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**University of Bath**

1  **2′-Deoxyadenosine 5′-diphosphoribose is an endogenous TRPM2 superagonist**

2  Running Head: 2′-Deoxy-ADPR is an endogenous superagonist of TRPM2

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16

17  **Abstract**

18  Transient receptor potential melastatin 2 (TRPM2), is a ligand-gated $Ca^{2+}$-permeable non-

19  selective cation channel. While physiological stimuli, e.g. chemotactic agents, evoke

20  controlled $Ca^{2+}$ signals via TRPM2, pathophysiological signals, such as reactive oxygen

21  species or genotoxic stress result in prolonged TRPM2-mediated $Ca^{2+}$ entry and consequently

22  apoptosis. To date, adenosine 5′-diphosphoribose (ADPR, **1**) has been assumed to be the main

23  agonist for TRPM2. Here, we show that 2′-deoxy-ADPR **2** was a significantly better TRPM2

24  agonist, inducing 10.4-fold higher whole cell currents at saturation. Mechanistically, this

25  increased activity was caused by decreased rate of inactivation and higher average open

26  probability. Using high performance liquid chromatography (HPLC) and mass spectrometry,

27  endogenous 2′-deoxy-ADPR was detected in Jurkat T-lymphocytes. Consistently, cytosolic

28  nicotinamide mononucleotide adenylyltransferase 2 (NMNAT-2) and nicotinamide adenine

29  dinucleotide (NAD)-glycohydrolase CD38 sequentially catalyzed synthesis of 2′-deoxy-

30  ADPR from nicotinamide mononucleotide and 2′-deoxy-ATP *in vitro*. Thus, 2′-deoxy-ADPR

31  is an endogenous TRPM2 superagonist that may act as cell signaling molecule.

32

33  **Introduction**

34  The *C*-terminal domain of TRPM2 is homologous to ADPR pyrophosphatase NUDT9. This

35  discovery resulted in the identification of adenosine 5′-diphosphoribose (ADPR, **1**) as its

36  agonist[1]. An increase in cellular ADPR to activate TRPM2 is thought to proceed by

37  hydrolysis of β-NAD either by NAD-glycohydrolase CD38 or via concerted action of poly-

38  ADPR polymerases (PARPs) and poly-ADPR glycohydrolase (PARG) (reviewed in [2]). The

39  half maximal effective concentration ($EC_{50}$) for activation of TRPM2 by ADPR (reviewed in

40  [3]) implies that a significant amount of cellular NAD must be converted to ADPR to achieve

41  activation. While this may make sense for the induction of apoptosis or cell death[4,5], it appears

42  rather unlikely to be the case for physiological activation of TRPM2, e.g. in chemotaxis[6],

43  insulin secretion[7], or thermosensation[8,9]. Alternative activation mechanisms have been

44  described, including direct activation by hydrogen peroxide[10], by intracellular $Ca^{2+}$ ions[11] or

45  by *O*-acetyl-ADPR[12].

46  In summary, we show that among various ADPR analogues only 2′-deoxy-ADPR **2**, 3′-deoxy-

47  ADPR **17**, 2′-phospho-ADPR **15**, and 2-F-ADPR **13** activated TRPM2. 2′-Deoxy-ADPR **2**

48    activated TRPM2 with similar potency but higher efficacy than ADPR, making 2′-deoxy-

49    ADPR a TRPM2 superagonist. The massive increase in macroscopic current induced by 2′-

50    deoxy-ADPR was due to decelerated TRPM2 inactivation and a higher average open

51    probability, while the single channel conductance remained unaffected. Further, we

52    demonstrate formation of 2′-deoxy-ADPR *in vitro* in two steps: from NMN and 2′-deoxy-

53    ATP to 2′-deoxy-NAD as catalyzed by NMNAT-2, and from 2′-deoxy-NAD to 2′-deoxy-

54    ADPR catalyzed by CD38. Finally, we proved the presence of endogenous 2′-deoxy-ADPR

55    and 2′-deoxy-NAD and present evidence for hydrogen peroxide-evoked increase of 2′-deoxy-

56    ADPR in Jurkat T cells. Importantly, 2′-deoxy-ADPR is not only a significantly better agonist

57    regarding TRPM2 activation than ADPR, but in addition does not require any NAD

58    consumption for its synthesis. 2′-deoxy-ADPR thus exhibits many of the properties expected

59    of a second messenger.

60

61    **Results**

62    *2′-Deoxy-ADPR as a TRPM2 superagonist*

63    Our interest in 2′-deoxy-ADPR as a potential TRPM2 agonist began when we probed the

64    structural requirements for activation of the channel. We assessed the agonist activity of

65    ADPR  analogues (Supplementary Results, Supplementary Fig. 1–4) we had previously

66    evaluated as potential TRPM2 inhibitors[13]. These analogues feature modifications in the

67    purine base, the adenosine ribose, the pyrophosphate group and the terminal ribose. Published

68    $EC_{50}$ values for the activation of TRPM2 by ADPR are in the micromolar range (between

69    1 µmol/L and 90 µmol/L)[3] indicating an interaction of rather low affinity. We therefore

70    anticipated that many of the analogues might activate TRPM2. To our surprise most of the

71    analogues had no, or negligible, agonist activity (Fig. 1). Among the ADPR analogues with

72  modifications at the purine ring only 2-F-ADPR retained partial agonist activity (Fig. 1).

73  These results clearly demonstrate the requirement of a combination of terminal ribose,

74  pyrophosphate, and adenosine motifs for activation of TRPM2.

75  The part of ADPR most permissive to modifications was the adenosine ribose (Fig. 2). 2′-

76  Phospho-ADPR showed partial agonist activity albeit at higher concentrations ($EC_{50}$

77  110 µmol/L) and with reduced efficacy (0.56 nA ± 0.64 nA, best-fit value ± standard error

78  (SE)) compared to ADPR (1.60 nA ± 0.47 nA, best-fit value ± SE) (Fig. 2a), in good

79  agreement with a recent study[14]. The most interesting finding, however, came from our

80  attempt to establish the role of the 2′- and 3′-hydroxyl groups of the adenosine ribose. While

81  3′-deoxy-ADPR showed an $EC_{50}$ comparable to that of ADPR ($EC_{50}$ 46 µmol/L and

82  28 µmol/L, respectively) and elicited only a slightly reduced maximum current (1.23 nA ±

83  0.52 nA, best-fit value ± SE), 2′-deoxy-ADPR activated TRPM2 at somewhat lower

84  concentrations than ADPR (Fig. 2a). Most importantly, 2′-deoxy-ADPR induced 10.4-fold

85  higher currents in the whole cell configuration (Fig. 2a). Whole cell patch clamp experiments

86  with the human T cell line Jurkat that endogenously expresses TRPM2[4,15–17] confirmed the

87  induction of significantly higher currents by 2′-deoxy-ADPR as compared to ADPR. In

88  contrast to HEK293 cells, Jurkat cells in the majority of experiments did not tolerate 2′-

89  deoxy-ADPR concentrations > 100 µmol/L, likely due to the high $Ca^{2+}$ and $Na^+$ influx (Fig.

90  2b). Under physiological ionic conditions, in these experiments the I-V curve obtained from

91  voltage ramps was nearly linear and was characterized by a reversal potential near 0

92  (Supplementary Fig. 5b), regardless of the activating agonist. These results clearly

93  demonstrate that 2′-deoxy-ADPR is a TRPM2 superagonist.

94  The increase in whole cell current after activation by superagonists might be explained either

95  by the preferential occupation of higher conductance states, as has been shown for the

96  insecticide clothianidin, a superagonist of the nicotinic acetylcholine receptor[18], by induction

97    of an otherwise inaccessible higher conductance state, or by a change in kinetics allowing the

98    channel to remain in the open state for longer periods of time, as is the case for 4,5,6,7-

99    tetrahydroisoxazolo[5,4-*c*]pyridin-3(2*H*)-one (THIP), a superagonist of the $\alpha_4\beta_3\delta$ GABA

100   receptor[19]. To distinguish between these possibilities we performed recordings on excised

101   inside/out patches from cells expressing either wild type hTRPM2 (Fig 3a+b+c) or hTRPM2

102   with modified selectivity filter (T5L) that shows increased affinity to permeating cations

103   resulting in higher single channel conductivity[20]. The results showed no significant difference

104   in single channel conductance (Fig. 3c) upon activation by either ADPR or 2′-deoxy-ADPR.

105   Instead, the inactivation of TRPM2 following 2′-deoxy-ADPR activation was significantly

106   slower compared to ADPR (Fig 3d+e+f; 2′-deoxy ADPR: 0.050 s$^{-1}$ ± 0.003 s$^{-1}$ vs ADPR:

107   0.037 s$^{-1}$ ± 0.003 s$^{-1}$, best-fit values ± SE, extra sum-of-squares F test, p=0.001). Further, the

108   average open probability was 37% higher (Fig. 3d+g) for activation by 2′-deoxy-ADPR

109   (median 0.92, interquartile range (IQR) 0.80 to 0.97) compared to ADPR (median 0.67, IQR

110   0.17 to 0.74, Mann-Whitney test, p< 0.0001). In addition, we also observed an approx. 5-fold

111   increase in the number of channels simultaneously in the open state when excised patches

112   were exposed to 2′-deoxy-ADPR (Fig. 3d+h), at least partially attributable to the increased

113   open probability. Whole cell patch clamp experiments with different $Ca^{2+}$ concentrations in

114   the pipette buffer solution demonstrated a reduced $Ca^{2+}$-sensitivity of activation of TRPM2 by

115   2′-deoxy-ADPR when compared to activation by ADPR (Fig. 2c). Whereas the maximum

116   current induced by ADPR (as obtained from the concentration-response curve) increased by

117   140% (from 0.67 nA ± 1.07 nA to 1.60 nA ± 0.47 nA) when the free $[Ca^{2+}]$ was raised from

118   50 nmol/L to 200 nmol/L, the maximum current induced by 2′-deoxy-ADPR increased only

119   by 16% (from 14.3 nA ± 1.45 nA to 16.7 nA ± 0.74 nA, best-fit values ± SE). At

120   (supraphysiological) saturating concentrations for $Ca^{2+}$ and ADPR the open probability of

121   human TRPM2 approaches a value of one[21]. It therefore seems likely that in intact cells

122 activation by 2′-deoxy-ADPR is less sensitive to $Ca^{2+}$, with the concentration-response for

123 $Ca^{2+}$ shifted to the left, allowing for higher open probabilities and larger whole cell currents at

124 physiological $Ca^{2+}$ concentrations.

125 *Biosynthetic pathways for 2'-deoxy-ADPR*

126 Superagonist behavior has previously been observed with pharmacological modulators of

127 ligand-gated ion channels usually having little or no resemblance to the natural ligands.

128 However, 2′-deoxy-ADPR is closely related to ADPR. Thus, we hypothesized that 2′-deoxy-

129 ADPR might be an endogenous modulator of TRPM2.

130 A conceivable endogenous pathway to 2′-deoxy-ADPR would be the hydrolysis of 2′-deoxy-

131 NAD by either CD38 or the PARP/PARG system. 2′-Deoxy-NAD can be synthesized by

132 nicotinamide mononucleotide adenylyltransferase (NMNAT, EC 2.7.7.1) catalyzing the

133 condensation of β-nicotinamide mononucleotide (NMN) and 2′-deoxy-ATP, as has been

134 shown for NMNAT from yeast[22] and human NMNAT-1[23]. There are three different isoforms

135 of NMNAT with distinct subcellular localization that contribute to different NAD pools[24].

136 Here we demonstrate for the first time that NMNAT-2, the isoform that presumably maintains

137 the cytoplasmic NAD pool[24], also catalyzed the conversion of 2′-deoxy-ATP and NMN to 2′-

138 deoxy-NAD (Fig. 4a; Supplementary Fig. 6). At a saturating concentration of 500μmol/L β-

139 NMN the $K_{0.5}$ for 2′-deoxy-ATP is 3.28 mmol/L ± 0.55 mmol/L (Michaelis-Menten constant

140 $K_M$ for ATP 611 μmol/L ± 31 μmol/L, both best-fit values ± SE). Interestingly, the enzyme

141 did not only show cooperativity (Hill coefficient $n_H$ 1.4 ± 0.1, best-fit value ± SE) with regard

142 to 2'-deoxy ATP, but was also inhibited by high concentrations of the substrate, which is

143 clearly not the case for ATP (Fig. 4b).

144 Next we explored potential pathways for generation of 2'-deoxy-ADPR via both the

145 PARP/PARG and CD38 pathways. Since it was shown in isolated nuclei that 2'-deoxy-ADPR

146   was incorporated into poly-ADPR polymers of nuclear proteins[25], we analyzed whether poly-

147   ADP ribosylated proteins actually contain 2'-deoxy-ADPR residues. Poly-ADP-ribosylated

148   proteins were isolated from HEK293 cells previously exposed to $H_2O_2$[5,26]. An increase of the

149   amount of poly-ADP ribosylated proteins, compared to unstimulated controls was confirmed

150   by western blot analysis (Supplementary Fig. 7, upper inset). The immunoprecipitate was then

151   hydrolyzed by recombinant human PARG. Analysis of products by HPLC showed significant

152   amounts of ADPR and a small amount of AMP, most likely due to spontaneous degradation

153   of ADPR  Supplementary Fig. 7, central panel), but despite the high sensitivity of the method

154   (40 fmol at signal-to-noise (S/N) ratio $\geq 3$) no 2'-deoxy-ADPR was detected (Supplementary

155   Fig. 7, central and lower panel). Thus, it is unlikely that release of 2′-deoxy-ADPR from poly-

156   ADP-ribosylated proteins by PARG significantly contributes to TRPM2 activation.

157   Next we tested whether CD38 might produce 2'-deoxy-ADPR. CD38 is well known to be

158   expressed as ecto-enzyme (type II orientation), but recently evidence for type III orientation

159   with the active site facing the cytoplasm was presented[27], thereby potentially allowing

160   turnover of cytosolic 2′-deoxy-NAD. Recombinant soluble human CD38 completely

161   hydrolyzed 2'-deoxy-NAD to a product that co-eluted with chemically synthesized 2'-deoxy-

162   ADPR (Fig. 4c). Product identity was confirmed by spiking with authentic 2'-deoxy-ADPR

163   (Supplementary Fig. 8a) and hydrolysis by pyrophosphatase to 2'-deoxy-AMP

164   (Supplementary Fig. 8b). Further, type III CD38 enzyme activity, though small in comparison

165   to ecto-enzyme (type II) CD38 enzyme activity, was clearly detected in Jurkat T cells (Fig

166   4d). While in CD38^{-/-} cells measurable NAD glycohydrolase was not detected (Fig. 4d, left),

167   specific inhibition of ecto-CD38 by 2'-deoxy-2'-fluoro arabinosyl NAD (araF-NAD)[28],

168   forming a stable covalent inhibitor-CD38 complex, almost completely abolished type II CD38

169   activity (Fig. 4d, middle, 1st araF-NAD addition). Permeabilization of such cells with saponin

170   allowed to access type III CD38 and to determine its activity (Fig. 4d, right). Specificity was

171 demonstrated by a 2$^{nd}$ addition of araF-NAD fully blocking type III CD38 (Fig. 4d, right).

172 Kinetic analysis using NAD vs 2'-deoxy-NAD as substrates resulted in 9.2-fold higher affinity

173 of CD38 for 2'-deoxy-NAD and comparable values for maximal velocity V$_{max}$

174 (Supplementary Fig. 8c). Collectively, strong evidence is presented that 2'-deoxy-NAD serves

175 as a substrate for type III CD38 to produce 2'-deoxy-ADPR, while 2'-deoxy-NAD can be

176 produced from the abundant cellular metabolites nicotinamide mononucleotide and 2'-deoxy-

177 ATP by cytosolic NMNAT (Supplementary Fig. 9).

178 *2′-Deoxy-ADPR and 2′-deoxy-NAD present in Jurkat cells*

179 To detect endogenous 2′-deoxy-ADPR and 2′-deoxy-NAD in Jurkat T cells, we used two

180 consecutive reversed phase (RP)-HPLC separations on C8 and C18 columns using a volatile

181 ammonium acetate buffer to allow for subsequent high resolution mass spectrometry (Fig. 5).

182 Fractions pre-fractionated on a C8 column (Fig. 5a) and co-eluting with 2′-deoxy-ADPR and

183 2′-deoxy-NAD on a C18 column (Fig. 5b+c) showed molecular ions corresponding to 2′-

184 deoxy-ADPR (observed mass [M-H]$^-$ 542.0722, calculated mass: 542.0695) and 2′-deoxy-

185 NAD (observed mass [M-H]$^-$ 646.1104, calculated mass: 646.1069) (Supplementary Fig. 10

186 a+b). The main electrospray ionization (ESI) fragmentation products of 2′-deoxy-ADPR and

187 2′-deoxy-NAD, 2′-deoxy-AMP ([M-H]$^-$ 330.0633) and 2′-deoxy-ADP ([M-H]$^-$ 410.0304),

188 were prominent in both samples. Since it is unlikely that both products co-eluted with 2′-

189 deoxy-ADPR and 2′-deoxy-NAD during two-dimensional HPLC, these molecular ions are

190 most likely to result from fragmentation of 2′-deoxy-ADPR and 2′-deoxy-NAD during mass

191 spectrometry. The HPLC system based on ammonium acetate eluents was suitable for

192 quantification of endogenous 2′-deoxy-NAD (Fig. 5c+g), amounting to $5.05 \pm 0.58$

193 pmol/10$^7$ cells (mean $\pm$ SEM, n=12, significantly different from a theoretical median of 0,

194 Wilcoxon Signed Rank Test). The concentration of 2′-deoxy-NAD was not significantly

195 affected by exposure of the cells to hydrogen peroxide ($5.67 \pm 0.78$ pmol/10$^7$ cells, mean $\pm$

196   SEM, n=11) (Fig. 5c+g). To investigate any role of CD38 in the synthesis of 2′-deoxy-ADPR

197   in Jurkat T cells, CD38 was knocked-out using CRISPR (Clustered Regularly Interspaced

198   Short Palindromic Repeats)/Cas9 technology[29,30]. P10 membranes of the resulting CD38^{-/-} cell

199   line did not show NAD glycohydrolase activity and were negative for CD38 in western blot

200   analysis (Supplementary Fig 11). Neither was the endogenous concentration of 2′-deoxy-

201   NAD significantly affected by knock-out of CD38 nor by hydrogen peroxide stimulation (Fig.

202   5 d+f+g). In addition, we also analyzed cellular ADPR and NAD contents under these

203   conditions (Supplementary Fig 12). As expected, NAD was present in much higher quantities

204   $(7.58 \pm 0.42$ nmol/$10^7$ cells, mean±SEM, n=12), but was not affected by knockout of NAD

205   glycohydrolase CD38 $(6.42 \pm 0.30$ nmol/$10^7$ cells, mean±SEM, n=15). Exposure to hydrogen

206   peroxide did, at least during the 5 min of the experiment, not affect NAD (p=0.78 for wild

207   type and p=0.62 for CD38^{-/-} cells). ADPR was found in quantities $(292 \pm 79$ pmol/$10^7$ cells,

208   mean±SEM, n=11) consistent with our previous results[4], but was neither significantly affected

209   by knockout of CD38 nor by exposure to hydrogen peroxide.

210   In contrast to almost baseline-separated 2′-deoxy-NAD (Fig. 5c+f), HPLC using volatile

211   ammonium acetate buffer (ideally suited for subsequent mass spectrometry) did not allow for

212   baseline separation of 2′-deoxy-ADPR (Fig. 5b+e). Though 2′-deoxy-ADPR was qualitatively

213   identified (i) by its retention time, (ii) by using samples from cell extracts spiked with a small

214   amount of authentic 2′-deoxy-ADPR (Fig. 5b+e), and (iii) by mass spectrometry

215   (Supplementary Fig. 10a), the separation on a C18 column was not sufficient to quantify 2′-

216   deoxy-ADPR reliably (Fig. 5b+e). However, we noted that the peak containing 2′-deoxy-

217   ADPR increased after stimulation with hydrogen peroxide. Further, comparison of the

218   chromatograms of samples from wildtype (Fig. 5b) and CD38^{-/-} cells (Fig. 5e) shows that the

219   peak containing 2′-deoxy-ADPR was absent in the samples from CD38^{-/-} cells regardless of

220    whether they have been exposed to $H_2O_2$ or not, suggesting that 2′-deoxy-ADPR is produced

221    from 2′-deoxy-NAD by CD38.

222    Using ion-pair instead of conventional RP-HPLC, two consecutive separations on C8 and C18

223    columns (Fig. 6) resulted in sufficient separation of endogenous 2′-deoxy-ADPR for

224    quantification (Fig. 6a, lower panel). Here, 2′-deoxy-ADPR was identified using authentic 2′-

225    deoxy-ADPR as standard or in a spiked sample (Fig 6a). Recovery of 2′-deoxy-ADPR was

226    determined using 1, $N^6$-etheno-adenosine (Supplementary Fig 13). Any trials to identify 2′-

227    deoxy-ADPR by mass spectrometry in this HPLC system failed due to the presence of the ion

228    pair reagent in the collected samples; furthermore, several procedures tested to deplete the ion

229    pair reagent were not successful. However, using this ion-pair RP-HPLC separations on C8

230    and C18 columns, 2′-deoxy-ADPR was detected in Jurkat cells and determined to be

231    35 pmol/$10^7$ cells (median, interquartile range (IQR): 17 pmol/$10^7$ cells to 50 pmol/$10^7$ cells,

232    significantly different from a theoretical median of 0, Wilcoxon Signed Rank Test). As

233    hydrogen peroxide treatment results in TRPM2 activation, we then tested the impact of $H_2O_2$

234    on the endogenous 2′-deoxy-ADPR concentration (Fig. 6b+c). After 5 min the amount of 2′-

235    deoxy-ADPR rose significantly by 2.71 fold to 95 pmol/$10^7$ cells (median, IQR: 42

236    pmol/$10^7$ cells to 116 pmol/$10^7$ cells).

237    **Discussion**

238    It is widely accepted that TRPM2 can be activated downstream of reactive oxygen species

239    like hydrogen peroxide[10,31]. Despite reports of direct activation of the channel by hydrogen

240    peroxide[32], this has mostly been attributed to the generation of ADPR by the consecutive

241    action of PARP and PARG[5,33]. Especially during certain cell cycle phases, 2′-deoxy-ADPR as

242    an additional mechanism might contribute to this process: hydrogen peroxide-induced DNA

243    damage can cause a replication arrest during S phase, which would result in an accumulation

244  of 2′-deoxy-ATP, because deoxy-nucleoside triphosphates will no longer be consumed by the

245  replication machinery. In addition, the activation of PARPs following DNA damage will

246  result in consumption of NAD for poly-ADP ribosylation. The consequent drop in cellular

247  NAD will increase its synthesis, which ultimately results in decreased cellular ATP pools. The

248  increased ratio of 2′-deoxy-ATP to ATP then facilitates synthesis of 2′-deoxy-NAD by

249  NMNAT and subsequent hydrolysis to 2′-deoxy-ADPR by CD38. The latter reaction is

250  preferred due to the 9.2-fold higher affinity of CD38 for 2'-deoxy-NAD, as compared to NAD

251  (see Supplementary Fig. 8c). The increase in cellular 2′-deoxy-ADPR then either mediates

252  TRPM2 activation on its own or contributes to TRPM2 activation mediated by ADPR derived

253  from PARP/PARG activity.

254  How do the nucleotide determinations from cell extracts translate into cytosolic, or even sub-

255  plasmalemmal, concentrations? Assuming a mean diameter of Jurkat cells of 11.5 μm[34], a

256  cellular 2′-deoxy-ADPR concentration of 4.4 μmol/L can be calculated. However, the

257  lymphocyte is not simply a sphere filled with aqueous liquid, but contains solid material, e.g.

258  organelles, DNA, proteins. This solid portion is difficult to calculate, but well known for

259  Jurkat cells is the nucleus volume, amounting to 55% of the total volume[34]. Using this portion

260  to distinguish the cytosolic from the intra-nuclear space, the cytosolic concentration of 2′-

261  deoxy-ADPR amounts to 9.8 μmol/L, a value found in the lower part of the concentration-

262  response curve (Fig. 2). Upon stimulation by hydrogen peroxide this cytosolic concentration

263  within 5 min rises to 26.6 μmol/L. If we further assume that the concentration of 2′-deoxy-

264  ADPR in the cytosol is not evenly distributed, but is increased at the site of its biosynthesis,

265  the sub-plasmalemmal space where the catalytic center of type III CD38 is localized[27], then

266  the local concentrations are readily in the dynamic range of the concentrations response

267  curve(s) shown in Fig. 2, especially at slightly elevated free cytosolic $Ca^{2+}$ concentrations,

268  e.g. 200 nM (Fig. 2c). Besides 2′-deoxy-ADPR, we also established the presence of

269  endogenous 2′-deoxy-NAD, the potential precursor of 2′-deoxy-ADPR. The cytosolic

270  concentration of 2′-deoxy-NAD is low at 1.41 μmol/L, in comparison to the cellular

271  concentration of NAD (955 μmol/L). This indicates that 2′-deoxy-NAD represents a

272  nucleotide with specialized function(s), very much in contrast to NAD that serves as (co)-

273  substrate for a plethora of enzymatic reactions. The kinetic parameters obtained for NMNAT-

274  2 (Fig. 2c) suggest that inside the cell mainly ATP is used to maintain the cellular NAD pool.

275  However, upon stimulation with hydrogen peroxide, small amounts of 2′-deoxy-ATP appear

276  to flux via 2′-deoxy-NAD into 2′-deoxy-ADPR; substrate inhibition of NMNAT-2 by 2′-

277  deoxy-ATP, but not ATP, indicates that the pathway towards 2′-deoxy-ADPR is a highly

278  regulated process. It has not escaped our notice that the presence of endogenous 2′-deoxy-

279  NAD also in principle presents the possibility for generation of 2′-deoxy-cADPR by CD38, in

280  addition to 2′-deoxy ADPR. While 2′-deoxy-cADPR was shown to be equipotent to cADPR

281  in $Ca^{2+}$ signaling in sea urchin[35], like its 3′-deoxy congener, it was not active in Jurkat T

282  cells[36] excluding the need to consider 2′-deoxy-cADPR more widely in a signaling context.

283  Our results might solve an important conceptual problem concerning activation of TRPM2:

284  Cellular NAD serves many functions, most prominently as redox coenzyme, as co-substrate

285  for sirtuins, and as precursor for nicotinamide adenine dinucleotide phosphate (NADP) and

286  the second messengers cyclic adenosine diphosphoribose (cADPR) and nicotinic acid adenine

287  dinucleotide phosphate (NAADP, reviewed in[24]). NAD depletion appears therefore

288  incompatible with life and has been shown to ultimately result in cell death[37]. We therefore

289  consider it unlikely that during physiological activation the amount of ADPR necessary for

290  TRPM2 activation, especially at resting $Ca^{2+}$ concentrations, is generated by turnover of

291  nuclear, cytosolic or mitochondrial NAD pools. In contrast, a small cytosolic pool of 2′-

292  deoxy-NAD might be maintained, for instance by NMNAT, independently of NAD, since 2′-

293  deoxy-NAD can neither be phosphorylated by NAD kinase to yield 2′-deoxy-NADP nor is it a

294    good substrate for NAD dependent dehydrogenases[22,38,39]. Thus, formation of 2′-deoxy-ADPR

295    from 2′-deoxy-NAD by type III CD38 does not interfere with energy metabolism and other

296    NAD functions. Previous work demonstrating that CD38 hydrolyzed 2′-deoxy-NAD with

297    similar $V_{max}$ as NAD[40] is confirmed here; however, a 9.2-fold higher affinity for 2′-deoxy-

298    NAD as compared to NAD was determined (Supplementary Fig. 8c). Thus, we present here a

299    possible mechanism for the generation of 2′-deoxy-ADPR via conversion of cytosolic 2′-

300    deoxy-NAD by CD38 in a type III orientation. However, since the endogenous concentration

301    of 2′-deoxy-NAD is small and the relative activity of CD38 in the type III orientation that we

302    observed was low, generation of 2′-deoxy-ADPR may be accomplished via alternative

303    pathways.

304    Taken together, we demonstrate that 2′-deoxy-ADPR is the most efficient endogenous agonist

305    of TRPM2 described to date. In addition, 2′-deoxy-ADPR appears to be the first superagonist

306    for an ion channel that can be synthesized by the enzymatic machinery of most cell types. Our

307    results suggest the possibility that TRPM2 can be activated in context-specific manner. In

308    pathological cell death in neurodegenerative diseases[41], stroke[42], or myocardial infarction[43],

309    ADPR may be generated by PARP/PARG[33] or by CD38 thereby not only triggering TRPM2

310    activation, but also depleting cellular NAD and ATP pools. In contrast, since physiological

311    processes, e.g. neutrophil chemotaxis, require an intact cytoplasmic NAD pool, 2′-deoxy-

312    ADPR may activate TRPM2 without consumption of NAD, thus acting independently of

313    energy metabolism. Taken together, the confluence of properties associated with 2′-deoxy-

314    ADPR may point to a physiological role for the molecule as a novel second messenger

315    activating TRPM2.

316

317    **Acknowledgements**

322

323

324    **Author contributions**

325    AHG, BVLP and RF designed the study and individual experiments. CM and JMW

326    synthesized and purified the ADPR analogues. TK, RF and MDR performed

327    electrophysiological characterization of ADPR analogues. MDR carried out the single channel

328    recordings. AMWP and AB performed the enzyme assays with NMNAT and CD38. RW

329    analyzed nucleotide products from poly ADP-ribosylated proteins. RF and AH prepared

330    TRPM2 T5L and CD38 expression vectors and generated the TRPM2 T5L cell line. AB,

331    AWMP and MJ established the HPLC method for determination of endogenous 2′-deoxy-

332    ADPR. AB and MJ quantitatively analyzed endogenous nucleotides. AB determined substrate

333    saturation plots for NMNAT-2 and sCD38. JMW performed the HRMS analysis of 2′-deoxy-

334    ADPR and 2′-deoxy-NAD. VW generated the CD38$^{-/-}$ Jurkat cell line and produced and

335    purified soluble recombinant CD38. MF characterized the CD38$^{-/-}$ Jurkat cell line. AR, AB

336    and FG determined the activity of CD38 in type III orientation. All authors wrote the

337    manuscript.

338

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452

**Figure legends for main text**

454 Fig. 1 **ADPR analogues activate TRPM2 in whole cell patch clamp experiments.** Outward

455 currents at +15 mV were recorded as detailed in Methods section. Pipette concentration for

456 ADPR and ADPR analogues was 100 μmol/L in most cases; exceptions are indicated. Data

457 for 30 μmol/L 2′-deoxy-ADPR are from the same experiment as in Fig 2a. Shown are

458 maximum currents from individual patched cells, with the total number of cells indicated.

459 Recordings have usually been performed on multiple days. The median current from all cells

460 of one condition is indicated by a horizontal line. Since in some cases the number of data

461 points was too small to test for normality, data were analyzed by a nonparametric one-way

462 ANOVA (Kruskal−Wallis test) followed by comparison against buffer control, applying

463 Dunn's correction for multiple testing. Results significantly different from buffer control

464 (p<0.05) are indicated by an asterisk. The pipette solution for squaryl and triazole compounds

465 contained 0.1% DMSO; thus, 0.1% DMSO was also used for control conditions. (ADPR -

466 adenosine 5′-diphosphoribose; AMP - adenosine 5′-monophosphate; ASqR - adenosine

467 squaryl ribose; ATPR - adenosine 5′-triphosphate ribose; IDPR - inosine-5′-diphosphoribose;

468 Sal-AMS - salicyl-adenosine monosulfamide, 8-pCPT-AMP - 8-(4-

469 Chlorophenylthio)adenosine-5′-*O*-monophosphate; 8-Ph-ATrR - 8-Phenyl-adenosine-1,4-

470 triazole ribose). Abbreviations for ligands evaluated are consistent with those in reference [13].

471 Analogue structures shown are examples, see Supplementary Fig. 1-4 for all structures.

472

473 Fig. 2 **Modifications of the proximal ribose affect the ability of ADPR to activate**

474 **TRPM2. a**, Concentration response relationship for activation of TRPM2 by ADPR, 2′-

475 deoxy-ADPR, 3′-deoxy-ADPR and 2′-phospho-ADPR in HEK293 cells expressing hTRPM2.

476 Pipette solution contained 200 nmol/L free $Ca^{2+}$. In the NMDG-based bath solution TRPM2

477 showed characteristic reversal potential and outward rectification (Suppl. Fig. 5a). Currents

478 are displayed as mean±SEM (n= 6–12 for ADPR, 5–6 for 2′-phospho-ADPR, 5–12 for 2′-

479 deoxy-ADPR, 5–9 for 3′-deoxy-ADPR). **b**, Concentration response relationship for activation

480 of endogenous TRPM2 in Jurkat cells. In the NaCl-based bath solution the current showed the

481 reversal potential and I-V relationship of a non-selective cation channel (Suppl. Fig. 5b).

482 Currents are displayed as mean ±SEM (n=2–13 for ADPR, 2–21 for 2′-deoxy-ADPR). Result

483 from a single cell with  250 µmol/L 2′-deoxy ADPR in parenthesis (not included in the

484 concentration response curve). The current axis is shown in reversed orientation to facilitate

485 comparison with panel a. **c**, $Ca^{2+}$-dependence of TRPM2 activation by ADPR and 2′-deoxy

486 ADPR. Concentration response curves for ADPR and 2′-deoxy-ADPR with 50 nmol/L free

487 $Ca^{2+}$ in the pipette solution are shown in addition to the curves with 200 nmol/L from panel a.

488 Currents are displayed as mean±SEM (at 50 nM $Ca^{2+}$ n=2–6 for ADPR, 3–7 for 2′-deoxy-

489 ADPR for 200 nM $Ca^{2+}$ see panel **a**). In all panels n refers to the number of cells recorded for

490 the respective condition, recordings have been performed over multiple days.

491

492 Fig. 3 **Impact of 2′-deoxy-ADPR on hTRPM2 in excised inside-out patches. a**, Current

493 steps of hTRPM2 activated by 100 µmol/L ADPR at different potentials. **b**, I-V relationship

494 from patches containing TRPM2 wt or TRPM2 T5L activated by either 100 µmol/L ADPR

495 or 2'-deoxy-ADPR in the presence of 1 µmol/L free $Ca^{2+}$ (n=6-17 patches for TRPM2 wt and

496 n=2–3 patches for TRPM2 T5L). **c**, Single channel slope conductance γ obtained by linear

497 regression of the data in panel b. (unpaired, two-tailed T-tests of the mean±SEM obtained

498 from linear regression, p values are indicated) **d**, Representative continuous recordings of

499 excised inside out patches with the bath solution containing either 100 µmol/L ADPR or 2′-

500 deoxy-ADPR. **e-h**, **Quantitative analysis of continuous recordings from excised inside-out**

501 **patches e**, Histograms of time to inactivation of wild type TRPM2 after activation by ADPR

502    or 2′-deoxy ADPR with exponential fit. **f**, Decay constants for the time to inactivation. (extra

503    sum-of-squares F-Test, p=0.0012). **g**, Average open probability after activation by ADPR or

504    2′-deoxy-ADPR. Whiskers indicate min and max, horizontal bar represents the median

505    (Mann-Whitney test, p < 0.0001). **h**, Maximum number of simultaneously active channels in

506    an excised patch after application of either ADPR or 2′-deoxy-ADPR. (unpaired two-tailed T-

507    test, < 0.0001). **a-h** If not indicated otherwise, data are shown as mean±SEM. Numbers

508    indicated in the bars refer to the number of patches recorded, usually over multiple days.

509    Significant differences are indicated by asterisks.

510

511    Fig. 4 **NMNAT-2 and CD38 synthesize 2′-deoxy-ADPR *in vitro***. **a**, β-NMN and 2′-deoxy-

512    ATP were incubated with recombinant hNMNAT-2 and reaction products analyzed by HPLC.

513    2′-deoxy-AMP and 2′-deoxy-ADP were impurities contained in commercial 2′-deoxy-ATP. **b**,

514    Saturation plot for ATP and 2′-deoxy-ATP as substrates of NMNAT-2; β-NMN was used at

515    290µmol/L for both ATP and 2′-deoxy-ATP. Initial reaction rate was calculated from the

516    amount of product formed, determined by HPLC (mean±SD (n=3 independent experiments

517    with the concentration range shown for ATP, for 2′-deoxy-ATP 3 additional experiments were

518    performed for 3 mM, 5 mM, 7 mM and 9 mM, therefore n=6 for 3 mM and 9 mM)). Data for

519    ATP were fitted to a Michaelis-Menten model. Data up to 3 mmol/L 2′-deoxy-ATP were

520    fitted to a Hill model. At higher concentrations the reaction rate dropped rapidly, indicating

521    substrate inhibition (dashed line). **c**, Recombinant hCD38 hydrolyzed 2′-deoxy-NAD (from

522    panel a) to yield the TRPM2 agonist 2′-deoxy-ADPR (a+c are representative for three

523    independent experiments). **d,** CD38 activity was determined by incubation of Jurkat cells with

524    1,N$^6$-etheno-NAD and HPLC analysis of product 1,N$^6$-etheno-ADPR at different time points.

525    To analyze CD38 activity in type III orientation (red symbols), CD38 activity on the cell

526 surface was inhibited by covalently bound araF-NAD (blue symbols turned into gray). To

527 obtain access to type III CD38, the plasma membrane was selectively permeabilized using

528 saponin. After determination of type III CD38 activity, cells were incubated with araF-NAD

529 again (red symbols turned into gray). Data are shown as mean±SEM. The number of

530 experiments is indicated. For conditions tested in a one-way ANOVA for repeated

531 measurements, using Tukey's corrections for multiple testing, the corrected p values are

532 shown.

533

534 Fig. 5 The **increase in 2′-deoxy-ADPR in Jurkat cells exposed to hydrogen peroxide**

535 **depends on CD38**. Wild type and CD38$^{-/-}$ Jurkat cells were either exposed to 100 µmol/L

536 $H_2O_2$ for 5 min or left unstimulated. **a+d** Deproteinized extracts from cell samples were

537 separated by RP-HPLC on Phenomenex Luna C8 column and fractions co-eluting with

538 authentic ADPR, NAD, 2′-deoxy-ADPR or 2′-deoxy-NAD were collected. **b+c+e+f** Fractions

539 from the pre-fractionation on C8 column were subjected to a second dimension of RP-HPLC

540 on Multohyp BDS-C18 5µ column. To correctly assign peaks, each sample was split and one

541 half was spiked with the respective nucleotide (gray tracings). Representative chromatograms

542 for each condition are shown. Since we could not achieve baseline separation for 2′-deoxy-

543 ADPR, it was not analyzed quantitatively, however, the peak of 2′-deoxy-ADPR clearly

544 increased upon hydrogen peroxide stimulation. In the chromatograms from CD38$^{-/-}$ cells the

545 respective peak was not detected, regardless of stimulation. **g**, Quantitative analysis of the

546 impact of $H_2O_2$ on intracellular 2′-deoxy-NAD. Independent experiments were initiated on 3

547 separate days over the course of one month, with each experiment consisting of multiple

548 parallel cell preparations. 2–6 data points were obtained per experimental day. Results from

549 single experiments are indicated as filled circles (horizontal lines indicate the mean) with the

550 same shade of gray signifying individual experiments from a single day. Data are normally

551 distributed in each group. Analysis by one-way ANOVA did not reveal significant differences

552 for 2′-deoxy-NAD (p=0.074).

553

554 Fig. 6 **Concentration of 2′-deoxy ADPR in Jurkat cells increased after exposure to**

555 **hydrogen peroxide**. **a**, Deproteinized cell extracts from either unstimulated Jurkat cells or

556 Jurkat cells exposed to 100 µmol/L $H_2O_2$ were applied to a Phenomenex Luna C8 column and

557 a fraction co-eluting with chemically synthesized 2′-deoxy ADPR ($R_t$ between 24 and 25 min)

558 was collected **a + b,** The fractions were further chromatographed on a Multohyp BDS-C18 5µ

559 column. To confirm identity of the peak co-eluting with standard  2′-deoxy ADPR (dashed

560 tracing), samples were also spiked with 15.6 nmol 2′-deoxy ADPR (gray tracing). The slight

561 enhancement of the spiked peak is in agreement with the amount of 2′-deoxy-ADPR added as

562 spike (see standard trace for comparison). **d**, Quantitative analysis of the impact of $H_2O_2$ on

563 intracellular 2′-deoxy ADPR (per $10^7$ cells). Results from single experiments are indicated as

564 gray symbols (horizontal lines indicating median value) with symbols of same shape and gray

565 level signifying experiments from the same day. The number of independent preparations and

566 the number of days (12 samples from 3 days for $H_2O_2$ stimulated cells vs 47 samples from 7

567 days for unstimulated cells) are indicated in the chart. Due to non-normal distribution, data

568 were analyzed by a nonparametric Mann-Whitney test.

569

570 **Online Methods**

571 *Chemical Synthesis and Purification of ADPR Analogues*

572 Chemical synthesis, purification and characterization of 2′-deoxy-ADPR, 3′-deoxy-ADPR and

573 all other non-commercial ADPR analogues, apart from hydroxyl-ethoxy-ethyl-ASq, was as

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574 described previously[13,44,35]. Analogue identity was confirmed by $^1$H-NMR and HRMS. Purity

575 was checked by HPLC (>95%) and re-checked after storage.

576 *Synthesis of hydroxyl-ethoxy-ethyl-ASq*

577 *3-(2-(2-hydroxyethoxy)ethylamino)-4-ethoxycyclobut-3-ene-1,2-dione*

578 To a solution of 2-(2-aminoethoxy)ethanol (95 µL, 0.951 mmol) and DIPEA (81 µL, 0.467

579 mmol) in EtOH (8 mL) was added diethylsquarate (128 µL, 0.865 mmol). After 1h, the

580 solvent was removed under reduced pressure and the residue was purified on an Isco

581 chromatographic system (DCM-acetone, 8:2 v/v) to yield the desired product as a colorless oil

582 (165 mg, 91%). $^1$H (400 MHz, $d_4$-MeOH) δ 4.79 (q, 2H, J = 7.1 Hz, OC$\underline{H}_2$-Me), 3.85-3.61

583 (m, 8H, 4 x CH$_2$) and 1.51 (t, 3H, $J$ = 7.1 Hz, CH$_3$). $^{13}$C (100 MHz, $d_4$-MeOH) δ 189.9 (C-2),

584 184.9 (C-1), 177.9 (C-3), 175.1 (C-4), 73.5, 70.9 (both CH$_2$), 70.7 (Et: CH$_2$), 62.2, 45.5 (both

585 CH$_2$) and 16.2 (Et: CH$_3$). HRMS (ES$^+$) calcd for $C_{10}H_{16}NO_5$ 230.1023 (MH)$^+$ found

586 230.1031.

587 *3-(2´,3´-O-Isopropylidene-5´-amino-5´-deoxyadenosine)-4-(2-(2-hydroxyethoxy)ethyl-*

588 *amino)cyclobut-3-ene-1,2-dione*

589 To a solution of 3-(2-(2-hydroxyethoxy)ethylamino)-4-ethoxycyclobut-3-ene-1,2-dione (60

590 mg, 0.287 mmol) and DIPEA (27 µL, 0.154 mmol) in EtOH-DMF (1:1 v/v, 1 mL) was added

591 2′,3′-isopropylidene-5′-amino-5′-deoxyadenosine (96 mg, 0.315 mmol). After 26h, the solvent

592 was removed under reduced pressure and the residue was purified on an Isco chromatographic

593 system (DCM-MeOH, 8:2 v/v) to yield the desired product as a white foam (104 mg, 74%).

594 $^1$H (400 MHz, $d_6$-DMSO) δ 8.30 (s, 2H, H-8 and H-2), 6.24 (d, 1H, $J_{1',2'}$ = 2.6 Hz, H-1′), 5.55

595 (dd, 1H, $J_{2',3'}$ = 6.4 and $J_{2',1'}$ = 2.6 Hz, H-2′), 5.16 (dd, 1H, $J_{3',2'}$ = 6.4 and $J_{3',4'}$ = 3.7 Hz, H-3′),

596 4.44-4.41 (m, 1H, H-4′), 4.07-4.04 (m, 1H, H-5′a), 3.94 (dd, 1H, $J_{5'b,5'a}$ = 14.1 and $J_{5'b,4'}$ = 6.6

597 Hz, H-5′b), 3.78 (brs, 2H, CH$_2$-O), 3.72-3.70 (br, 2H, CH$_2$-NH), 3.64 (brs, 2H, CH$_2$-O), 3.61-

598    3.59 (m, 2H, $CH_2$-OH), 1.65 (s, 3H, $CH_3$) and 1.44 (s, 3H, $CH_3$). $^{13}$C (100 MHz, $d_6$-DMSO) δ

599    184.2 (C-2), 183.3 (C-1), 169.6 (both C=C), 157.6 (C-6), 154.3 (C-2), 150.3 (C-4), 142.0 (C-

600    8), 120.7 (C), 115.9 (C-5), 91.5 (C-1′), 87.0 (C-4′), 85.1 (C-2′), 82.9 (C-3′), 73.6, 71.4, 62.1,

601    46.7 (all $CH_2$), 45.1 (C-5′), 27.6 and 25.6 (both $CH_3$). HRMS (ES$^+$) calcd for $C_{21}H_{28}N_7O_7$

602    490.2045 (MH)$^+$ found 490.2027.

603    *3-(5′-amino-5′-deoxyadenosine)-4-(2-(2-hydroxyethoxy)ethylamino)cyclobut-3-ene-1,2-dione*

604    *(23)*

605    3-(2′,3′-*O*-Isopropylidene-5′-amino-5′-deoxyadenosine)-4-(2-(2-hydroxyethoxy)ethylamino)-

606    cyclobut-3-ene-1,2-dione (80 mg, 0.163 mmol) was stirred in a 75% aq. TFA solution (5 mL)

607    at rt for 1h. The solvents were evaporated under reduced pressure and the residue co-

608    evaporated with MeOH to remove any residual TFA. The remaining residue was purified on

609    an Isco purification system (DCM-MeOH, 8:2 v/v) to yield the desired compound as a white

610    solid (60 mg, 82%). $^1$H (400 MHz, $d_6$-DMSO) δ 8.39 (s, 1H), 8.22 (s, 1H), 7.57 (br s, 2H),

611    7.37 (br s, 2H, $NH_2$), 5.97 (d, 1H, $J_{1',2'}$ = 5.8 Hz, H-1′), 5.62 (d, $J$ = 6.1 Hz, 2′-OH), 5.44 (d, $J$

612    = 4.8 Hz, 3′-OH), 4.75 (ddd, 1H, $J_{2',3'}$ = 6.4, $J_{2',OH}$ = 6.1, $J_{2',1'}$ = 5.8 Hz, H-2′), 4.67 (t, $J$ = 5.3,

613    OH), 4.21 (ddd, 1H, $J_{3',2'}$ = 6.4, $J_{3',OH}$ = 4.8, $J_{3',4'}$ = 4.0 Hz, H-3′), 4.08-4.05 (m, 2H, H-4′, H-

614    5′a), 3.85-3.80 (m, 1H, H-5′b), 3.69 (brs, 2H, $CH_2$-O), 3.56-3.49 (m, 6H, 3 × $CH_2$). $^{13}$C (125

615    MHz, $d_6$-DMSO) δ 182.6 (C-2), 182.4 (C-1), 167.8 (both C=C), 156.1 (C-6), 152.7 (C-2),

616    149.4 (C-4), 139.8 (C-8), 119.2 (C-5), 87.4 (C-1′), 83.6 (C-4′), 72.7 (C-2′), 72.1 ($CH_2$), 70.8

617    (C-3′), 70.0, 60.1 (both $CH_2$), 45.5 (C-5′), 43.2 ($CH_2$). HRMS (ES$^+$) calcd for $C_{18}H_{24}N_7O_7$

618    450.1737 (MH)$^+$ found 450.1730.

619

620    *Commercial ADPR Analogues*

621     2′-phospho-ADPR (**15**), 1,N[6]-etheno-ADPR (**12**), and 8-(4-Chlorophenylthio)adenosine-5′-

622     mono-phosphate (8-pCPT-AMP, **32**) were purchased from Biolog.

623     *Cell Culture*

624     Jurkat subclone JMP with high expression of CD3 was originally generated at University of

625     Erlangen, Medical Faculty, Erlangen, Germany. They were recently authenticated as Jurkat by

626     short tandem repeats (STR) profiling and tested negative for contamination with rodent cells

627     (DSMZ service for the authentication of human cell lines). Jurkat cells were cultured as

628     described before[45]. Briefly, cells were kept in RPMI-1640 with GlutaMax-I and 25 mM 4-(2-

629     hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) supplemented with 7.5% newborn

630     calf serum (FCS) and penicillin (100 units/mL)/streptomycin (100 µg/mL). Cell density was

631     kept between $0.3 \times 10^6$ and $1.0 \times 10^6$/ml. For determination of endogenous concentrations of 2′-

632     deoxy-ADPR which required large amounts of cells, Jurkat cells were cultivated in spinner

633     bottles at a cell density of up to $1.0 \times 10^6$ cells/mL.

634     HEK293 Tet-On cells were obtained from Clontech/Takara. Wild type HEK293 cells were

635     kindly provided by Prof. Dr. Manfred Jücker (Department of Biochemistry and Signal

636     Transduction, University Medical Centre Hamburg-Eppendorf, Germany). HEK293 cells

637     were kept in DMEM medium with 4.5 g/L D-glucose and without pyruvate supplemented

638     with 10% fetal calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. For

639     maintenance of HEK293 cell lines TRPM2#24 and EGFP#8 400 µg/mL G418-sulfate was

640     added to the medium. For the maintenance of HEK293 Tet-On cell line expressing the

641     TRPM2 T5L mutant medium was supplemented with 100 µg/mL G418 sulfate and 25 µg/mL

642     hygromycin B. All cells were kept at 37°C and 5% $CO_2$. HEK293 cells are a well-established

643     system for the electrophysiological characterization of recombinant ion channels. They

644     express very little endogenous ion channels that might confound measurements and patch

645     clamp experiments are straightforward since cells tend to seal very well. To avoid the risk of

646 using cross-contaminated cells the parental wild type HEK293 cells and the HEK293 Tet-On

647 cell line expressing the T5L mutant of hTRPM2 were authenticated by STR profiling and

648 tested negative for contamination with rodent cells (DSMZ service for the authentication of

649 human cell lines). All cells were tested for mycoplasma contamination on a regular basis

650 using an enzymatic assay (MycoAlert™ Mycoplasma Detection Kit, Lonza).

651 *Generation of HEK293 Cell Lines*

652 Generation of HEK293 cells with stable expression of hTRPM2 and EGFP (TRPM2 #24) has

653 been described before[13]. The complete open reading frame of wild type hTRPM2 was

654 amplified from the expression vector pIRES2-EGFP-TRPM2 described previously[45] using the

655 primers 5′-GGAATTCCGCCACCATGGAGCCCTCAGCCCTG-3′ and 5′-

656 TACTGTCGACTCAGTAGTGAGCCCC-3′. *Eco*RI and *Sal*I sites in the primers allowed for

657 integration of the amplicon into the respective sites of the multiple cloning site of pTRE-tight

658 (Clontech/Takara). The mutation T5L described by Tóth and coworkers[20] was introduced by

659 QuikChange mutagenesis (Stratagene/Agilent) using the primers 5′-

660 GGCCAGATCCCGCTGGACGAGATCGACGGTGTGAACTTC-3′ and 5′-

661 GAAGTTCACACCGTCGACTCGTCCAGCCGGGATCTGGCC-3′. The complete open

662 reading frame was confirmed by DNA sequencing (MWG Eurofins). HEK 293 Tet-On cells

663 (Clontech/Takara) were co-transfected with pTRE-tight-TRPM2 T5L and pTK-Hyg

664 (Clontech/Takara). Transfectants were selected by adding 25 µg/mL hygromycin B to the

665 culture medium. Clonal cell lines for further analysis were generated from surviving cells by

666 limiting dilution and chosen according to their response to hydrogen peroxide as described

667 before[13]. While two clones showed a calcium signal in response to hydrogen peroxide, neither

668 was inducible by doxycycline. Excised inside/out patches from both clones showed ADPR

669 inducible currents with a higher single channel conductance than wild type TRPM2 as was

670 expected for the T5L mutant.

671 *Whole Cell Patch Clamp Analysis of TRPM2 Activation*

672 Whole cell patch clamp experiments were performed as described previously[13,46]. Briefly,

673 HEK293 cells with stable expression of human TRPM2 were seeded to 35 mm dishes. The

674 day after medium was removed and replaced by bath solution (140 mM N-methyl-D-

675 glucamine (NMDG), 5 mM KCl, 3.3 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM D-glucose, 10 mM

676 HEPES, pH 7.4 adjusted with HCl). Patch pipettes with a resistance of 1.5 to 3.5 MΩ were

677 pulled from thin-walled 1.5 mm diameter borosilicate (1.10 mm x 1.50 mm x 80 mm) glass

678 capillaries and filled with pipette solution (120 mM KCl, 8 mM NaCl, 1 mM $MgCl_2$, 10 mM

679 HEPES, 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA),

680 the concentration of free $Ca^{2+}$ ions in the pipette solution was buffered to 50 nM or 200 nM

681 by addition of either 2.3 mM or 5.6 mM $CaCl_2$ as calculated by Maxchelator:

682 http://maxchelator.stanford.edu/CaEGTA-NIST.htm). Currents were compensated for fast and

683 slow capacity transients and recorded using an EPC10 amplifier and PatchMaster software

684 (HEKA Elektronik). Series resistance was compensated by 70%. Voltage ramps from −85 mV

685 to +20 mV in 140 ms were applied every 5 s over a period of 450 s starting from a holding

686 potential of −50 mV. For analysis the maximum outward current at +15 mV during the course

687 of experiment was extracted from all experiments. In the NMDG-based bath solution the I-V

688 curve of TRPM2 showed characteristic reversal potential and outward rectification

689 (Supplementary Results, Supplementary Fig. 5a), facilitating differentiation between

690 activation of the non-selective cation channel and leaky cells/patches. If the patch ruptured

691 before peak current was obtained or if significant changes in series resistance occurred during

692 the measurement, recordings were excluded from analysis.

693 Jurkat cells were seeded to 35 mm dishes coated with poly-L-Lysine (MW 70–150kDa,

694 Sigma Aldrich). After cells had adhered to the dish, medium was removed and replaced by

695 bath solution (140 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES, and 5

696     mM glucose adjusted to pH 7.4 with NaOH). Pipettes were prepared as described above and

697     filled with pipette solution (140 mM KCl, 2 mM MgCl$_2$, 2.5 mM EGTA, and 10 mM HEPES

698     adjusted to pH 7.4 with KOH, the concentration of free Ca$^{2+}$ ions in the solution was buffered

699     to 50 nM by addition of 0.59 mM CaCl$_2$ as calculated by MaxChelator). Currents were

700     recorded as described above. Voltage ramps from –85 mV to +65 mV in 200 ms were applied

701     every 10 s over a period of 300 s starting from a holding potential of -60 mV.

702     *Electrophysiological Recordings from Excised Inside/Out-Patches*

703     Cells with stable expression of either wild type hTRPM2 or hTRPM2 T5L were seeded to

704     35 mm dishes 24–48 h before use. Before the experiments medium was exchanged for a

705     buffer based on sodium gluconate (140 mmol/L Na gluconate, 2 mmol/L MgCl$_2$, 7.6 mmol/L

706     CaCl$_2$, 10 mmol/L EGTA, 10 mM HEPES pH7.2, free Ca$^{2+}$ concentration 1 μmol/L). Patch

707     pipettes with resistances of 10–15 MΩ were pulled from thick-walled borosilicate glass

708     capillaries (0.86 mm x 1.50 mm x 80 mm) using a Sutter P-97 and filled with the same

709     solution (symmetrical solutions). 100 nmol/L Tetrodotoxin (TTX) was added to the pipette

710     solution to block endogenous voltage-gated sodium channels[47]. For activation of the channel

711     either ADPR or 2′-deoxy-ADPR was added to the bath solution. After excision of a patch

712     with one or a few channels the fast capacity transients were compensated. For the

713     determination of the average open probability the patch membrane was held at a potential of –

714     60 mV and the current was continuously recorded for a maximum of 30 min. For the

715     determination of the slope conductance the potential across the patch membrane was

716     increased by 20 mV steps every 20 s starting from –60 mV with a delay of 100 ms between

717     steps. Data were sampled at 10 kHz and recorded to disk using the EPC10 amplifier and

718     PatchMaster software (v2.32, HEKA Elektronik). For analysis of the single channel data

719     recordings were filtered to remove 50 Hz mains hum by an electrical interference filter and

720     low-pass-filtered (1 kHz) to remove high-frequency noise. To determine single channel

721 conductance Gaussian distributions were fitted to current histograms of the recordings and

722 voltage steps were calculated as difference between the centers of the distributions. To

723 determine average open probabilities continuous recordings were filtered and base-line

724 corrected and exported to a script for trapezoid integration of the area under the curve written

725 in Python (www.python.org, v2.7) and using the numpy/scipy libraries (www.scipy.org ,

726 numpy v1.8.1 and scipy v0.12.0) for numerical computations. At points of permanent

727 inactivation of channels the recording was split into sections and the sections were separately

728 integrated. The resulting integrals were divided by number of channels, duration and average

729 height of voltage steps at –60 mV. The number of simultaneously open channels in a patch

730 (Fig 3h) was determined either from the steps in the recording or for recordings where

731 identification of individual states was not possible the number of channels was calculated by

732 dividing peak current by single channel current for –60 mV. For display purposes (Fig 3a+d)

733 data was filtered with a 100 Hz low pass filter and down-sampled by a factor of 10.

734 *Production of Recombinant Human CD38*

735 A fragment of the open reading frame of human CD38 encoding amino acids P44 to I300 was

736 amplified using the primers 5′-

737 ATGGTACGTCTCAGGCCCCGAGGTGGCGCCAGCAGTG-3′ and 5′-

738 ATGGTACGTCTCAGCGCTGATCTCAGATGTGCAAGATGAATC-3′. The amplicon was

739 digested with *Esp*3I and inserted into the *Bsa*I site of the multiple cloning site of the

740 eukaryotic expression vector pEXPR-iba42 (Iba). The resulting reading frame encodes an *N*-

741 terminal BM40 signal sequence followed by a 6xHis-Tag, the extracellular part of CD38 and

742 a *C*-terminal Strep-Tag. This open reading frame was confirmed by DNA sequencing (MWG

743 Eurofins). HEK293 cells were transfected with the expression vector and transfectants were

744 selected by addition of 400 µg/mL G418-sulfate to the culture medium. The bulk of surviving

745 cells was continuously kept as described above and the supernatant medium was collected

746 each time cells were sub-cultured. Soluble recombinant CD38 secreted by the cells was

747 purified from the pooled supernatant by affinity chromatography using Ni-NTA agarose

748 (Qiagen). Imidazole was removed from elution fractions by dialysis and the protein solution

749 concentrated by ultrafiltration.

750 *Enzymatic Synthesis of 2'-deoxy-NAD*

751 5 mmol/L β-NMN and 5 mmol/L 2′-deoxy ATP were incubated at 37°C with 1 μg/mL

752 recombinant human NMNAT-2 (R&D Systems) overnight. The enzyme was removed

753 afterwards by passing the sample through a centrifugal filter device with 10 kDa cutoff. The

754 sample volume was reduced using a SpeedVac evaporator. Separation of the product 2'-

755 deoxy-NAD from byproducts and remaining substrates was achieved by HPLC using a

756 250 mm×10 mm Luna C8 5 µm column (Phenomenex) equipped with a 10 mm×10 mm guard

757 cartridge containing a C8 ODS filter element (Phenomenex) at a flow rate of 2.5 mL/min with

758 the buffer (50 mmol/L ammonium acetate, 0.05% acetic acid, pH 5.4) containing increasing

759 amounts of methanol as outlined below. The fraction co-eluting with chemically synthesized

760 2'-deoxy-NAD was collected, dried using a SpeedVac evaporator, reconstituted in water and

761 checked for purity by HPLC as outlined below. Concentration of the solution was adjusted

762 against chemically synthesized 2'-deoxy-NAD.

763 *HPLC-based Enzyme Assays for NMNAT-2 and CD38*

764 Recombinant human NMNAT-2 (R&D Systems) at a concentration of 5 ng/μL was incubated

765 for 15 min at 37°C with 290μmol/L β-NMN and 2.5 mmol/L 2′-deoxy-ATP in assay buffer

766 (50 mmol/L Tris, 10 mmol/L $MgCl_2$, pH 7.6). Afterwards the enzyme was removed by

767 passing the samples through centrifugal filter devices with 10 kDa cutoff. Reaction products

768 were analyzed via reversed-phase ion pair (RP)-HPLC on a Multohyp BDS-C18 5μ column

769 (250 mm x 4.6 mm, particle size 5 μm, CS Chromatographie Service) equipped with a

770 security guard cartridge (4 mm x 3.0 mm) containing a C18 ODS filter element (Phenomenex)

771  at a flow rate of 0.8 mL/min with the buffer (20 mmol/L KH$_2$PO$_4$, 5 mmol/L

772  tetrabutylammonium dihydrogen phosphate, pH 6) containing increasing amounts of

773  methanol. The methanol gradient was: 0 min (15%), 3.5 min (15%), 11 min (31.25%), 15 min

774  (31.25%), 25 min (50%), 27 min (50%), 29 min (15%) and 38 min (15%). The DAD (Diode-

775  Array detector, Agilent Technologies) was set to 260 nm for detection of nucleotides. Peak

776  integration was performed using ChemStation Software (Rev. C.01.05, Agilent

777  Technologies).

778  Peaks co-eluting with chemically synthesized 2′-deoxy-NAD were collected and methanol

779  was removed on a SpeedVac evaporator. Afterwards pH was adjusted to 7.2 and the MgCl$_2$

780  concentration to 1 mmol/L. Part of the sample was incubated for 1 h at 37°C with

781  0.33 Units/mL nucleotide pyrophosphatase from *Crotalus adamanteus*. The remainder of the

782  sample was incubated with a recombinant soluble form of human CD38 for 15 min at 37°C.

783  The putative 2′-deoxy-ADPR peak from the CD38 reaction was collected for pyrophosphatase

784  digest. In all cases the products were analyzed via reversed-phase ion pair HPLC as described

785  above.

786  *Substrate saturation plots*

787  To create substrate saturation plots of human NMNAT-2 for the substrates ATP and 2'-deoxy-

788  ATP, 30 ng recombinant NMNAT-2 (R&D Systems) was incubated in 100 µl reaction buffer

789  (25 mmol/L KH$_2$PO$_4$, 5 mmol/L DTT, 20 mmol/L MgCl$_2$, 0.5 mg/ml bovine serum albumin

790  (BSA), adjusted to pH 7.5 with KOH) with different concentrations of the substrates (ATP, 2′-

791  deoxy-ATP). For soluble recombinant human CD38, 0.5 ng or 1.0 ng of the enzyme

792  (produced as described above) was incubated in 100 µl reaction buffer (110 mmol/L KCl,

793  5 mmol/L KH$_2$PO$_4$, 10 mmol/L NaCl, 2 mmol/L MgCl$_2$, 20 mmol/L HEPES, pH 7.2 mit

794  KOH) with different concentrations of either NAD or 2'-deoxy-NAD as substrate for 20 min

795    at 37°C. Reactions were stopped by cooling the samples rapidly to nearly 0°C and removing

796    the enzyme by centrifugation through a 10 kDa filter device (VivaSpin, Sartorius) at 4°C. The

797    amount of product formed was determined by HPLC analysis as described above using

798    external standards for quantification. In case of CD38 substrate solutions contained minor

799    contaminations of either ADPR / 2'-deoxy-ADPR that were substracted before kinetic

800    parameters for the enzyme/substrate pairs were calculated using GraphPad Prism (v6.03,

801    GraphPad Software).

802    *Immunoprecipitation and western blot analysis of poly-ADP-ribosylated proteins*

803    HEK-293 cells were seeded to 150 mm dishes. Four days later at 80-90% confluence medium

804    was replaced by pre-warmed buffer (15 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCl,

805    1 mmol/L $MgCl_2$, 10 mmol/L Glucose, 1.8 mmol/L $CaCl_2$, pH 7.4) and hydrogen peroxide

806    was added to a final concentration of 1 mmol/L. After 5 min buffer was replaced by pre-

807    warmed phosphate buffered saline (137.93 mmol/L NaCl, 2.67 mmol/L KCl, 8.06 mmol/L

808    $Na_2HPO_4$, 1.47 mmol/L $KH_2PO_4$, 0.493 mmol/L $MgCl_2$, 0.901 mmol/L $CaCl_2$, pH 7.38) and

809    cells were washed twice. Lysis of cells and isolation of poly ADP-ribosylated proteins was

810    based on Gagné et al[48]. Dishes were put on ice and 2 mL lysis buffer (40 mmol/L HEPES,

811    120 mmol/L NaCl, 1 mmol/L EDTA, 0.3% CHAPS (w/v), pH 7.5) with protease inhibitor

812    (Roche complete, EDTA-free) and PARG inhibitor ADP-HPD (Enzo Life Sciences) was

813    added to the cells which then were detached from the dishes and kept on ice for 15 min and on

814    overhead rotation at 4°C for an additional 20 min. Non-lysed cells were removed by

815    centrifugation. Paramagnetic beads were washed twice with sodium acetate buffer (0.1 mol/L

816    Na acetate, pH 5.0) and incubated 30 min at 4°C with either anti-pADPR antibody (10H,

817    Abcam # ab14459) or mouse normal IgG (Thermo Fisher Scientific) in PBS-T (PBS, 0.02%

818    Tween20 (v/v)) on overhead rotation. Non-specific binding was prevented by incubation with

819    blocking buffer for 1 h at room temperature. Directly before addition to the lysed cells beads

820 were washed with lysis buffer with protease inhibitor. Beads were incubated with the cell

821 lysate for 2 h at 4°C on overhead rotation and washed five times with lysis buffer. For

822 Western blot analysis poly-ADP-ribosylated proteins were eluted from the beads by

823 incubation in sample buffer (0.22 mol/L Tris, 22.6% (v/v) glycerol, 4% (w/v) SDS, 5.3%

824 (v/v) 2-mercapto ethanol, pH6.8) at 65°C for 5 min. Afterwards proteins were separated by

825 10% SDS-PAGE and transferred to a PVDF membrane (Merck, Darmstadt) by tank blotting.

826 The membrane was blocked in TBS-MT (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.6, 0.1%

827 Tween20 (v/v), 5% dry milk protein(w/v)) and incubated overnight at 4°C with the primary

828 anti-PAR antibody (pADPR antibody from rabbit, R&D Systems/Bio-Techne #4336-APC-

829 050) diluted 1:1000 in TBS-MT. The secondary goat anti-rabbit antibody conjugated to

830 horseradish peroxidase (HRP) (Dianova #111-035-045) was applied in 1:10 000 dilution in

831 TBS-MT. HRP was detected by chemoluminescence using an Image Quant

832 LAS4000/LAS3000 (GE Healthcare Life Sciences) after incubation of the membrane in

833 SuperSignal Working Solution (Thermo Fisher Scientific) for 5 min.

834 *PARG hydrolysis of poly-ADP-ribosylated proteins and HPLC analysis of products*

835 Beads with poly-ADP ribosylated proteins were washed in phosphate buffer (20 mmol/L

836 $KH_2PO_4$, pH 7.2), resuspended in 100 μl phosphate buffer + 10 mmol/L $MgCl_2$ and after

837 addition of 100 ng recombinant human PARG (Adipogen International) incubated for 2.5 h at

838 37°C. After removal of beads and PARG by passing the samples through centrifugal filter

839 devices with 10kD cutoff, 1 μL citric acid, 1 μL chloroacetaldehyde and 33 μL water were

840 added to 85 μL of the sample and incubated for 40 min at 80°C resulting in conversion of the

841 adenine nucleotides to the respective fluorescent 1, $N^6$-etheno compounds. Analysis of the

842 converted nucleotides was based on the method described by Bobalova et al.[49] HPLC analysis

843 was performed on either a 1200 Series system or a 1260 Infinity system (both Agilent

844 Technologies) using a 250 mm×4.6 mm Multohyp BDS-C18 5μ column (CS

845    Chromatographie Service) equipped with a 4.0mm×3.0mm guard cartridge containing a C18

846    ODS filter element (Phenomenex) at a flow rate of 0.8 mL/min with the buffer (100 mmol/L

847    $KH_2PO_4$, pH 6) containing increasing amounts of methanol. The gradient was: 0 min (0%

848    methanol), 22.5 min (35% methanol), 25 min (35% methanol), 29 min (0% methanol) and

849    38 min (0% methanol). Fluorescence of 1, $N^6$-ethenoadenosine was detected at an emission

850    wavelength of 410 nm after excitation at 230 nm. Peaks were integrated using ChemStation

851    Software (Rev. C.01.05) from Agilent Technologies. Quantification was performed using

852    external standards.

853    *Assay for type III CD38 activity using permeabilized cells*

854    $10^6$ wildtype Jurkat cells or CD38$^{-/-}$ Jurkat cells were washed, resuspended in 200 μl reaction

855    buffer (140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $NaH_2PO_4$, 1 mmol/L $MgSO_4$, 1 mmol/L

856    $CaCl_2$, 5.5 mmol/L Glucose, 20 mmol/L HEPES, pH7.4 with NaOH) and incubated with 1,

857    $N^6$-etheno-NAD at a final concentration of 50 µmol/L. Reaction was stopped after 0, 5, 10, 15

858    and 30 min by spinning down the cells and passing the supernatant through a centrifugal filter

859    device with 10kD cutoff. Samples were analysed by HPLC using fluorescence detection as

860    described below, to determine the amount of 1, $N^6$-etheno-ADPR formed. Reaction rates were

861    calculated from the increase in product by linear regression. To block the activity of CD38 on

862    the cell surface, cells were incubated in 1 µmol/L araF-NAD in reaction buffer for either

863    40 min or 90 min at RT. Excess araF-NAD was removed by washing the cells in reaction

864    buffer prior to the determination of the CD38 activity. To selectively permeabilize the plasma

865    membrane $10^6$ cells were washed once in intracellular buffer (120 mmol/L KCl, 10 mmol/L

866    NaCl, 1.2 mmol/L $MgCl_2$, 0.533 mmol/L $CaCl_2$, 10 mmol/L HEPES, 1 mmol/L EGTA, pH7.2

867    with KOH) and resuspended in intracellular buffer with 90 µg/mL saponin. After incubation

868    at 37°C for 5 min cells were washed in nominally $Ca^{2+}$ free intracellular buffer (120 mmol/L

869    KCl, 10 mmol/L NaCl, 1.2 mmol/L $MgCl_2$, 10 mmol/L HEPES, pH7.2 with KOH) twice

870      before CD38 activity was determined using 1, $N^6$-etheno-NAD as described above. To test

871      whether residual activity was due to CD38, permeabilized cells were also incubated with

872      1 µmol/L araF-NAD for 40 min at RT. Excess araF-NAD was removed by washing the cells

873      prior to the determination of the CD38 activity as described above.

874

875      *Quantification of endogenous 2'-deoxy-ADPR by HPLC*

876      $1 \times 10^8$ Jurkat cells at a cell density of $1 \times 10^6$ cells/mL were harvested from suspension, washed

877      twice in $Ca^{2+}$ measurement buffer (140 mmol/L, NaCl, 5 mmol/L KCl, 1 mmol/L $MgSO_4$,

878      1 mmol/L $CaCl_2$, 1 mmol/L $NaH_2PO_4$, 5.5 mmol/L D-glucose and 20 mmol/L HEPES, pH

879      7.4) and resuspended in 5 mL of the same buffer. After 25 min at 25°C hydrogen peroxide

880      was added to a final concentration of 100 µmol/L. An additional 2 or 5 minutes later cells

881      were collected by centrifugation and nucleotides extracted from the cells by addition of

882      trichloroacetic acid, freeze/thawing and sonication. Denatured proteins and nucleic acids were

883      removed by centrifugation and 25 pmol 1, $N^6$-ethenoadenosine was added to the supernatant

884      as internal standard. Trichloroacetic acid was removed by four cycles of extraction with

885      diethyl ether. To remove residual diethyl ether samples were dried using a SpeedVac

886      evaporator and reconstituted in water. After samples were filtered through 0.2 µm syringe

887      filter devices, samples were again dried and reconstituted in 15% methanol/water. HPLC

888      analysis was performed on either a 1200 Series system or a 1260 Infinity system (both Agilent

889      Technologies). The first step of chromatographic separation was run on a 250 mm×10 mm

890      Luna C8 5 µm column (Phenomenex) equipped with a 10 mm×10 mm guard cartridge

891      containing a C8 ODS filter element (Phenomenex) at a flow rate of 2.5 mL/min with the

892      buffer (20 mmol/L $KH_2PO_4$, 5 mmol/L tetrabutylammonium dihydrogen phosphate, pH 6)

893      containing increasing amounts of methanol. The gradient was: 0 min (15% methanol),

894      3.5 min (15% methanol), 11 min (31.25% methanol), 15 min (31.25% methanol), 25 min

895     (50% methanol), 27 min (50% methanol) , and 29 min (15% methanol). Adenine nucleotides

896     were detected using the DAD (photo diode array detector) at 260 nm. Fluorescence of 1, $N^6$-

897     ethenoadenosine was detected at an emission wavelength of 410 nm after excitation at

898     275 nm. Two fractions of roughly 2.5 mL were manually collected around the retention times

899     of 1, $N^6$-ethenoadenosine ($R_t$ 13.5 min) and 2′-deoxy-ADPR ($R_t$ 24.5 min). Fractions were

900     dried on a SpeedVac evaporator and reconstituted in 15% methanol/water. After

901     reconstitution samples were split into twin samples. For the 2′-deoxy-ADPR fractions one of

902     the samples was spiked with 15.625 pmol 2′-deoxy-ADPR. The second step of

903     chromatographic analysis was run on a 250 mm×4.6 mm Multohyp BDS-C18 5µ column (CS

904     Chromatographie Service) equipped with a 4.0 mm×3.0 mm guard cartridge containing a C18

905     ODS filter element (Phenomenex) at a flow rate of 0.8 mL/min using the same buffer system

906     and detection as above. The gradient was: 0 min (15% methanol), 3.5 min (15% methanol),

907     11 min (31.25% methanol), 15 min (31.25% methanol), 25 min (50% methanol), 27 min

908     (50% methanol) , and 29 min (15% methanol). Peaks were integrated using ChemStation

909     Software (Rev. C.01.05) from Agilent Technologies. Samples with peaks unsufficiently

910     resolved for proper integration were excluded. Quantification was performed using external

911     standards. Recovery was determined from the internal standard 1,$N^6$-ethenoadenosine. Results

912     for 2′-deoxy-ADPR were corrected for recovery.

913

914     *HPLC of endogenous 2'-deoxy-ADPR and 2'-deoxy-NAD for HRMS and quantification of*

915     *endogenous 2'-deoxy-NAD, NAD and ADPR*

916     $1\times10^8$ wild type Jurkat cells or CD38$^{-/-}$ Jurkat cells per sample were prepared as described

917     above and either left unstimulated or stimulated for 5 min with 100 µmol/L hydrogen

918     peroxide. After deproteination and extraction of the trichloroacetic acid by diethyl ether,

919     samples were dried on a SpeedVac evaporator to remove residual diethyl ether and

920  reconstituted in water. After samples were filtered through 0.2 μm syringe filter devices,

921  samples were again dried on a SpeedVac evaporator and reconstituted in 3% methanol/water.

922  HPLC analysis was performed on either a 1200 Series system or a 1260 Infinity system (both

923  Agilent Technologies). The first step of chromatographic separation was run on a

924  250 mm×10 mm Luna C8 5 μm column (Phenomenex) equipped with a 10 mm×10 mm guard

925  cartridge containing a C8 ODS filter element (Phenomenex) at a column temperature of 20°C

926  and a flow rate of 2.5 mL/min with the buffer (50 mmol/L ammonium acetate, 0.05% acetic

927  acid, pH 5.4) containing increasing amounts of methanol. The gradient was: 0 min

928  (3% methanol), 5 min (3% methanol), 35 min (50% methanol), 38 min (50% methanol), 45

929  min (3% methanol), and 50 min (3% methanol). Adenine nucleotides were detected as

930  described above. Five fractions of roughly 2.5 mL were manually collected around the

931  retention times of ADPR ($R_t$ 8.2 min), NAD ($R_t$ 12.9 min), 2′-deoxy-ADPR ($R_t$ 13.9 min), 2′-

932  deoxy-NAD ($R_t$ 16.5 min), and 1, $N^6$-ethenoadenosine ($R_t$ 24.5 min). All fractions were dried

933  on a SpeedVac evaporator. The fractions containing ADPR, NAD and 1, $N^6$-ethenoadenosine

934  were reconstituted in buffer (20 mmol/L $KH_2PO_4$, 5 mmol/L tetrabutylammonium dihydrogen

935  phosphate, pH 6 containing 15% methanol) whereas the fractions containing 2′-deoxy-ADPR

936  and 2′-deoxy-NAD were reconstituted in 3% methanol/water. After reconstitution all samples

937  except the ones for 1, $N^6$-ethenoadenosine were split into twin samples. One half of the twin

938  sample was spiked with an appropriate amount of the respective nucleotide (ADPR with 100

939  or 250 pmol, NAD with 5 nmol, 2′-deoxy-ADPR with 62.5 pmol and 2′-deoxy-NAD with

940  15.63 pmol). The samples with ADPR, NAD, and 1, $N^6$-ethenoadenosine were run on a

941  Multohyp BDS-C18 5μ column using the same (ion pair) conditions as described above. For

942  the samples with 2′-deoxy-ADPR and 2′-deoxy-NAD which were to be submitted to HRMS

943  the second step of chromatographic analysis was run on a 250 mm×4.6 mm Multohyp BDS-

944  C18 5μ column (CS Chromatographie Service) equipped with a 4.0 mm×3.0 mm guard

945 cartridge containing a C18 ODS filter element (Phenomenex) at a column temperature of

946 25°C and a flow rate of 0.8 mL/min with the buffer (50 mmol/L ammonium acetate, 0.05%

947 acetic acid, pH 5.4) containing increasing amounts of methanol. The gradient was: 0 min

948 (3% methanol), 7 min (3% methanol), 32 min (50% methanol), 35 min (50% methanol), 41

949 min (3% methanol), and 45 min (3% methanol). Adenine nucleotides were detected as

950 described above. Separate fractions of roughly 500 µL were manually collected for 2′-deoxy-

951 ADPR ($R_t$ 10.85 min) and 2′-deoxy-NAD ($R_t$ 17.2 min) around the retention times of the

952 respective standards. Fractions were dried on a SpeedVac evaporator and submitted to

953 HRMS. Quantification using external standards was done as described above. Recovery was

954 determined from the internal standard 1, $N^6$-ethenoadenosine. Since recovery was always

955 around 100% results were not corrected during these experiments.

956 *High resolution mass spectrometry(HRMS)*

957 HRMS was carried out using ultrahigh resolution ESI-QTOF on a maXis HD Bruker

958 Daltonics instrument in negative mode with direct injection (50 µL). Spectra were acquired in

959 full scan mode in the mass range 100 – 750 m/z and the baseline subtracted (MilliQ water).

960 *Generation of CD38$^{-/-}$ Jurkat cell line using CRISPR/Cas9*

961 Knock-out of CD38 in Jurkat subclone JMP was performed by CRISPR/Cas9 using the

962 expression plasmids pX330-Puro-T2A-hCas9 and pCAG-EGxxFP, which were kindly

963 provided by Prof. Dr. Alexander Flügel (Department of Neuroimmunology, University

964 Medical Center Göttingen, Göttingen, Germany). The sgRNA sequence

965 (CCACCGCGAGCACCACGACG) was designed with the GenScript gRNA design tool

966 (http://www.genscript.com/gRNA-design-tool.html). The adapted expression plasmids were

967 co-transfected into Jurkat cells by electroporation. EGFP positive cells from the transfection

968 were sorted and individualized by FACS (FACS Sorting Core Unit, University Medical

969      Centre Hamburg-Eppendorf, Germany). Resulting Jurkat clones were characterized regarding

970      CD38 protein expression and NAD glycohydrolase activity.

971      *Preparation of membrane fractions from Jurkat cells*

972      Membrane fractions from Jurkat cells were prepared as described previously[50,51]. Briefly,

973      either $5 \times 10^7$ wild type Jurkat cells or CD38$^{-/-}$ Jurkat cells were suspended in lysis buffer

974      (110 mmol/L NaCl, 20 mmol/L HEPES, pH 7.4) with protease inhibitor mix (Roche) and

975      disrupted using a dounce homogenizer. After removal of undisrupted cells and intact nuclei,

976      membranes were enriched by centrifugation at 10 000xg. The membrane pellet was

977      resuspended in lysis buffer and protein content determined by Bradford protein assay (Bio-

978      Rad) against BSA as standard.

979      *Assay of NAD glycohydrolase activity*

980      NAD glycohydrolase activity in P10 membranes from wild type Jurkat cells or CD38$^{-/-}$ Jurkat

981      cells was determined as described previously[50,51]. Briefly, 20 µg of P10 membrane proteins

982      from wild type Jurkat cells or CD38$^{-/-}$ Jurkat cells were incubated with 1, $N^6$-etheno-NAD

983      (100 µmol/L) in buffer (140 mmol/L, NaCl, 5 mmol/L KCl, 1 mmol/L MgSO$_4$, 1 mmol/L

984      CaCl$_2$, 1 mmol/L NaH$_2$PO$_4$, 5.5 mmol/L D-glucose and 20 mmol/L HEPES, pH 7.4). In the

985      course of the reaction fluorescence is increasing due to the higher fluorescence of the product

986      1, $N^6$-etheno-ADPR compared to the substrate. The increase in fluorescence was followed

987      (excitation at 300 nm, emission at 410 nm) using an Infinite M200 micro plate reader (Tecan).

988      The amount of product formed was calculated from the fluorescence readings using a 1, $N^6$-

989      etheno-ADPR standard curve.

990      *Western Blot analysis of CD38 activity*

991      CD38 expression in Jurkat cells was analyzed as described recently[50,51]. Briefly 60 µg of P10

992      membrane proteins from wild type and CD38$^{-/-}$ Jurkat cells were separated on a 12% non-

993 reducing SDS-PAGE (0.4% SDS) and subsequently transferred to a PVDF membrane

994 (Immobilon-FL, Millipore). The membrane was probed with a primary antibody against

995 human CD38 (1:200, mouse monoclonal antibody AT-1, Santa Cruz Biotechnology #sc-7325)

996 and a secondary goat anti-mouse antibody conjugated to horse radish peroxidase (1:10 000,

997 Santa Cruz Biotechnology #sc-2302). Incubation of the membrane in SuperSignal Working

998 Solution (Thermo Fisher Scientific) for 5 min allowed for detection of the secondary antibody

999 by chemiluminescence using an Image Quant LAS4000/LAS3000 (GE Healthcare Life

1000 Sciences).

1001 *Statistical Analysis*

1002 Statistical analysis was performed using GraphPad Prism (v 6.03, GraphPad Software).

1003 Quantitative data were tested for normality using D'Agostino-Pearson Omnibus Test

1004 ($\alpha$=0.05). If all groups were normally distributed, data is reported as mean±SEM (standard

1005 error of the mean) and parametric tests were used (unpaired, two-tailed T-test or one-way

1006 ANOVA). In case of non-normal distribution or if the sample size for groups was too small to

1007 test for normality, data is reported as median with interquartile range and non-parametric tests

1008 (Mann-Whitney test, Kruskal-Wallis test) were chosen. If non-normal data were to be

1009 compared against a hypothetical value (especially against 0) a Wilcoxon Signed Rank Test

1010 was used. Post-hoc tests were performed as Holm-Sidak or Dunn's test with p-values

1011 corrected for multiple testing.

1012 Linear and non-linear regression analysis was performed using GraphPad Prism (v 6.03,

1013 GraphPad Software). For fitting of concentration-response curves (Fig. 2) a four parameter

1014 logistic model has been chosen. Conditions were: bottom value constrained to 0 and Hill

1015 coefficient shared between data sets. For the analysis of channel inactivation (Fig. 3e+f) a

1016 histogram of the frequency distribution with a bin width of 5 s was generated and a one phase

1017 exponential decay function fitted to the data by non-linear regression. To test whether

1018   parameters of fit differ between conditions extra-sum-of-squares F tests were used. In case of

1019   the slope conductance (Fig. 3c) data were fitted to a linear equation (without intercept). To

1020   compare the slope conductance between channel variants and agonists the results from the

1021   linear regression (slope±SEM) were tested by multiple unpaired, two-tailed T-tests assuming

1022   normal distribution of the residuals and without correction for multiple testing.

1023   An a priori power analysis to determine sample sizes has not been done. For all statistical tests

1024   a significance level $\alpha$ of 0.05 was adopted.

1025

**Methods-only references**

1026     **Methods-only references**

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1048

1049    **Statement on image integrity**

1050    The authors confirm that the paper complies with Nature Publishing policy concerning image

1051    integrity.

1052    **Statement on competing financial interests**

1053    The authors declare that they do not have any competing financial interests.

1054    **Code Availabilty**

1055    Codes can be obtained upon request from the corresponding author (guse@uke.de).

1056    **Data Availability Statement**

1057    Any materials, associated protocols, and other supporting data may be obtained from the

1058    corresponding author (guse@uke.de).

1059

1060

**a  Adenine Modifications**

**b  Adenosine Ribose Modifications**
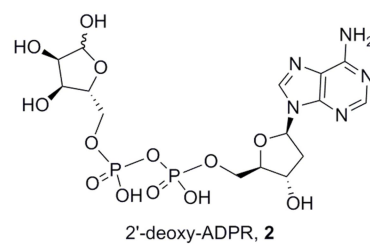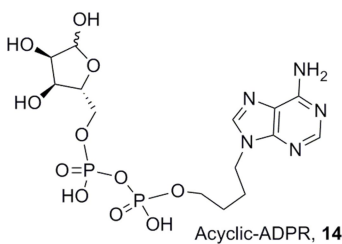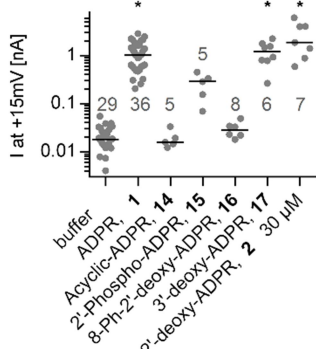
**c  Pyrophosphate Modifications**
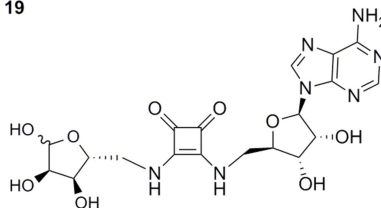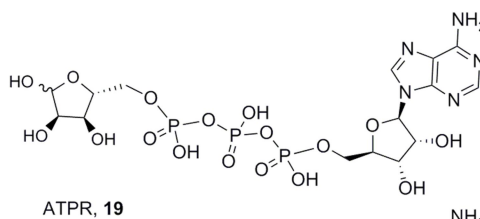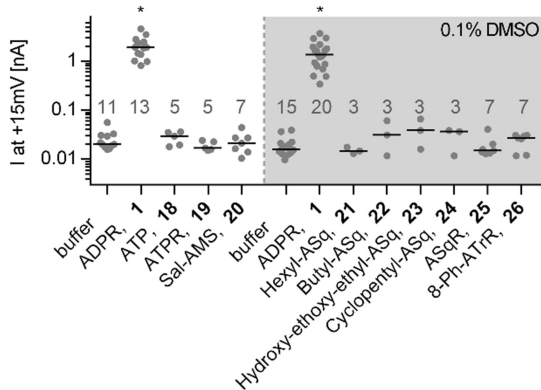
**d  Terminal Ribose Modifications**

ADPR

1,N⁶-etheno-ADPR, **12**

2-F-ADPR, **13**

Acyclic-ADPR, **14**
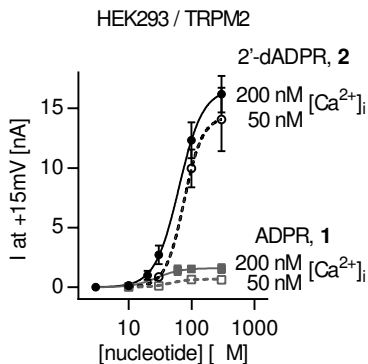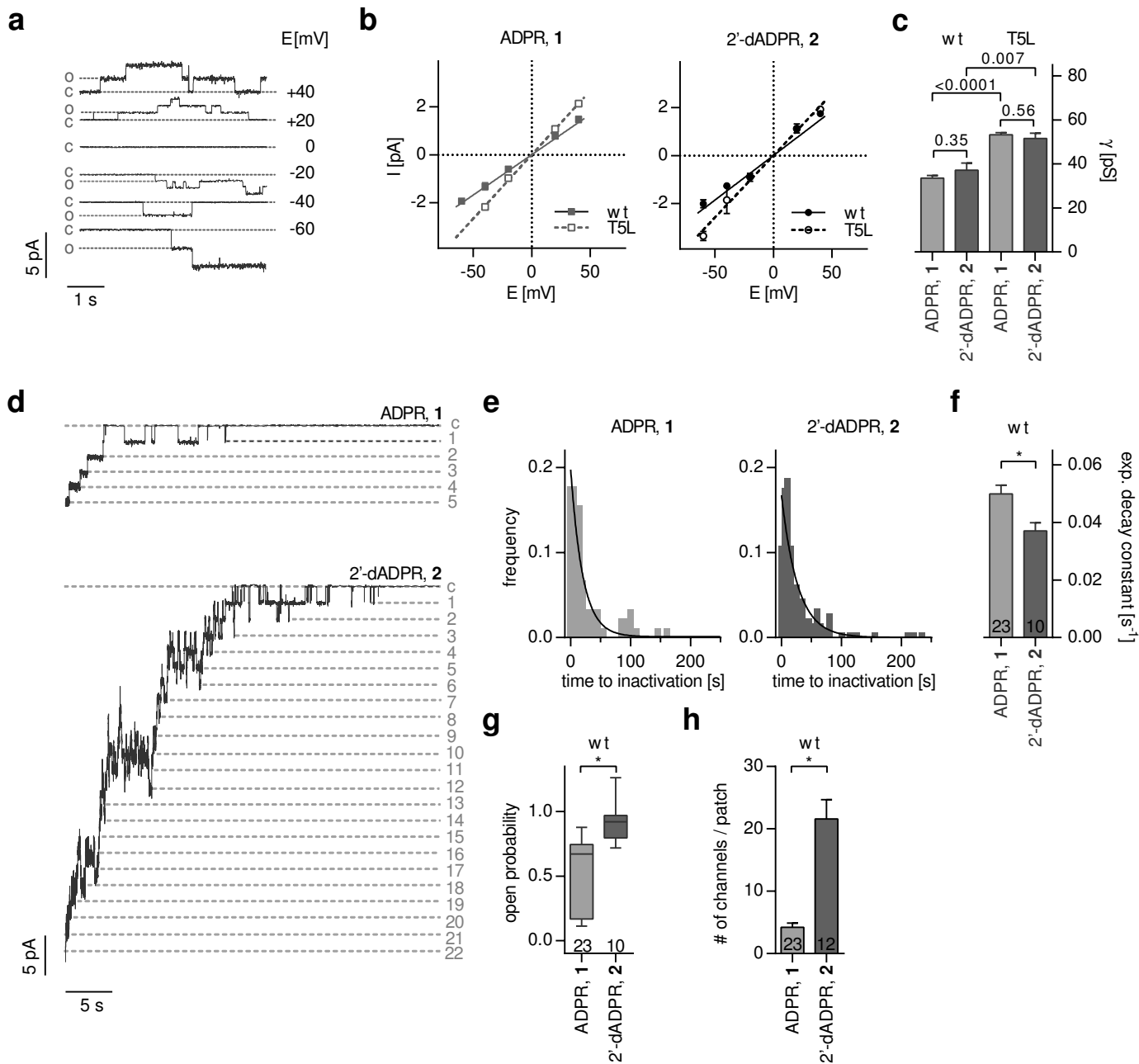
2'-deoxy-ADPR, **2**

ATPR, **19**

ASqR, **25**

2'-deoxy-AMP, **33**

Cyclopentyl-ADP, **34**
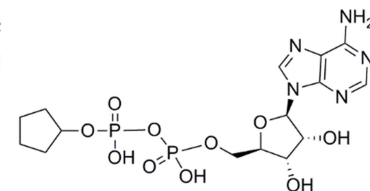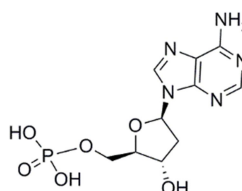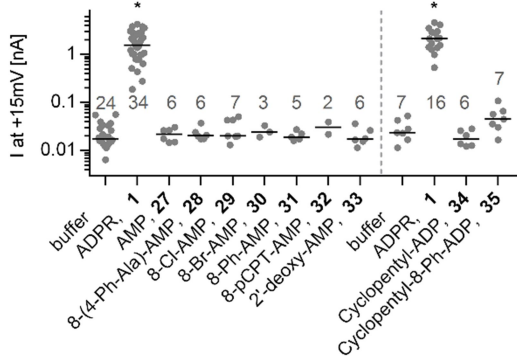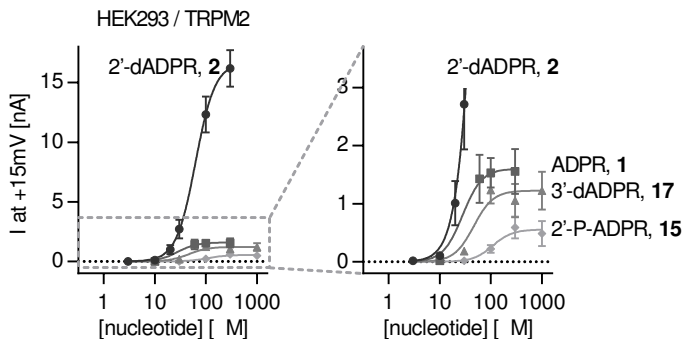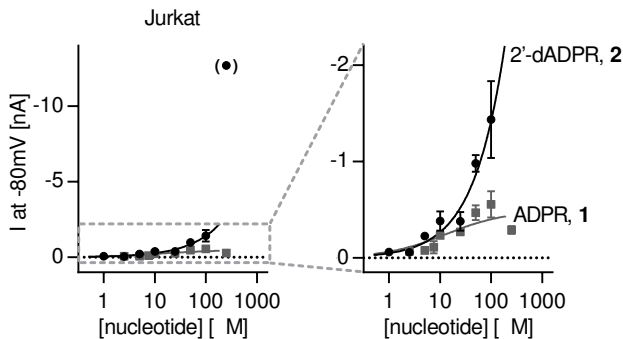
Figure 2

# Figure 3

Figure 4

Figure 5

# Figure 6



**a** unstim.

**b** H₂O₂

**c**

TRPM2

CD38

2'-dADPR    2'-dNAD

ADPR    PPi

NMNAT-2

CD38

β-NAD    β-NMN    2'-dATP

nucleus

2'-dADPR