
<td>0.45</td>
</tr>
<tr>
<td>NMDG</td>
<td>91 ± 4 (4)</td>
<td>−5.4 ± 0.45 (7)</td>
<td>0.74</td>
<td>NMDG</td>
<td>84 ± 4 (4)</td>
<td>−4.8 ± 0.35 (4)</td>
<td>0.84</td>
</tr>
<tr>
<td>Gluconate</td>
<td>48 ± 6 (6)</td>
<td>−8.8 ± 0.6 (6)</td>
<td>0.96</td>
<td>Gluconate</td>
<td>42 ± 6 (3)</td>
<td>−8.3 ± 0.34 (4)</td>
<td>0.90</td>
</tr>
</tbody>
</table>

* Mean ± SEM for number of cells in parentheses.
ADP-ribose (Fig. 4A). The current-voltage relation remained linear throughout this period, and reversed close to 0 mV (−2.7 ± 0.6 mV, n = 11) (Fig. 4B). With 1 μM ADP-ribose, the development of the current was fitted by an exponential with a time constant of 1 min (Fig. 4C). The lowest concentration of ADP-ribose that was effective was 100 nM, and maximum value of the current was reached with 100 μM; the EC50 was 10 μM (Fig. 4D). Changing the level of intracellular calcium buffering did not obviously alter the effectiveness of ADP-ribose to activate this current. In our control, strongly buffered solution (10 mM EGTA, 0 mM calcium, 1 mM EGTA, 2 mM calcium, −100 nM [Ca2+]i), ADP-ribose (300 μM) elicited a current of 703 ± 119 pA (n = 3); with a higher level of intracellular calcium (11 mM EGTA, 2 mM calcium, −100 nM [Ca2+]i), this was 811 ± 149 pA (n = 5). No similar current developed when the recording electrode contained ADP (100 μM), ADP-glucose (100 μM), cyclic ADP-ribose (100 μM), AMP (100 μM), ATP magnesium salt (100 μM), d-ribose (100 μM), d-ribose 5-phosphate (100 μM), βNADH (300 μM or 1 mM), βNADP+ (300 μM), or βNADPH (300 μM) (n = 3 cells in all cases). However, βNAD+ at a concentration of 1 mM (n = 4) evoked a current similar to that observed with ADP-ribose, although 100 and 300 μM were ineffective. The peak current elicited by βNAD+ (1 mM) was 758 ± 428 pA (n = 4), which is not different from that seen with ADP-ribose (Fig. 4).

The relative permeability of the current induced by ADP-ribose (300 μM) was examined by measuring the reversal potential in different extracellular solutions. From the values given in Table I, it can be seen that the relative permeability to chloride, NMDG, gluconate, and calcium ions did not differ markedly from those determined for the currents elicited by depolarization.

The current evoked by ADP-ribose was also very sensitive to block by extracellular lanthanum (Fig. 3A), with an IC50 of 10 μM. It was also blocked by SKF96365 (100 μM, 77 ± 4%, 5 of 5 cells tested; 10 μM, 70 ± 7%, 7 of 12 cells tested) and by intracellular either ATP (5 mM, inhibited by 66 ± 11%, n = 5) or ADP (10 mM, inhibited by 76 ± 3%, n = 4) (Fig. 3B). These relative inhibitions were not different from those observed for the current activated by depolarization (ANOVA, p > 0.05).

A membrane current with similar properties was also elicited by applying xanthine (0.1 mM) and xanthine oxidase (0.05 U/ml), to generate superoxide anions; this current was completely blocked by 100 μM lanthanum (Fig. 5A). Application of xanthine alone had no effect. In parallel experiments, we used intracellular sodium imaging as an index of depolarization of undisturbed macrophages. In these cells, H2O2 (10 mM) evoked a progressive increase in intracellular sodium concentration ([Na+]i) (Fig. 5B). The time course of the [Na+]i increase was similar to that of the inward current observed with xanthine/xanthine oxidase, or depolarization, and the increase in [Na+]i was also strongly reduced by lanthanum (1 mM) (Fig. 5B).

**Occlusion of the two currents**

The similar properties of the two currents led us to hypothesize that sustained depolarization and intracellular ADP-ribose were
activating the same conductance. We recorded from 10 cells with electrodes containing ADP-ribose (300 μM); after 4 min, when the inward current reached its steady value, we applied a depolarization to +10 mV for an additional 4 min. This depolarization evoked no additional current. In time-matched controls, the depolarization elicited large inward currents recorded at −80 mV (Fig. 6).

Discussion
We found that sustained depolarization of rat peritoneal macrophages activates a large inward current. The current is carried by a conductance that is nonselective for cations, and that also has quite significant permeability to chloride and gluconate ions. The permeability to chloride ions was surprising, and for this reason we compared the results directly with those for the permeability of another cation-selective membrane channel. In the low chloride solutions that we have used ([Na]o = 172 mM, [Cl]o = 148 mM, [Na]i = 44 mM, [Cl]i = 41 mM), the observed reversal potential for the P2X2/3 receptor corresponds to a very low chloride permeability (PCl/PNa of ~0.02). The estimates of permeability to NMDG, calcium, and gluconate should be considered as less reliable than those for PCl/PNa. This is because in those cases there are three permeant ions present, and the estimate involves using the value previously determined for PCl/PNa.

Likewise, the reversal potential observed with extracellular NMDG indicates that the P2X2/3 receptor is essentially impermeant (P[NMDG]/PNa < 0.03), whereas both the currents presently studied in macrophages have significant permeability to NMDG. From experiments of the type described, it is difficult to exclude the possibility that the sustained depolarization is activating more than one set of ionic channels; for example, one permeable to anions and another permeable to cations. All that can be said in this case is that each has a very similar, and linear, current-voltage relation and each has a similar sensitivity to block by lanthanum. We shall return later to discuss the question of how a sustained depolarization might lead to the activation of such an ionic conductance(s).

There are many similarities between these permeation properties and those reported by Holevinsky and Nelson (1) for the current following phagocytosis of fluorescein-labeled immune complexes recorded from macrophages derived from human blood monocytes. That was a large current (typically ~1 nA), similar in amplitude to the currents that we presently describe; this is in marked contrast to the very small (~50 pA) current elicited by thapsigargin in the same cells (10). The underlying conductance had P[NMDG]/PNa of 0.69 in the work of Holevinsky and Nelson (1), which is close to our present estimate. Furthermore, they also reported that the underlying channels had significant anion permeability, and that glutamate appeared to be more permeable than chloride. That current was also completely blocked by 1 mM lanthanum, although the effects of lower concentrations were not examined. Holevinsky and Nelson (1) found that a current with similar properties could be activated by xanthine plus xanthine oxidase, although not by xanthine alone, and they suggested that the release of free radicals following phagocytosis activated the underlying conductance. Holevinsky and Nelson (1) did not investigate whether a sustained depolarization could elicit such a current, as we have reported; they did report that, under current clamp, the current led to a sustained depolarization of the cells.

It has recently become clear that oxidants such as H2O2 can indeed activate a cation conductance in certain immune cells (12). In the monocytic cell line U937, as well as Jurkat T lymphocytes and the eosinophil line EOL1, this can be mimicked by intracellular βNAD+, but not by βNADH (12, 13). This implies that βNAD+, formed by the action of free radicals on intracellular
βNADH, mediates the effect. The molecular identity of the underlying channel has been approached in two ways. First, heterologous expression of TRPM2 cDNA results in the appearance of channels permeable to cations that can be activated by intracellular NAD (1 mM) (12, 13). Second, treatment of U937 monocytes with antisense oligonucleotides that reduced the expression of TRPM2 (measured by Western blotting and immunocytochemistry) also reduced the calcium entry and cell death elicited by H2O2 (12). These experiments strongly suggest that one or more TRPM2 subunits are a key component of the channel expressed by these immune cell lines that is activated by oxidative damage (12, 13).

In contrast, it has recently been found that H2O2 can activate heterologously expressed TRPM2 channels by a mechanism that appears not to involve changes in ADP-ribose or NAD levels (23). The homomeric channels formed by expression of TRPM2 subunits are directly activated also by ADP-ribose (13, 15), although not by several closely related analogs (cyclic ADP-ribose, βNADH, βNADP+, βNADPH, ATP, and ADP) (12, 13, 15). The activation results from binding of the ADP-ribose to a Nudix domain in the C terminus of the protein, because it is prevented by point mutations of key residues in this motif (12, 15). The concentration of ADP-ribose causing 50% activation of the whole cell current is ~30–100 μM (15). When activated by intracellular diffusion of ADP-ribose during the course of whole cell recordings, these TRPM2 currents show close to linear I/V relations and other properties that resemble the current first described by Wilding et al. (24) in ascidian oocytes.

The current that we now report in rat peritoneal macrophages, which is activated by ADP-ribose, has some, but not all, of the same features. These include the effectiveness of ADP-ribose during the course of whole cell recordings, these TRPM2 currents show close to linear I/V relations and other properties that resemble the current first described by Wilding et al. (24) in ascidian oocytes.
1–10 μM) on the depolarization-evoked and ADP-ribose-evoked current in peritoneal macrophages, but the sensitivity of TRPM2 currents to lanthanum has not yet been studied systematically.

In contrast, other currents have been described with properties that more closely resemble the macrophase currents observed in the present study. For example, heterologous expression of PKD1 and PKD2, genes named for the polycystic kidney disease that arises when they are mutated in humans (26), gives rise to a similar conductance (27). Those channels have PNa,NAD/PNa,0 of 0.51. The PCl/PNa cannot be calculated directly from the data of Hanaoka et al. (27) (they had three permeant ions present: cesium, sodium, and chloride); however, the 45 mV shift in reversal potential would indicate PCl/PNa, of −0.3. The lanthanum sensitivity of the PKD1/2 current (50% inhibition by −50 μM) is also not very different from that which we observed in peritoneal macrophages. It is not clear how polycystin channels could be activated by βNAD+ and ADP-ribose, because their amino acid sequence does not contain a Nudix domain. Further work, such as recordings of single channel activity and antisense oligonucleotide approaches, will be needed to establish the molecular composition of the macrophage channel.

The occlusion experiment illustrated in Fig. 5 strongly suggests that the two currents that we describe pass through the same channels (although it is also formally possible that intracellular ADP-ribose could in some way inhibit the effect of depolarization to activate a separate population of channels). This is consistent with the overall similarity in the properties of the currents with respect to permeability (Table 1) and lanthanum blockade (Fig. 3). This raises the question deferred earlier: what might be the mechanism by which sustained depolarization activates this cell conductance? Speculations might include that depolarization evokes the release by which sustained depolarization activates this cell conductance? This is consistent with the two currents that we describe pass through the same channel (such as phagocytosis) would be further enhanced. Such a positive feedback process, in which activation of the current leads to depolarization of the macrophage and depolarization leads to further activation, might contribute to the eventual cell death that readily follows exposure to exogenous oxidants.

Acknowledgments

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References


