



**HAL**  
open science

## Endogenous ADP-ribose enables calcium-regulated cation currents through TRPM2 channels in neutrophil granulocytes

Inka Heiner, Jörg Eisfeld, Maike Warnstedt, Natalia Radukina, Eberhard Jüngling, Andreas Lückhoff, Andreas Lückhoff

► **To cite this version:**

Inka Heiner, Jörg Eisfeld, Maike Warnstedt, Natalia Radukina, Eberhard Jüngling, et al.. Endogenous ADP-ribose enables calcium-regulated cation currents through TRPM2 channels in neutrophil granulocytes. *Biochemical Journal*, 2006, 398 (2), pp.225-232. 10.1042/BJ20060183 . hal-00478527

**HAL Id: hal-00478527**

**<https://hal.science/hal-00478527>**

Submitted on 30 Apr 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Endogenous ADP-ribose enables calcium-regulated cation currents through TRPM2 channels in neutrophil granulocytes**

**Inka Heiner, Jörg Eisfeld, Maike Warnstedt, Natalia Radukina, Eberhard Jüngling, Andreas Lückhoff**

**From the Institute of Physiology, Medical Faculty, RWTH Aachen, Pauwelsstr. 30, D-52057 Aachen, Germany**

Address correspondence: Andreas Lückhoff, Institut für Physiologie, Medizinische Fakultät der RWTH Aachen, Pauwelsstr. 30, D-52057 Aachen, phone +49-241-8088812, Fax +49-241-8082434; E-mail: luckhoff@physiology.rwth-aachen.de

**Short title:** ADP-ribose in neutrophils and Ca<sup>2+</sup>-dependent TRPM2 activation

**Keywords:** ADP-ribose cyclic ADPR-ribose TRPM2 calcium influx HPLC patch-clamp fMLP

**Abbreviations:** ADPR, ADP-ribose; cADPR, cyclic ADPR; fMLP, N-formyl-methionyl-leucyl-phenylalanine; FMN, riboflavin-5'-monophosphate; NAD, nicotinamide dinucleotide; NMDG, N-methyl-D-glucamine; PARP, Poly(ADPR) polymerase; PCA, perchloric acid; TBAHS tetrabutylammonium dihydrogensulfate

## SUMMARY

TRPM2 is a  $\text{Ca}^{2+}$ -permeable cation channel gated by ADP-ribose (ADPR) from the cytosolic side. To test whether endogenous concentrations of intracellular ADPR are sufficient for TRPM2 gating in neutrophil granulocytes, we devised an HPLC protocol to determine ADPR contents in perchloric acid cell extracts. The reversed phase ion-pair HPLC protocol with a  $\text{Mg}^{2+}$  containing isocratic eluent allows baseline resolution of one ADPR peak. Intracellular ADPR concentrations were about  $5 \mu\text{M}$  in granulocytes and not significantly altered by stimulation with the chemoattractant peptide fMLP. We furthermore analyzed intracellular concentrations of cyclic ADPR (cADPR) with a cyclase assay involving enzymatic conversion of cADPR to NAD and fluorometric determination of NAD. Intracellular cADPR concentrations were about  $0.2 \mu\text{M}$  and not altered by fMLP. In patch-clamp experiments, ADPR ( $0.1$  to  $100 \mu\text{M}$ ) was dialyzed into granulocytes to analyze its effects on whole-cell currents characteristic for TRPM2, in the presence of a low ( $< 10 \text{ nM}$ ) or a high ( $1 \mu\text{M}$ ) intracellular  $\text{Ca}^{2+}$  concentration. TRPM2 currents were significantly larger in high than in low  $\text{Ca}^{2+}$  (e.g.  $-225 \pm 27.1$  vs.  $-7 \pm 2.0 \text{ pA/pF}$  at  $5 \mu\text{M}$  ADPR) but no currents at all were observed in the absence of ADPR (ADPR concentration  $\leq 0.3 \mu\text{M}$ ). cADPR ( $0.1$ ,  $0.3$  and  $10 \mu\text{M}$ ) was without effect even in the presence of subthreshold ADPR ( $0.1 \mu\text{M}$ ). We conclude that ADPR enables an effective regulation of TRPM2 by cytosolic  $\text{Ca}^{2+}$ . Thus, ADPR and  $\text{Ca}^{2+}$  in concert behave as a messenger system for agonist-induced influx of  $\text{Ca}^{2+}$  through TRPM2 in granulocytes.

## INTRODUCTION

TRPM2 is a non-selective cation channel expressed in a wide range of human tissues, including the brain, bone marrow, spleen and pancreas [1-3]. We have recently shown its expression in neutrophil granulocytes with RT-PCR analysis [4]. Moreover, we have provided functional evidence for its expression by demonstrating channels in neutrophils [4] that exhibit the same characteristic biophysical properties of channels found when TRPM2 is heterologously expressed in HEK 293 cells [5]. Thus, TRPM2 is so far the only  $\text{Ca}^{2+}$ -permeable channel in neutrophils identified by its functional properties that can be attributed to a particular channel protein.

Gating of TRPM2 channels can be induced by ADP-ribose (ADPR) [5]. Further activators of TRPM2 that either alone, or in cooperation with ADPR, or through the formation of ADPR may lead to the opening of the channel include cyclic ADPR (cADPR) [6], NAD [7,8],  $\text{H}_2\text{O}_2$  [8,9], and  $\text{Ca}^{2+}$  ions [10]. For gating by ADPR, the NUDT9 homology region of TRPM2 is decisive. Most likely, binding of ADPR to a defined part within the homology region is involved [11]. The NUDT9 homology region is localized within the cytosolic C-terminal tail of TRPM2. Thus, TRPM2 may be activated by an endogenous intracellular ligand, raising the question whether ADPR has a role as a second messenger that regulates cation influx through TRPM2.

However, actual intracellular concentrations of ADPR are not known in granulocytes. When we started our study, no analytical method for the determination of ADPR levels had been reported. Furthermore, metabolic pathways through which the formation of ADPR may be induced are poorly understood, particularly with respect to their regulation. One possible source of ADPR in many cells are ADPR polymers, formed in the nucleus by Poly(ADPR) polymerases (PARPs). The polymers may be degraded to ADPR by glycohydrolases. PARP activation can be elicited by many factors, such as radiation, oxidative stress or some toxic substances. There have been many enzymes described with a PARP domain, numbered from PARP-1 to PARP-18 [12]. The majority of PARP activity is attributed to PARP-1. Strikingly, neutrophil granulocytes are within the few cells that do not express PARP-1 [13]. Therefore, activation of TRPM2 in neutrophils by ADPR derived from ADPR polymers seems unlikely. As alternative pathway for ADPR production, the membrane-associated protein CD38 may be considered in neutrophil granulocytes. This is an enzyme with glycohydrolase activity that degrades NAD and related compounds [14]. In the past, major relevance of CD38 has been attributed to its generation of cADPR [15]. cADPR is an established second messenger that mediates  $\text{Ca}^{2+}$  release from intracellular stores by action on the ryanodine receptor, a  $\text{Ca}^{2+}$  release channel in the membrane of calcium stores [16]. Moreover, cADPR in concentrations of 10  $\mu\text{M}$  has been reported to potentiate the effects of ADPR [6]. However, cADPR contributes only marginally to the CD38 products. Within the products of CD38-catalyzed metabolism of NAD, the overwhelming majority (96 %) is comprised by ADPR [17]. Thus, CD38 may be viewed as an ADPR synthase. Yet it has to be taken into account that CD38 is an ectoenzyme [18] that catalyzes the conversion of extracellular

NAD to extracellular ADPR. At present, it has not been clarified whether neutrophil CD38 may provide intracellular ADPR formation as well or whether effective transport systems exist that shuffle the substrate and the product of CD38 through the plasma membrane.

The aim of the present study was a determination of the concentrations of ADPR and of cADPR in neutrophil granulocytes, to answer the question whether these concentrations are sufficient for TRPM2 gating. Both nucleotides, in the determined physiological concentrations, were then dialyzed into neutrophils and the currents through TRPM2 were monitored. Furthermore, we tested whether stimulation with the chemoattractant peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), known to induce  $\text{Ca}^{2+}$  influx [19], led to increases in the concentrations of the nucleotides. We report the modification of an HPLC-based method, originally developed for the analysis of intracellular nucleotides [20], for ADPR determination, while cADPR was analyzed with the cyclase assay developed by Graeff and Lee [21]. The levels of ADPR but not of cADPR were in the range that allow intracellular  $\text{Ca}^{2+}$  an effective regulation of TRPM2 channels in cooperation with ADPR. Since intracellular  $\text{Ca}^{2+}$  concentrations are raised by fMLP-induced release from stores, ADPR and  $\text{Ca}^{2+}$  in concert behave as messenger system for the induction of cation influx through TRPM2.

## EXPERIMENTAL

### Materials

Nucleotide standards, nucleotide pyrophosphatase from *Crotalus adammentus* venom, alkaline phosphatase, NADase from *Neurospora crassa*, ADP-ribosyl-cyclase, nicotinamide, alcohol dehydrogenase, resazurin, diaphorase and N-formyl-methionyl-leucyl-phenylalanine (fMLP) were from Sigma (Sigma, Taufkirchen, Germany), riboflavin-5'-monophosphate (FMN) and tri-*n*-octylamine were from Fluka (Fluka, Sigma-Aldrich, Steinheim, Germany). 1,1,2-trichlorotrifluoroethane was from Riedel-de Haën (Seelze, Germany). Perchloric acid (PCA), methanol (LiChrosolv) and tetrabutylammonium dihydrogensulfate (TBAHS) were from Merck (Darmstadt, Germany).

### Neutrophil isolation

Neutrophil granulocytes were prepared from peripheral blood of healthy volunteers as described [4].

### Cell extracts

Neutrophils ( $5$  to  $7 \times 10^7$ ) were stored in test tubes (15 ml, Greiner, Frickenhausen, Germany) at room temperature for up to 30 min and then kept at  $37^\circ\text{C}$  for 2 or 5 min. During this time, some aliquots of the cell suspensions were stimulated with fMLP ( $1 \mu\text{M}$ ). Thereafter, the tubes were placed in an ice-

salt bath for 3 min prior to centrifugation (1000 g, 4°C, 2 min). Pellets were resuspended in ice-cold 0.4 N perchloric acid, followed by three freeze-thaw-cycles (liquid nitrogen / 37°C) and short ultrasonic disruption. Cell debris was removed at 550 g for 5 min at 4°C. For ADPR determinations, supernatants were divided into two identical halves, to one of which an ADPR standard was added (spiking). Extracts were titrated to pH 5-6 with potassium carbonate. Precipitated potassium chlorate was removed by centrifugation (10,000 g, 4°C, 10 min). Finally, the supernatants were filtered through disposable 0.2 µm filters (Spin X, Corning Costar, Wiesbaden, Germany). For cADPR determinations, perchloric acid was removed by mixing 1 vol. of the supernatants with a solution containing 3 vol. of 1,1,2-trichlorotrifluoroethane and 1 vol. tri-*n*-octylamine and vortexing the mixture for 1 min. Following a centrifugation step for 10 min at 1500 g, the probes were maintained on ice until the aqueous phase easily separated from the organic phase. The aqueous layer containing the cADPR was removed and adjusted to a pH of 7-8 with identical volume of sodium phosphate (20 mM, pH 8).

### **Enzymatic degradation of ADPR**

Neutralized extracts or standard ADPR were incubated with nucleotide pyrophosphatase (1.8 U/ml, 2 mM MgCl<sub>2</sub>, 37°C, 30 min). The enzyme was removed by centrifugation of the samples through Centriscart I filter devices (10 kDa MWCO, Sartorius, Göttingen, Germany).

### **HPLC Analysis**

Neutralized neutrophil extracts were analyzed by reversed phase HPLC protocol modified from a procedure developed for the analysis of purine nucleotides in tissue extracts [20,22]. A LaChrom Elite HPLC system (Merck, Hitachi) was used with a LiChrospher RP-8 column (250 x 4 mm, 5 µm particle size, Merck). A volume of 20-40 µl of the samples (corresponding to the extract of 2x10<sup>6</sup> to 4x10<sup>6</sup> cells) or standards were injected with an autosampler (LaChrom Elite L-2200, Merck, Hitachi) that kept the samples at 4°C. An isocratic eluent was used containing methanol (6 % w/v) and (in mM): KH<sub>2</sub>PO<sub>4</sub> 130, TBAHS 3, MgSO<sub>4</sub> 1; pH 5.9. The flow rate was 1.0 ml/min. Absorbance was measured at 270 nm with an UV-absorbance detector (LaChrom Elite L-2400, Merck, Hitachi). Data were processed by the EZChrom Elite data acquisition system. The recovery of the extraction was calculated by comparing an ADPR standard with spiked and non-spiked samples.

### **cADPR assay**

cADPR concentrations were determined in standards and in cell extracts with the method described by Graeff and Lee ([21]), based on the conversion of cADPR to NAD with commercially available

ADP-ribosyl-cyclase and the fluorometrical determination of the NAD concentration with the resazurin/resorufin indicator reaction. In short, 100  $\mu$ l of the cell extracts were incubated overnight at room temperature in an enzyme mixture containing (final concentrations): nucleotide pyrophosphatase (0.44 U/ml), alkaline phosphatase (12.5 U/ml), NADase (0.0625 U/ml),  $MgCl_2$  (2.5 mM), and sodium phosphate (20 mM, pH 8). Enzymes were removed by filtration with Vivaspine 500 filters (10 kDa MWCO, Vivascience, Hannover, Germany) and samples were recovered in the filtrate after centrifugation at 4°C and 3000 g for 30 min. Conversion of cADPR to NAD was initiated by incubating 100  $\mu$ l of the probes with 50  $\mu$ l of a reagent for 15 min at room temperature. The reagent contained ADP-ribosyl-cyclase (0.3  $\mu$ g/ml), nicotinamide (30 mM), sodium phosphate (100 mM, pH 8). Afterwards, 100  $\mu$ l of the cycling reagent were added, containing ethanol (2%), alcohol dehydrogenase (100  $\mu$ g/ml), resazurin (20  $\mu$ M), diaphorase (10  $\mu$ g/ml), FMN (10  $\mu$ M), nicotinamide (10 mM), BSA (100  $\mu$ g/ml) and sodium phosphate (100 mM, pH 8). The samples were then put into glass tubes (0.3 ml, CS Chromatographie Service, Langerwehe, Germany) and the increase in the resorufin fluorescence (with excitation at 544 nm and emission at 590 nm) was monitored for 65 min in a fluorescence spectrophotometer (Cary Eclipse, Varian, Darmstadt, Germany). The cADPR concentration of the cell extracts was calculated from the fluorescence increase over time in comparison to that of cADPR standards. As negative controls, probes without cyclase addition were used.

#### **cADPR purification**

Since cADPR stock solutions contained about 20 % ADPR as contamination, ADPR was removed by incubation with nucleotide pyrophosphatase as described above for extracts.

#### **Electrophysiology**

Human granulocytes were studied with the patch-clamp technique in the whole-cell mode, using an EPC 9 amplifier equipped with a personal computer with Pulse 8.5 and X Chart software (HEKA, Lamprecht, Germany). Pipets were made of borosilicate glass; the pipet tips were coated with dental wax (Moyco, Philadelphia, USA) to reduce thermic noise for the resolution of single channel events in the whole-cell mode. The bath solution was as described [4], with  $Na^+$  as main cation sometimes replaced by 150 mM N-methyl-D-glucamine (NMDG). The pipet solution contained 145 mM Cs-glutamate, 8 mM NaCl, 2 mM  $MgCl_2$ , 10 mM HEPES, pH 7.2 (CsOH) and the  $Ca^{2+}$  concentration was adjusted to either <10 nM (10 mM Cs-EGTA) or 1  $\mu$ M (0.886 mM  $CaCl_2$ , 1 mM Cs-EGTA).  $Ca^{2+}$  concentrations were calculated using the MAXC program (<http://www.stanford.edu/~cpatton/maxc.html>). The weakly  $Ca^{2+}$ -buffered pipet solution contained 50  $\mu$ M Cs-EGTA. ADPR was added from a stock solution to final concentrations of 0.1 to 100  $\mu$ M. cADPR was added from a freshly prepared stock solution to final nominal concentrations of either 10, 0.3 or 0.1  $\mu$ M. Neutrophils were held at -60 mV and current-voltage (I-V) relations were obtained

from voltage ramps (from -90 to +60 mV over 400 ms). These experiments were carried out at room temperature.

## RESULTS

To verify whether the chosen HPLC system enabled quantitative determinations of ADPR concentrations, the column was loaded with standards containing either of the following nucleotides in various mixtures: ATP, ADP, AMP, adenosine, GTP, cADPR, NAD, nicotinamide, and ADPR. These compounds were readily separated, resulting in defined peaks in the chromatograms that allowed the identification of each of them by their individual retention times (see supplementary Fig. 1). The area of the peak attributed to ADPR was linearly correlated with the ADPR content in the sample. The detection limit was in the range of 1 pmol ADPR.

Chromatograms of neutralized PCA extracts from neutrophil granulocytes displayed several more peaks than the standards, many of them not well separated (Fig. 1A). Importantly, however, there was one single baseline-resolved peak with the same retention time as that of ADPR standards. To test for the specificity of this peak attributed to ADPR, we spiked the extracts with ADPR, i.e. we added ADPR to the extracts prior to neutralization with potassium carbonate, thereby increasing their ADPR content by a defined amount. Only 50 % of the volume of each extract was spiked, whereas the same volume of water was added to the other half. The two chromatograms of representative twin samples (spiked and not spiked) from neutrophils are compared in Fig. 1A. Spiking resulted in the specific enlargement of the ADPR peak. From a comparison of the peak area of the unspiked sample with that of an ADPR standard, the amount of ADPR in the unspiked sample after neutralization is calculated as 7.6 pmol. The comparison with the peak area of the spiked sample allows to calculate a recovery rate during the sample preparation of 98 %. The same recovery rate was found when five fold higher amounts of ADPR were used for spiking. As further test for the specificity of the ADPR peak, we incubated standards as well as twin samples of cell extracts with pyrophosphatase that converts ADPR to AMP and ribose-5-phosphate. Fig. 1B shows that pyrophosphatase incubation completely removed the ADPR peak, in standards and extracts alike. At the same time, the AMP peak was considerably enlarged, indicating that ADPR as well as other nucleotides were degraded to AMP.

When neutrophil granulocytes were stimulated with fMLP (1  $\mu$ M, 2 min) prior to acidic precipitation, the ADPR concentrations in the extracts were not significantly (paired t-test) changed (Fig. 2; n = 6 experiments with extracts from stimulated vs. control cells). There was a tendency for a small reduction of ADPR contents by fMLP (Fig. 2B), in the range of the resolution of the method. Similar results were obtained after a stimulation of 5 min (data not shown). For comparison, we performed experiments on HEK 293 cells stimulated with hydrogen peroxide (10 mM, 10 min, n = 3). This treatment diminished the ADPR contents by about 60 % and strikingly altered several other



peaks, noticeably those attributable to ATP, ADP, and to further unidentified compounds (data not shown).

The HPLC data so far define the amounts of ADPR present in a given number of neutrophil granulocytes. When these figures are to be used for a calculation of intracellular ADPR concentrations, some uncertainties exist, most notably the cell size and the ADPR distribution space within the cells. The cell volume of neutrophil granulocytes has been estimated as 350 fl [23]. We derived similar values from the determination of the cytocrit of granulocyte suspensions. When this volume is assumed along with a homogenous intracellular ADPR distribution, the concentration of ADPR is  $5.0 \pm 1.8 \mu\text{M}$  (mean  $\pm$  SEM) in unstimulated neutrophil granulocytes and  $3.9 \pm 1.8 \mu\text{M}$  after fMLP stimulation. We estimate that these values have an uncertainty factor of 0.5 to 6, i.e. the actual concentrations may be in the range from 2 to 30  $\mu\text{M}$ .

To test for the functional significance of these ADPR concentrations for TRPM2 gating in neutrophil granulocytes, we performed patch-clamp experiments in which ADPR was infused into granulocytes through the patch pipet. We considered that intracellular  $\text{Ca}^{2+}$  is a cofactor of ADPR-dependent TRPM2 gating [10]. However, this finding has only been obtained with heterologously expressed TRPM2 channels which does not necessarily indicate that the same holds true in granulocytes. Therefore, the experiments were performed with two different  $\text{Ca}^{2+}$  concentrations in the pipet solution, either a very low one achieved by addition of EGTA (10 mM) to a nominally  $\text{Ca}^{2+}$ -free solution ( $\text{Ca}^{2+}$  concentration  $< 10 \text{ nM}$ ), or a concentration corresponding to that in stimulated cells, i.e. about 1  $\mu\text{M}$  accomplished by a mixture of EGTA and  $\text{CaCl}_2$  (see Experimental).

When ADPR (5  $\mu\text{M}$ ) was infused into granulocytes in the presence of low  $\text{Ca}^{2+}$ , the opening of a few channels was induced that could be resolved within the total whole-cell currents (Fig. 3A). The extremely long (several seconds) open times and the single channel current amplitudes (consistent with the reported conductance of 50-70 pS) are characteristic for TRPM2 channels [5] and provide strong evidence that ADPR gates TRPM2 channels in neutrophil granulocytes. Sizeable whole-cell currents, rather than only some single channel openings, were induced in granulocytes when ADPR (even at 1  $\mu\text{M}$ ) was infused along with  $\text{Ca}^{2+}$  (Fig. 3B). No single channel events can be resolved within large whole-cell currents. However, since the exchange of the cytosolic solutes with that of the pipet fluid is a time-dependent process, the currents typically developed gradually before a plateau was reached. During the initial phase of the current development, the consecutive opening of single channels was evident, again with the characteristic properties of TRPM2 (Fig. 3B). Whole-cell currents were blocked by the large cation NMDG (Fig. 3) reported to be mostly impermeant to TRPM2 [9].

Experiments with higher ADPR concentrations (30 or 100  $\mu\text{M}$ ) demonstrated again a critical role of  $\text{Ca}^{2+}$ . Although ADPR evoked whole-cell currents in the absence of  $\text{Ca}^{2+}$ , the currents were strikingly larger in amplitude in the presence of  $\text{Ca}^{2+}$  (1  $\mu\text{M}$ ) in the pipet solution (Fig. 4). The I-V

relation of the currents, with either  $\text{Na}^+$  or  $\text{NMDG}^+$  as main extracellular cation, was characteristic for TRPM2 currents [4,5,9].

Importantly, gating of TRPM2 channels was not evoked by  $\text{Ca}^{2+}$  alone. When a pipet solution was used that contained the high  $\text{Ca}^{2+}$  concentration (ADPR) but no ADPR, no single channel events and no increases in the whole-cell currents were observed (Fig. 3C). Thus, the presence of ADPR is essential for the effects of  $\text{Ca}^{2+}$  on TRPM2. The dependence of currents in granulocytes on the concentration of ADPR, in the presence of either a high or a low intracellular  $\text{Ca}^{2+}$  concentration, is summarized in Fig. 5. A minimal ADPR concentration of 1  $\mu\text{M}$  was required to induce currents in the presence of high  $\text{Ca}^{2+}$ . At higher ADPR concentrations, a saturation of the effects was observed. Over the whole ADPR concentration range studied, considerably and significantly lower currents were found for low than for high  $\text{Ca}^{2+}$ .

A modulator of ADPR-induced TRPM2 activation may be cADPR, implicated to be a second messenger regulated by receptor-dependent stimulation in other cells [24]. The cADPR concentration reported to enhance TRPM2 currents was 10  $\mu\text{M}$ . In our HPLC conditions, cADPR produces a peak at about 2.8 min retention time. For the range of this retention time, Fig. 6 shows an expanded view of two chromatograms of cell extracts, one from fMLP-stimulated cells and one from resting cells, along with a chromatogram of a cADPR standard. The two extracts produced almost identical chromatograms (in the shown retention time range) but no peak at the expected retention time of cADPR. We cannot exclude that one of the neighbouring peaks may represent cADPR; in this case, an fMLP-induced concentration increase could be ruled out. More likely, cADPR is not present intracellularly in sufficient concentrations to be resolved with our HPLC conditions. Therefore, we resorted to the enzymatic cADPR assay based on the conversion of cADPR to NAD and the fluorometrical determination of NAD [21] to assess intracellular cADPR concentrations. With standards nominally containing 0-10 nM cADPR, a calibration curve was obtained in which the cADPR concentration was linearly related to the increase in resorfin fluorescence (correlation coefficient  $r = 0.92$ , see supplementary Fig. 2). The cADPR concentrations found in extracts were between 1 and 9 nM ( $n = 6$ ). From the number of cells in each extract and the estimated cytosolic volume, the intracellular concentration was calculated to be  $221 \pm 84$  nM. Aliquots of the same granulocyte preparations were stimulated with fMLP (1  $\mu\text{M}$  for 3 min), resulting in an intracellular concentration of  $152 \pm 93$  nM ( $n = 6$ ). When cADPR was added to cell extracts in amounts corresponding to a raise in the intracellular concentration by 50 nM, a concentration increase in the expected range was found in extracts from unstimulated as well as from fMLP-stimulated cells. Since we discovered later (see below) that the standards may have contained, to a sizeable part, ADPR rather than cADPR, the actual intracellular cADPR concentrations are likely to be about 25 % lower.

As in the case of ADPR, we wished to examine whether cADPR in physiological concentrations had effects on whole-cell currents. First, we repeated the experiments performed by Kolisek et al. [6] in TRPM2-transfected HEK 293 cells in granulocytes, i.e. we used a weakly  $\text{Ca}^{2+}$ -buffered pipet solution with 10  $\mu\text{M}$  cADPR. In contrast to Kolisek et al., we observed large currents ( $-200.8 \pm 10.6$  pA/pF,  $n = 3$ ) as if ADPR rather than cADPR were infused (Fig. 7A). Similar results were obtained with cADPR (10  $\mu\text{M}$ ) in our standard high  $\text{Ca}^{2+}$  pipet solution. This prompted us to test for the purity of our cADPR solutions with an HPLC analysis (inset Fig. 7A) that demonstrated considerable contamination with ADPR that may be conservatively estimated to be at least 25 %, even when cADPR solutions are freshly prepared from cADPR salts. These results were confirmed in all of several cADPR batches tested. Therefore, the currents induced in our experiments with nominally 10  $\mu\text{M}$  cADPR are explained by contaminating ADPR. To analyze possible effects of cADPR, cADPR stock solutions were incubated with a nucleotide pyrophosphatase, resulting in the complete conversion of ADPR to AMP (inset Fig. 7B). It was confirmed that pyrophosphatase-treated cADPR stock remained free of ADPR for several days after freezing. With pipet solutions prepared from ADPR-free cADPR, no currents were induced in granulocytes ( $n = 4$ , data not shown), not even when ADPR was added in a subthreshold concentration (0.1  $\mu\text{M}$ ;  $n = 4$ ; Fig. 7B). Since AMP has been reported as an inhibitor of TRPM2 currents [6], although only at high concentrations (0.5 mM), we furthermore tested AMP (5  $\mu\text{M}$  which slightly exceeds the concentrations used in the experiments with ADPR-free cADPR) in the presence of ADPR (5  $\mu\text{M}$ ) which did not noticeably alter ADPR-induced TRPM2 currents in granulocytes ( $n = 4$ ; Fig. 7C). Finally, we tested cADPR in physiological concentrations. When cADPR was infused in concentrations of 0.1  $\mu\text{M}$  ( $n = 7$ ) or 0.3  $\mu\text{M}$  ( $n = 6$ ), along with ADPR in a subthreshold concentration (i.e. 0.1  $\mu\text{M}$ ), no current was induced in any experiment.

## DISCUSSION

In the present study, we provide measurements of intracellular ADPR concentrations, an activator of the  $\text{Ca}^{2+}$ -permeable cation channel TRPM2, in neutrophil granulocytes. As main finding, we report that the ADPR concentrations are in the range that allow intracellular  $\text{Ca}^{2+}$  an effective regulation of TRPM2 gating. Thus, even though ADPR concentrations were not increased by stimulation of the cells and  $\text{Ca}^{2+}$  by itself is inactive on TRPM2, ADPR and  $\text{Ca}^{2+}$  in combination can act as a messenger system. This messenger system is capable to induce  $\text{Ca}^{2+}$  influx through TRPM2 channels in response to stimuli like fMLP that lead to release of  $\text{Ca}^{2+}$  from intracellular stores. In contrast, no evidence was obtained for a regulation of TRPM2 by cADPR.

Measurements of ADPR became possible after we modified a reversed phase ion-pair HPLC protocol devised for the measurements of intracellular purine nucleotides [20]. An isocratic eluent was used that offers the advantage that small concentration changes can be more precisely assessed

than with a gradient elution. The separation of ADPR from ATP and ADP became possible due to the addition of  $Mg^{2+}$  as additional counterion in the eluent [22]. The protocol provides the baseline resolution of an ADPR peak, not only in standards but in extracts from granulocytes as well. A similar HPLC method, not known to us at the start of our study, was recently reported [25] and used to analyze ADPR concentrations in Jurkat cells [26]. In our protocol, the preparation of the samples from PCA extracts involves fewer and less time-consuming steps; the almost complete recovery rate indicates that no ADPR is lost during the preparation. The experiments with nucleotide pyrophosphatase provide further proof for the specificity of the ADPR peak, even though other nucleotides are certainly substrates for this enzyme.

From the ADPR contents measured in the cell extracts, a range of likely ADPR concentrations may be deduced under consideration of several uncertainties. A relatively safe assumption is the cell size of 350 fl, meaning that the average intracellular concentration is about 5  $\mu M$ . However, the distribution space of ADPR within the cell is largely unknown. Thus, the cytosolic concentration facing TRPM2 could be several times larger. Fortunately, our data indicate that no matter whether the actual ADPR concentration may be 2 or 30  $\mu M$ , the relevance of ADPR remains the same. This is because over this concentration range (and beyond), the concentration-effect curve of ADPR on TRPM2 currents reveals a uniform requirement for  $Ca^{2+}$  of ADPR-dependent TRPM2 gating (see Fig. 6). In the absence of  $Ca^{2+}$ , ADPR induced a few channel openings but no sizeable whole-cell currents. However, almost maximal TRPM2 currents were evoked in the presence of 1  $\mu M$   $Ca^{2+}$  when ADPR was infused at concentrations as low as 1  $\mu M$ . Further experiments demonstrate the essential role of ADPR for TRPM2 currents. Infusion of an ADPR-free solution prohibited any currents. This is explained by a rapid dilution of endogenous ADPR when the cell is dialyzed with the pipet fluid in the whole-cell configuration [27]. In this context, it is noteworthy that a pipet solution with 0.1  $\mu M$  ADPR was without effect on TRPM2 even in the presence of 1  $\mu M$   $Ca^{2+}$ , demonstrating that dilution down to and below this level occurs in patch-clamp experiments without ADPR.

We did not obtain a full concentration-effect curve for  $Ca^{2+}$  but confined the analysis to two concentrations, one extremely small and the other one in the range of peak intracellular concentrations after stimulation with fMLP. In between, accurate adjustment of  $Ca^{2+}$  concentrations with EGTA as chelator is problematic due to the equilibrium constant of EGTA/ $Ca^{2+}$ . Attempts to buffer  $Ca^{2+}$  in the concentration range of resting or mildly stimulated cells may result in severe errors deriving from small inaccuracies of the calculated EGTA and  $CaCl_2$  amounts, aggravated by small calcium contaminations of other salts and not completely quantifiable effects of other ions. Moreover, the buffering is weak in this  $Ca^{2+}$  concentration range. It is expected that other  $Ca^{2+}$  concentrations may exert intermediate effects in comparison to those observed with the two extreme concentrations but an exact quantification cannot be provided at present.

On the basis of our measurements of physiological ADPR levels, the cooperativeness of ADPR and  $Ca^{2+}$ , and the previous well-established findings that fMLP induced increases in the

cytosolic  $\text{Ca}^{2+}$  concentration due to release of  $\text{Ca}^{2+}$  from intracellular stores [19], we propose that ADPR in neutrophil granulocytes renders TRPM2 a  $\text{Ca}^{2+}$  dependent  $\text{Ca}^{2+}$  entry pathway that is activated in consequence of a PI response. In this model, there is no need for a stimulated ADPR formation because  $\text{Ca}^{2+}$  acts at basal ADPR concentrations. These basal concentrations, however, are essential because  $\text{Ca}^{2+}$  has no effect on TRPM2 by its own.  $\text{Ca}^{2+}$  influx through TRPM2 is a self-enhancing process, in line with the fact that fMLP leads to an irreversible activation of neutrophil granulocytes. Taken together, ADPR and  $\text{Ca}^{2+}$  in concert mediate the effects of extracellular ligands like fMLP. Thus, ADPR and  $\text{Ca}^{2+}$  as pair behave like a second messenger system, exhibiting relevant increases of the one player ( $\text{Ca}^{2+}$ ) that becomes effective by the other one (ADPR).

These considerations on the ADPR /  $\text{Ca}^{2+}$  messenger system do not include the possibility of ADPR concentration increases confined to small areas around the cell membrane and, especially, TRPM2 channels. One prominent enzyme that could be responsible for the formation of ADPR in neutrophil granulocytes is CD38 that exhibits the paradoxical property of being an ectoenzyme [18]. At present, one can only hypothesize whether CD38 in the plasma membrane has a role as source of intracellular ADPR, which would necessitate the presence of a transport of NAD (the substrate) to the outside and of ADPR (the product) to the inside [14]. However, if such transport systems exist, spatially increased ADPR concentrations would appear likely. The experimental demonstration of localized ADPR peaks would enlarge our view on the second messenger role of the ADPR /  $\text{Ca}^{2+}$  system but would probably not dramatically diminish the importance of  $\text{Ca}^{2+}$  because ADPR even at concentrations considerably higher than the average physiological ones is far more effective in the presence than in the absence of  $\text{Ca}^{2+}$ .

Other intracellular compounds may contribute to the  $\text{Ca}^{2+}$ -modulated activation of TRPM2 by ADPR. Most notably, cADPR and NAD may act as enhancers and AMP as inhibitor [6]. However, the required concentrations of cADPR were quite high. Kolisek et al used up to 1 mM cADPR, when the compound stimulated TRPM2 by its own, whereas a potentiation of ADPR-induced currents was reported by 10  $\mu\text{M}$  cADPR. Already our HPLC analysis of cell extracts allows to exclude intracellular concentrations of 10  $\mu\text{M}$ . This was confirmed with the cycling assay that revealed a concentration range of about 0.2  $\mu\text{M}$ . Our patch-clamp experiments in granulocytes with 10  $\mu\text{M}$  cADPR were complicated by ADPR contamination of the commercially available cADPR. After enzymatic removal of ADPR, no effect of cADPR could be confirmed, not even in the presence of sub-threshold ADPR concentrations.

Stimulation with fMLP did not lead to an increase in intracellular cADPR concentrations; there was rather a tendency towards a decrease. However, since cADPR levels were at the detection limit of the method, small and possibly spatially limited concentration increases cannot be excluded. Such increases could indirectly be relevant for TRPM2 if cADPR induced  $\text{Ca}^{2+}$  release from stores in granulocytes. Interestingly, 8-bromo-cADPR, a membrane permeant antagonist of cADPR, inhibited

Ca<sup>2+</sup> influx in response to fMLP [28]. These findings would be in line with Ca<sup>2+</sup> release and subsequent Ca<sup>2+</sup>-induced Ca<sup>2+</sup> influx as well as with direct activation of Ca<sup>2+</sup> entry pathways. On the basis of our experiments, a direct effect of cADPR on TRPM2 channels in neutrophil granulocytes appears unlikely.

In conclusion, our study provides evidence for a physiological role of TRPM2 gating by ADPR in neutrophil granulocytes. The role in these cells is different from that proposed for Jurkat cells [26] that display slight increases in their ADPR content in response to concavalin A, interpreted as a second messenger role of ADPR. In granulocytes, the regulatory function of ADPR depends on its cooperation with Ca<sup>2+</sup>. A second messenger role can be attributed only to ADPR and Ca<sup>2+</sup> in concert. In effect, basal levels of ADPR enable activation by Ca<sup>2+</sup> of TRPM2 which will induce further increases in the cytosolic Ca<sup>2+</sup> concentration due to Ca<sup>2+</sup> influx. This mechanism may be associated with the irreversible phase of neutrophil activation. Future studies in other cells may be on the question whether a decrease in ADPR concentrations is used to terminate Ca<sup>2+</sup> influx through TRPM2.

#### ACKNOWLEDGEMENTS

We thank Ilinca Ionescu for excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Project B5 of SFB542)

## REFERENCES

- 1 Nagamine, K., Kudoh, J., Minoshima, S., Kawasaki, K., Asakawa, S., Ito, F. and Shimizu, N. (1998) Molecular cloning of a novel putative Ca<sup>2+</sup> channel protein (TRPC7) highly expressed in brain. *Genomics* **54**, 124-131
- 2 Inamura, K., Sano, Y., Mochizuki, S., Yokoi, H., Miyake, A., Nozawa, K., Kitada, C., Matsushime, H. and Furuichi, K. (2003) Response to ADP-ribose by activation of TRPM2 in the CRI-G1 insulinoma cell line. *J.Membr.Biol.* **191**, 201-207
- 3 Uemura, T., Kudoh, J., Noda, S., Kanba, S. and Shimizu, N. (2005) Characterization of human and mouse TRPM2 genes: identification of a novel N-terminal truncated protein specifically expressed in human striatum. *Biochem.Biophys.Res.Commun.* **328**, 1232-1243
- 4 Heiner, I., Eisfeld, J., Halaszovich, C. R., Wehage, E., Jüngling, E., Zitt, C. and Lückhoff, A. (2003) Expression profile of the transient receptor potential (TRP) family in neutrophil granulocytes: evidence for currents through long TRP channel 2 induced by ADP-ribose and NAD. *Biochem.J.* **371**, 1045-1053
- 5 Perraud, A. L., Fleig, A., Dunn, C. A., Bagley, L. A., Launay, P., Schmitz, C., Stokes, A. J., Zhu, Q., Bessman, M. J., Penner, R., Kinet, J. P. and Scharenberg, A. M. (2001) ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature* **411**, 595-599
- 6 Kolisek, M., Beck, A., Fleig, A. and Penner, R. (2005) Cyclic ADP-ribose and hydrogen peroxide synergize with ADP-ribose in the activation of TRPM2 channels. *Mol.Cell* **18**, 61-69
- 7 Sano, Y., Inamura, K., Miyake, A., Mochizuki, S., Yokoi, H., Matsushime, H. and Furuichi, K. (2001) Immuncyte Ca<sup>2+</sup> influx system mediated by LTRPC2. *Science* **293**, 1327-1330
- 8 Hara, Y., Wakamori, M., Ishii, M., Maeno, E., Nishida, M., Yoshida, T., Yamada, H., Shimizu, S., Mori, E., Kudoh, J., Shimizu, N., Kurose, H., Okada, Y., Imoto, K. and Mori, Y. (2002) LTRPC2 Ca<sup>2+</sup>-permeable channel activated by changes in redox status confers susceptibility to cell death. *Mol.Cell* **9**, 163-173
- 9 Wehage, E., Eisfeld, J., Heiner, I., Jüngling, E., Zitt, C. and Lückhoff, A. (2002) Activation of the cation channel long transient receptor potential channel 2 (LTRPC2) by hydrogen peroxide. A splice variant reveals a mode of activation independent of ADP-ribose. *J.Biol.Chem.* **277**, 23150-23156

- 10 McHugh, D., Flemming, R., Xu, S. Z., Perraud, A. L. and Beech, D. J. (2003) Critical intracellular Ca<sup>2+</sup> dependence of transient receptor potential melastatin 2 (TRPM2) cation channel activation. *J.Biol.Chem.* **278**, 11002-11006
- 11 Kühn, F. J. and Lückhoff, A. (2004) Sites of the NUDT9-H domain critical for ADP-ribose activation of the cation channel TRPM2. *J.Biol.Chem.* **279**, 46431-46437
- 12 Ame, J. C., Spenlehauer, C. and de Murcia, G. (2004) The PARP superfamily. *Bioessays* **26**, 882-893
- 13 Sanghavi, D. M., Thelen, M., Thornberry, N. A., Casciola-Rosen, L. and Rosen, A. (1998) Caspase-mediated proteolysis during apoptosis: insights from apoptotic neutrophils. *FEBS Lett.* **422**, 179-184
- 14 Schuber, F. and Lund, F. E. (2004) Structure and enzymology of ADP-ribosyl cyclases: conserved enzymes that produce multiple calcium mobilizing metabolites. *Curr.Mol.Med.* **4**, 249-261
- 15 Partida-Sanchez, S., Cockayne, D. A., Monard, S., Jacobson, E. L., Oppenheimer, N., Garvy, B., Kusser, K., Goodrich, S., Howard, M., Harmsen, A., Randall, T. D. and Lund, F. E. (2001) Cyclic ADP-ribose production by CD38 regulates intracellular calcium release, extracellular calcium influx and chemotaxis in neutrophils and is required for bacterial clearance in vivo. *Nat.Med.* **7**, 1209-1216
- 16 Guse, A. H., Cakir-Kiefer, C., Fukuoka, M., Shuto, S., Weber, K., Bailey, V. C., Matsuda, A., Mayr, G. W., Oppenheimer, N., Schuber, F. and Potter, B. V. (2002) Novel hydrolysis-resistant analogues of cyclic ADP-ribose: modification of the "northern" ribose and calcium release activity. *Biochemistry* **41**, 6744-6751
- 17 Howard, M., Grimaldi, J. C., Bazan, J. F., Lund, F. E., Santos-Argumedo, L., Parkhouse, R. M., Walseth, T. F. and Lee, H. C. (1993) Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science* **262**, 1056-1059
- 18 De Flora, A., Franco, L., Guida, L., Bruzzone, S., Usai, C. and Zocchi, E. (2000) Topology of CD38. *Chem.Immunol.* **75**, 79-98
- 19 Krause, K. H., Campbell, K. P., Welsh, M. J. and Lew, D. P. (1990) The calcium signal and neutrophil activation. *Clin.Biochem.* **23**, 159-166



- 20 Jüngling, E. and Kammermeier, H. (1980) Rapid assay of adenine nucleotides or creatine compounds in extracts of cardiac tissue by paired-ion reverse-phase high-performance liquid chromatography. *Analytical Biochemistry* **102**, 358-361
- 21 Graeff, R. and Lee, H. C. (2002) A novel cycling assay for cellular cADP-ribose with nanomolar sensitivity. *Biochem.J.* **361**, 379-384
- 22 Folley, L. S., Power, S. D. and Poyton, R. O. (1983) Separation of nucleotides by ion-pair, reversed-phase high-performance liquid chromatography: Use of Mg(II) and triethylamine as competing hetaerons in the separation of adenine and guanine nucleotides. *Journal of Chromatography A* **281**, 199-207
- 23 Jankowski, A. and Grinstein, S. (1999) A noninvasive fluorimetric procedure for measurement of membrane potential. Quantification of the NADPH oxidase-induced depolarization in activated neutrophils. *J.Biol.Chem.* **274**, 26098-26104
- 24 Guse, A. H., da Silva, C. P., Berg, I., Skapenko, A. L., Weber, K., Heyer, P., Hohenegger, M., Ashamu, G. A., Schulze-Koops, H., Potter, B. V. and Mayr, G. W. (1999) Regulation of calcium signalling in T lymphocytes by the second messenger cyclic ADP-ribose. *Nature* **398**, 70-73
- 25 Gasser, A. and Guse, A. H. (2005) Determination of intracellular concentrations of the TRPM2 agonist ADP-ribose by reversed-phase HPLC. *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.* **821**, 181-187
- 26 Gasser, A., Glassmeier, G., Fliegert, R., Langhorst, M. F., Meinke, S., Hein, D., Kruger, S., Weber, K., Heiner, I., Oppenheimer, N., Schwarz, J. R. and Guse, A. H. (2006) Activation of T cell calcium influx by the second messenger ADP-ribose. *J.Biol.Chem.* **281**, 2489-2496
- 27 Pusch, M. and Neher, E. (1988) Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflugers Arch.* **411**, 204-211
- 28 Partida-Sanchez, S., Iribarren, P., Moreno-Garcia, M. E., Gao, J. L., Murphy, P. M., Oppenheimer, N., Wang, J. M. and Lund, F. E. (2004) Chemotaxis and calcium responses of phagocytes to formyl peptide receptor ligands is differentially regulated by cyclic ADP ribose. *J.Immunol.* **172**, 1896-1906

## FIGURE LEGENDS

### **Figure 1 Chromatograms of samples prepared by PCA extraction from neutrophil granulocytes**

**(A)** One extract (black) was spiked with ADPR (13 pmol). ADPR in extracts displayed the same retention time as ADPR in standards (shown in the supplementary Fig. 1 displaying an HPLC analysis of a mixture of several standard nucleotides). The ADPR content in the non-spiked extract (grey chromatogram) was calculated as 7.6 pmol. The inset shows an enlarged section of the chromatogram that contained the ADPR peak. **(B)** Effects of pyrophosphatase incubation on the ADPR peak in extracts (and in standards, see inset). Note that the AMP peak is considerably enlarged by pyrophosphatase treatment, indicating that ADPR is not an exclusive substrate of the enzyme. The minor differences in the retention time in both panels are due to different columns (although of the same type) and different equilibration times with elution buffer (1 vs. 2 days) in the chromatographies.

### **Figure 2 ADPR content in neutrophil granulocytes at basal conditions or after stimulation with fMLP**

**(A)** Chromatograms from two extracts of granulocytes from one donor. Cells were stimulated with fMLP (1  $\mu$ M for 2 min; black chromatogram) or were not stimulated (control, gray chromatogram). **(B)** Summary of the fMLP effects on the ADPR content. Data (mean  $\pm$  SEM) are from six granulocyte preparations from different donors in which 50 % of the cells were stimulated with fMLP and 50 % were used as unstimulated controls.

### **Figure 3 Whole-cell currents in neutrophil granulocytes induced by low ADPR concentrations and the effects of intracellular Ca<sup>2+</sup>**

The pipet solution contained ADPR (5 or 1  $\mu$ M) in the experiments shown in *A* and *B* and was free of ADPR in *C*. The intracellular (pipet) Ca<sup>2+</sup> concentration was low (< 10 nM) in **(A)** and high (1  $\mu$ M) in **(B)** and **(C)**. The holding potential was -60 mV. The Na<sup>+</sup> in the bath was replaced by the impermeable cation NMDG during time periods indicated by horizontal bars. The enlarged current tracings in the insets of **A** and **B** (scale bars 5 pA and 10 s, respectively) show single-channel openings resolved at low whole-cell current amplitudes.

**Figure 4 Whole-cell currents in neutrophil granulocytes induced by high ADPR concentrations and the effects of intracellular  $\text{Ca}^{2+}$**

The pipet solution contained 30  $\mu\text{M}$  ADPR (A-C) or 100  $\mu\text{M}$  ADPR (D) in the presence of either a low (<10 nM) or high (1  $\mu\text{M}$ )  $\text{Ca}^{2+}$  concentration. The  $\text{Na}^+$  in the bath was replaced by the impermeable cation NMDG during time periods indicated by horizontal bars in A and D. Current-voltage relations in low  $\text{Ca}^{2+}$  (B) and in high  $\text{Ca}^{2+}$  (C) were recorded with voltage ramps at time points indicated with numbers in A. Note the different scaling of the ordinate in B and C.

**Figure 5 Current densities in dependence of the ADPR concentration, in either low or high  $\text{Ca}^{2+}$**

Current densities (mean  $\pm$  SEM) at low  $\text{Ca}^{2+}$  (open circles) were significantly (Mann-Whitney U-test) lower than at high  $\text{Ca}^{2+}$  (closed circles) for each ADPR concentration. The number of experiments is indicated for each experimental condition.

**Figure 6 Expanded view of HPLC chromatograms of extracts and a standard around the cADPR peak**

The standard (dotted line) contained nominally 16  $\mu\text{M}$  cADPR. The two almost identical chromatograms were from two extracts from cells from one donor. One extract was from cells stimulated with fMLP (1  $\mu\text{M}$  for 2 min; black), the other one from unstimulated cells (grey).

**Figure 7 Effect of cADPR on whole-cell currents in neutrophil granulocytes in the presence of 1  $\mu\text{M}$  intracellular  $\text{Ca}^{2+}$**

(A) The pipet solution that contained nominally 10  $\mu\text{M}$  cADPR was prepared from a cADPR stock solution considerably contaminated with ADPR, as shown with HPLC (inset). Inward currents developing during infusion of contaminated cADPR were blocked by NMDG present in the bath during time periods indicated by horizontal bars.

(B) In contrast to (A), the pipet solution was prepared from a cADPR stock solution incubated with nucleotide pyrophosphatase (2.7 U/ml, 20 min, 37 °C), resulting in the complete conversion of ADPR to AMP (inset). ADPR was then added to the pipet solution at a sub-threshold concentration (0.1  $\mu\text{M}$ ). The absence of currents under these conditions was confirmed in 3 further experiments.

(C) To check for the effects of AMP, AMP (5  $\mu\text{M}$ ) was added to a pipet solution containing ADPR (5  $\mu\text{M}$ ). AMP did not prevent or noticeably alter ADPR-induced currents in three further experiments.

Figure 1

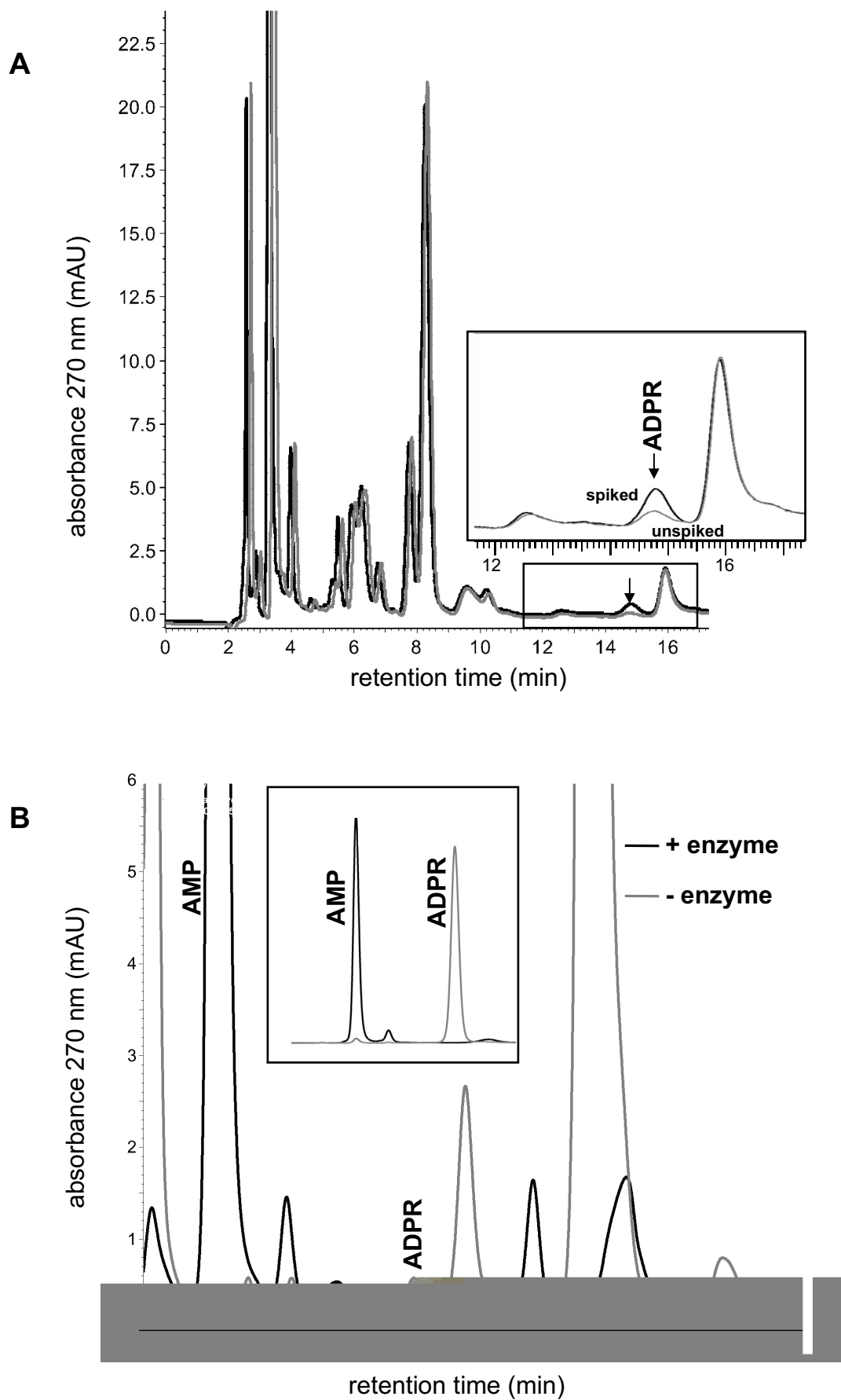
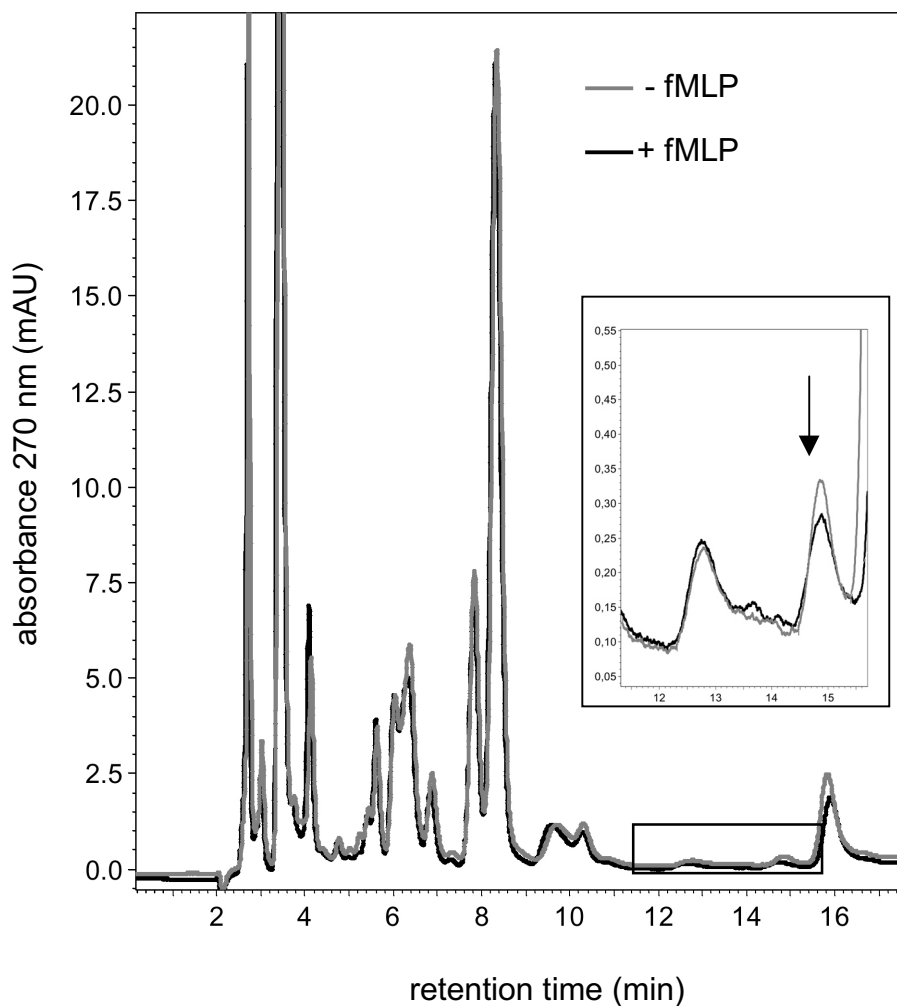


Figure 2

A



B

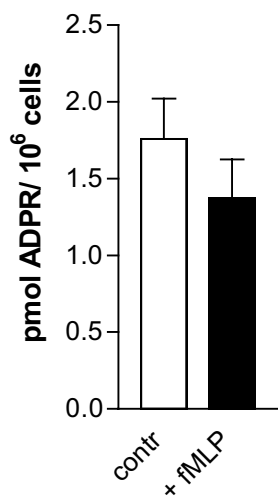


Figure 3

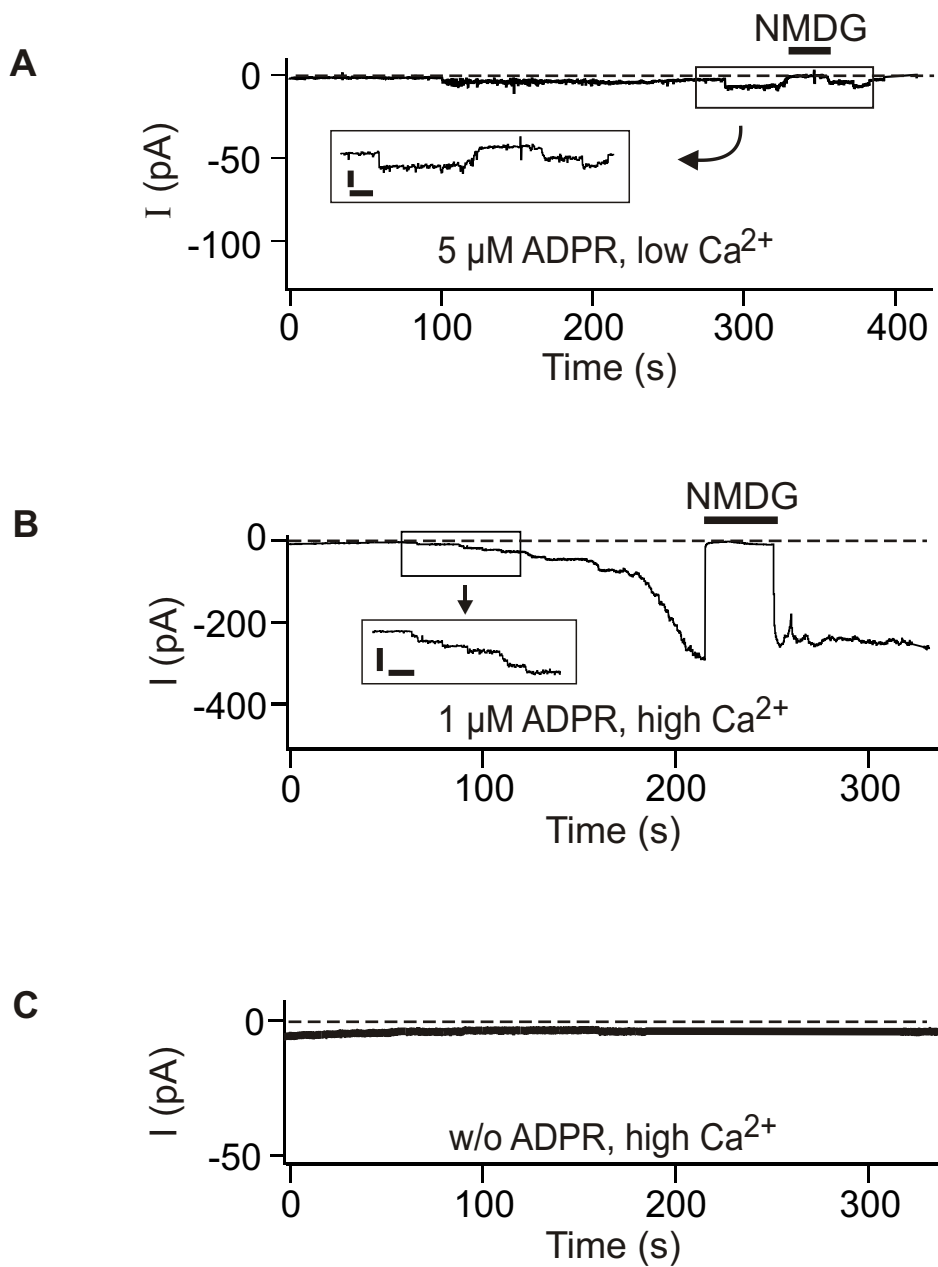
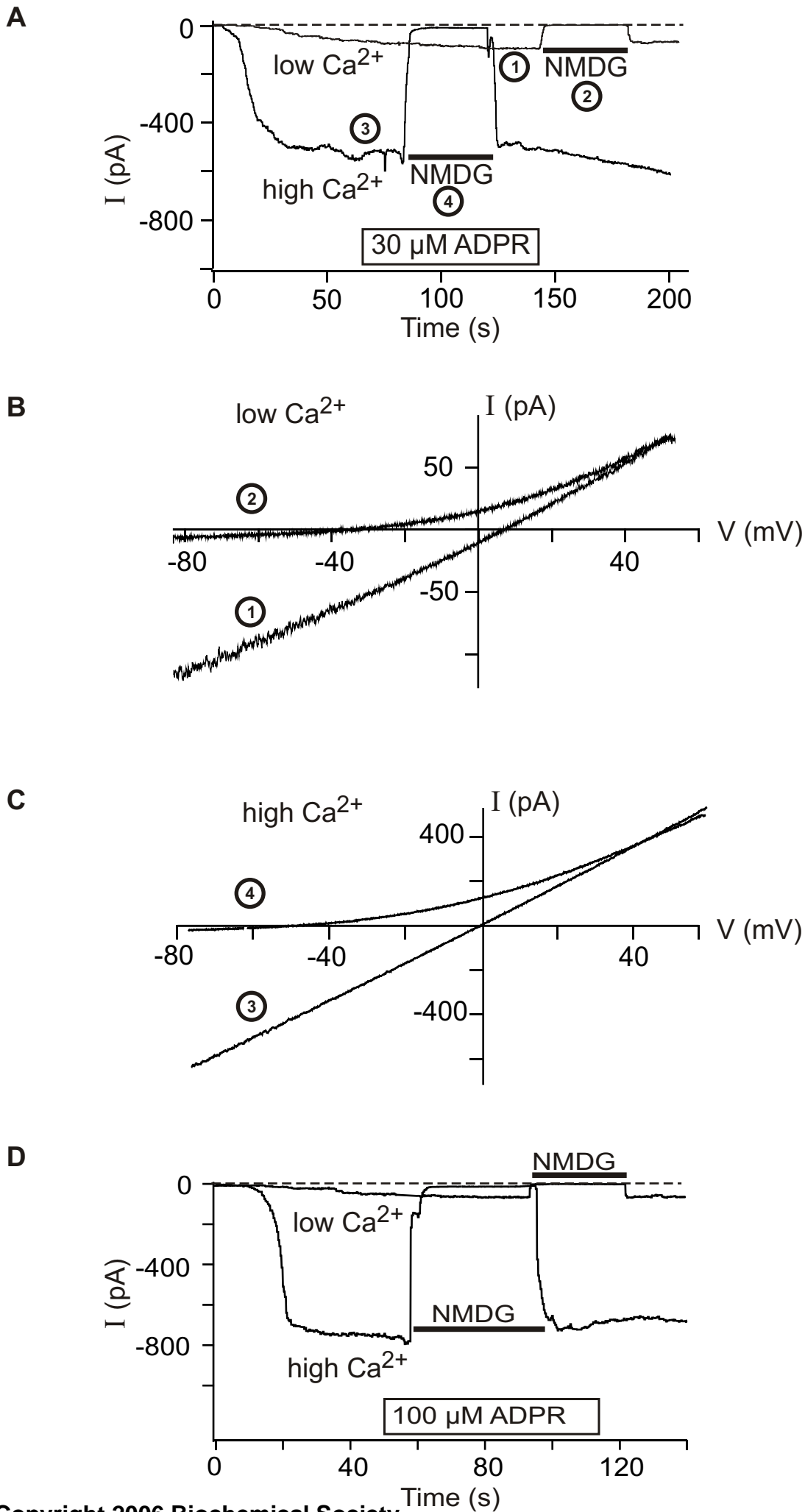


Figure 4



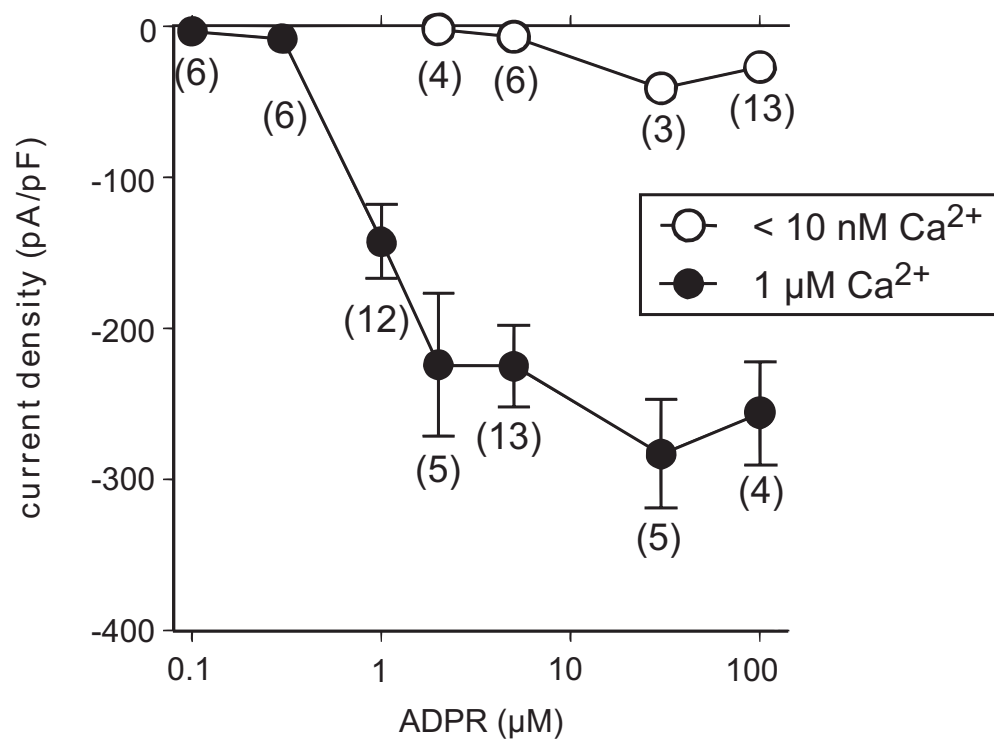




Figure 6

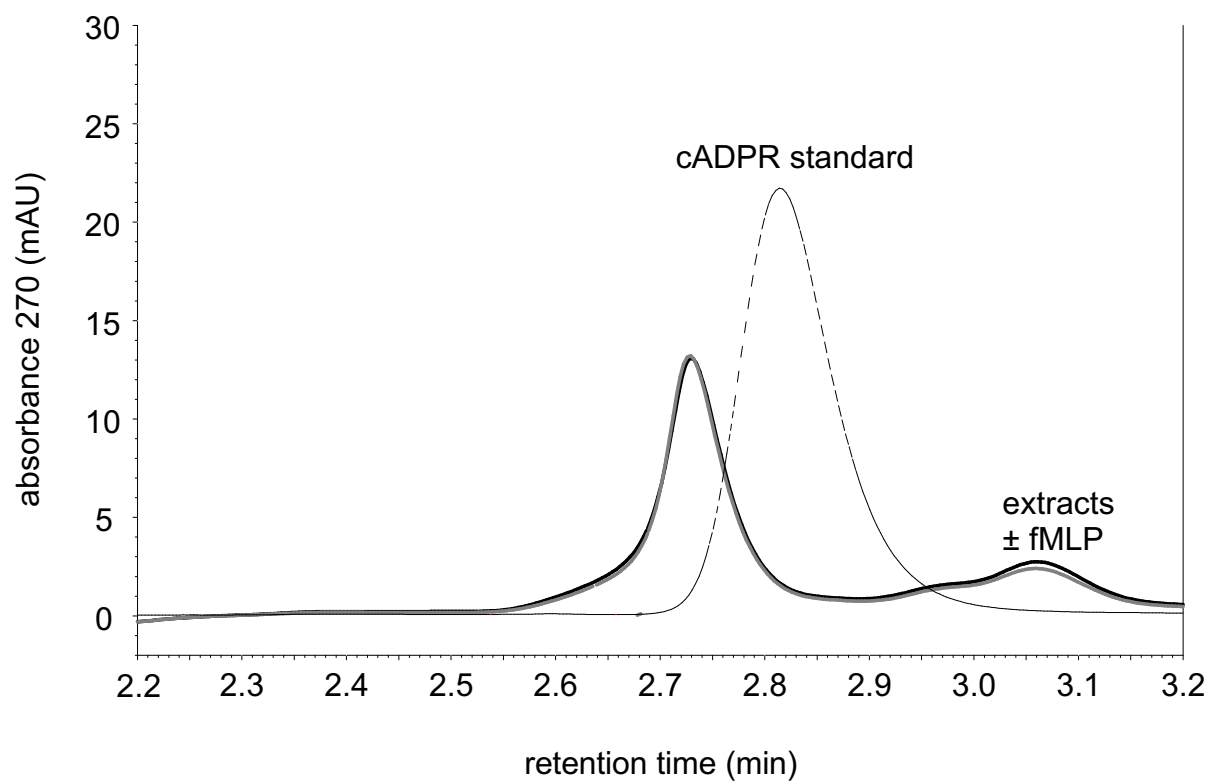


Figure 7

