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Biochimica et Biophysica Acta 1278 (1996) 125–130

BBA Biochimica et Biophysica Acta

A cation non-selective channel induced by extracellular ATP in macrophages and phagocytic cells of the thymic reticulum

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Received 5 April 1995; revised 19 July 1995; accepted 16 August 1995

Abstract

Extracellular ATP^{4-} can bind to P_{2Z} purinergic receptors inducing depolarization and cytoplasmic membrane permeabilization to small molecular weight solutes in macrophages, thymocytes, mast cells, phagocytic cells of the thymic reticulum and other cell types. An ATP^{4-}-induced cation current has been described in whole-cell records of some of these cells but it is currently not clear whether these currents and the phenomenon of membrane permeabilization are a consequence of only one type of P_{2Z}-associated channel/pore or two different phenomena triggered by one or more receptors. Here we use the outside-out patch-clamp technique to describe a single channel associated with this cation current in two murine phagocytic cells: intraperitoneal macrophages and phagocytic cells of the thymic reticulum. Multi channel currents could be readily observed in 77% of the outside-out patches of macrophages. Single channels of 7.8 pS could usually be resolved only in tail currents. Reversal potential measurements and ion replacement experiments indicated a lack of cation selectivity, similarly to what has already been described for the ATP^{4-}-induced whole-cell inward current. No large-conductance channels that could explain the permeabilization to small molecular weight solutes was observed under our experimental conditions. A single channel of approx. 5 pS was also observed in phagocytic cells of the thymic reticulum under similar conditions. We conclude that the channel here described is the main carrier of cation current usually associated with the binding of ATP^{4-} to P_{2Z} receptors in whole-cell and outside-out patch-clamp experiments.

Keywords: ATP; Purinergic receptor; Single channel; Macrophage; Phagocytic cell; (Thymic reticulum)

1. Introduction

It has long been recognized that extracellular nucleotides can influence many tissues and cell types (reviewed in [1–4]. ADP is a strong inducer of platelet aggregation and secretion while ATP can induce constriction and dilation of blood vessels, act as a neurotransmitter or a co-transmitter in certain synapses, and induce cation currents and permeabilization to low molecular weight solutes with a M_r of up to 900 in macrophages, mast cells and other cell types. Recently, extracellular ATP (ATP_o) has been implicated in the regulation of chloride transport in the airway epithelia [5–7] and ATP analogs have been shown to be effective in preventing endotoxic death [8].

It is currently accepted that most of these effects are mediated by at least 5 types of receptors: P_{2X}, P_{2Z}, and P_{2T} receptors are intrinsic cation channels, while P_{2Y} and P_{2U} activate a protein G-dependent IP_3/DG metabolic pathway and the release of intracellular Ca^{2+} stores [1,3,9]. It has also been proposed that P_{2Z} receptors can activate phospholipase D [10] and that P_{2T} receptors may induce a decrease in cAMP concentration [11].

P_{2Z} receptors are specifically activated by the fully ionized form of ATP (ATP^{4-}) and their presence have been described in several cell types, including macrophages, macrophage polykaryons, mast cells, thymocytes, some types of transformed fibroblasts, and megacaryocytes [1,3]. In all cell types were P_{2Z} receptors have been studied, extracellular ATP^{4-} induces membrane depolarization and permeabilization to small molecular

Abbreviations: ATP_o, extracellular ATP; P-TR, phagocytic cell of the thymic reticulum.

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SSDI 0005-2736(95)00200-6

weight solutes with a M_r of up to 900 [1,3]. However, while electrophysiological studies in macrophages [12–15] and mast cells [16] have clearly demonstrated the existence of a non-selective cation conductance, usually associated with P_{2Z} receptors, no evidence for a large ATP_o-induced channel that could explain permeabilization has been described. The cation current has not been resolved at the single channel level but analysis of whole-cell and outside-out tail currents has indicated that these channels should be smaller than 40 pS, possibly in the range of 1–10 pS [12,13,16]. Progress in this field has been difficult due to the expression of multiple types of P_2 purinergic receptor in one cell type, the lack of receptor-specific agonist and antagonists, and, possibly, also due to the large surface density of P_{2Z} receptors expressed in macrophages and mast cells patches.

In this study we use the outside-out patch-clamp technique to describe for the first time the ATP_o-induced cation non-selective single-channels of murine intraperitoneal macrophages and in phagocytic cells of the thymic reticulum (P-TR), a cell that is involved in T cell maturation and retains some functional properties and surface markers of both dendritic and mononuclear-phagocytic lineage cells [17–21]. A small cation channel with conductance in the range of 5–8 pS was found in both phagocytic cell types. Our results suggest that this channel is the main charge carrier involved in ATP_o-induced cation currents usually associated with P_{2Z} receptors.

2. Material and methods

2.1. Cells

Thioglycolate-elicited macrophages obtained from the intraperitoneal cavity of Swiss-Webster mice were transferred to RPMI-1640 medium containing 5% of heat-inactivated fetal calf serum, 2 g/l sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin, and plated in 35 mm Petri dishes as described [22,23]. All surgical manipulations were performed under ether anesthesia. After 1 h of incubation at 37°C in a 5% CO_2 humidified atmosphere, non-adherent cells were removed and the adherent cells were kept in the same conditions for 4 h to 20 days until use.

Non-epithelial phagocytic cells of the thymic reticulum (P-TR) were obtained from Swiss-Webster or Balb/c mice as previously described [17]. Briefly, the thymus was removed and minced into tiny fragments with fine scissors. These fragments were washed several times in RPMI 1640 medium to eliminate a maximum of lymphoid cells, and then incubated in complete medium (RPMI 1640 with 1% L-glutamine, 1% sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum), for 4 days at 37°C. The monolayers of adherent cells were then cultured for more 10–12 days until round cells with a hairy membrane profile started to proliferate and spontaneously detached from the culture plates. These cells were harvested and plated in 35 mm^2 plastic culture dishes to form P-TR-enriched secondary cultures. Experiments were performed 2–15 days later. Besides the selection provided by the methodology, P-TR were further characterized by dendritic morphology, phagocytic capacity, and ability to form rosettes with thymocytes, as well as Ia and CD11a expression [17,19]. The morphology was used to choose the cells used in patch clamp experiments.

2.2. Electrophysiological measurements

Whole-cell and outside-out patch clamping was performed at room temperature (23–30°C) using an EPC-7 amplifier (List Electronic, Darmstadt, Germany) according to standard techniques [24]. Cells were transferred to a culture chamber containing 8 ml of solution, built into the stage of a microscope and giga-ohm seal was formed after off-set potential compensation, using heat-polished micropipettes coated with Silgard® 184 (Dow Corning, Midland, MI, USA). Pipette resistance was in the range of 10–15 MΩ. Unless otherwise specified, the extracellular solution was a Hepes-buffered salt solution containing (in mM) 150 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 Na-Hepes, pH 7.4 (normal extracellular solution). The pipette solution contained (in mM) 150 KCl, 5 NaCl, 1 $MgCl_2$, 10 K_2-EGTA, and 10 K-Hepes, pH 7.2 (normal intrapipette solution). Ion-substitution experiments were performed using the solutions listed in Table 1. Current and voltage were simultaneously registered on a paper chart recorder (Mark VII WR 3310, Graphtec, Yokohama, Japan) and in a VCR tape after digitalization by a NEU-

Table 1
Composition of extracellular and intrapipette solutions [a]

Solution	Composition (mM)							
	KCl	NaCl	Tris-Cl	mannitol	$CaCl_2$	$MgCl_2$	EGTA	Hepes
Normal intrapipette	150	5	–	–	–	1	10	10
Normal extracellular	5	150	–	–	1	1	–	10
Low-potassium intrapipette	34	–	–	158	0.12	0.5	1.4	5
Tris extracellular	5	–	150	–	1	1	–	10

[a] pH was adjusted at 7.4 with NaOH for extracellular solutions and at 7.2 with KOH for intrapipette solutions. In some experiments the normal intrapipette solution contained only 0.1 mM EGTA.

ROCORDER (model DR-390, Neuro Data Instruments, NY, USA).

2.3. Reagents

ATP, EGTA, Tris, mannitol, culture media, and Hepes were purchased from Sigma (St. Louis, MO, USA).

2.4. ATP application

Pneumatic and iontophoretic applications of ATP were performed with micropipettes similar to the ones usually used for intracellular recordings (5 to 40 MΩ when filled with 3 M KCl), positioned 2 to 20 μm away from the cell surface. For pneumatic applications, pressure was manually controlled with the help of a 10 ml syringe and the same extracellular medium used in each experiment was used as solvent. ATP concentrations ranged from 100 μM to 10 mM as specified in the text and figure legends, and pH was adjusted to 7.4. Iontophoretic applications were achieved by applying voltage pulses ranging from -10 to -100 V and lasting 0.1 to 10 s. Distilled and deionized water was used as solvent, pH was not adjusted, and ATP concentration was 10 mM. In control experiments, none of the results here described were observed after the application of normal extracellular solution without ATP.

3. Results

3.1. Cation currents in outside-out patches of macrophages

We have previously described that an ATP_o-induced non-selective cation current and a Ca^{2+}-dependent K^+ current can be readily observed in macrophages under whole-cell patch-clamp conditions [13]. At high intracellular EGTA concentration however only the cation current is detected (Fig. 1A). Most of the outside-out patches obtained from responding cells (77%, $n = 168$) continue to display a current of similar pattern that can be repetitively stimulated by ATP_o applications (Fig. 1B–C). However, while the maximum amplitude the cation currents obtained in whole-cell records at -40 mV were around 8 nS, outside-out currents at the same transmembrane potential were usually smaller than 300 pS, a data consistent with a large reduction of the membrane area. The reversal potential of the outside-out currents are also similar to the whole-cell currents: it is close to 0 mV in normal conditions (Fig. 1B) but shifts to more negative values when extracellular Na^+ is replaced by Tris (Fig. 1C).

3.2. Single-channel currents associated with the ATP_o-induced cation current of macrophages

Resolution of the ATP_o-induced cation current at single channel level was achieved only by analyzing tail currents of patches that displayed the smallest outside-out current amplitudes upon ATP_o stimulation (Fig. 2). Single channel conductance of the most prominent channel was 7.8 ± 0.5 pS ($n = 10$) in normal solutions (Fig. 2A and C). The reversal potential of the channels was close to 0 mV ($+1 \pm 5$ mV, $n = 10$), indicating that they were either cation non-specific or chloride channels. After replacing the normal intrapipette solution by a low-potassium solution (Table 1), the reversal potential shifted to $+34 \pm 1$ mV ($n = 5$), a value consistent with the equilibrium potential of $+36$ mV calculated for the monovalent cations in this solution (Fig. 2B–C). A shift towards negative values (-45 mV) was also observed in one outside-out patch when Na^+ was replaced by Tris in the extracellular medium (not shown). We concluded that a non-selective monovalent-cation channel of 7.8 pS is the main carrier of the P_2 purinergic receptor-associated inward current observed in whole-cell and outside-out patches of macrophages.

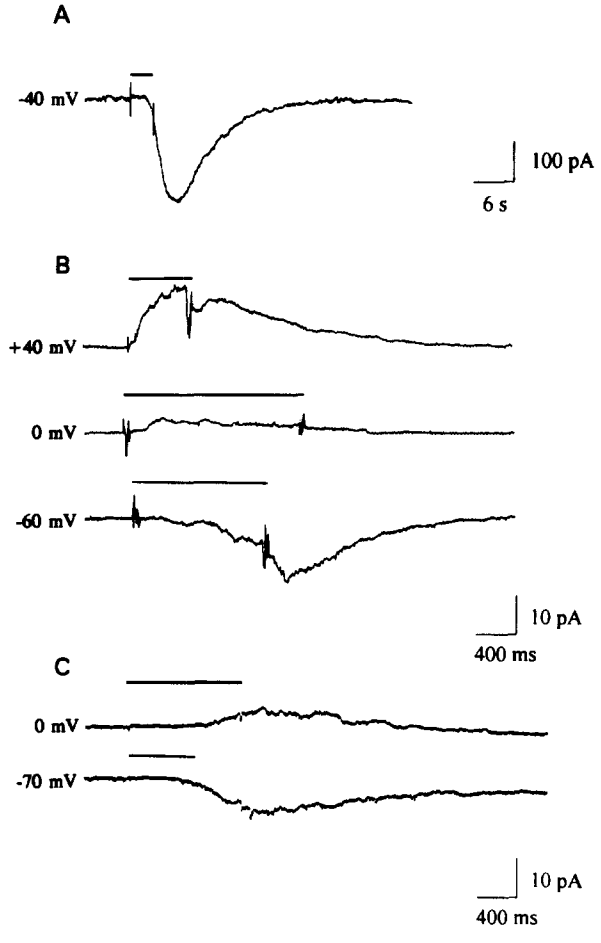

Fig. 1. ATP_o-induced currents in whole-cell and outside-out patches of macrophages. (A) A whole-cell record using normal extracellular and intrapipette solutions. (B) Outside-out record of an membrane patch excised from the same cell in (A) at three different holding potentials. (C) Outside-out record of another macrophage using normal intrapipette solution and the Tris extracellular solution. Iontophoretic applications of ATP are indicated by horizontal bars.

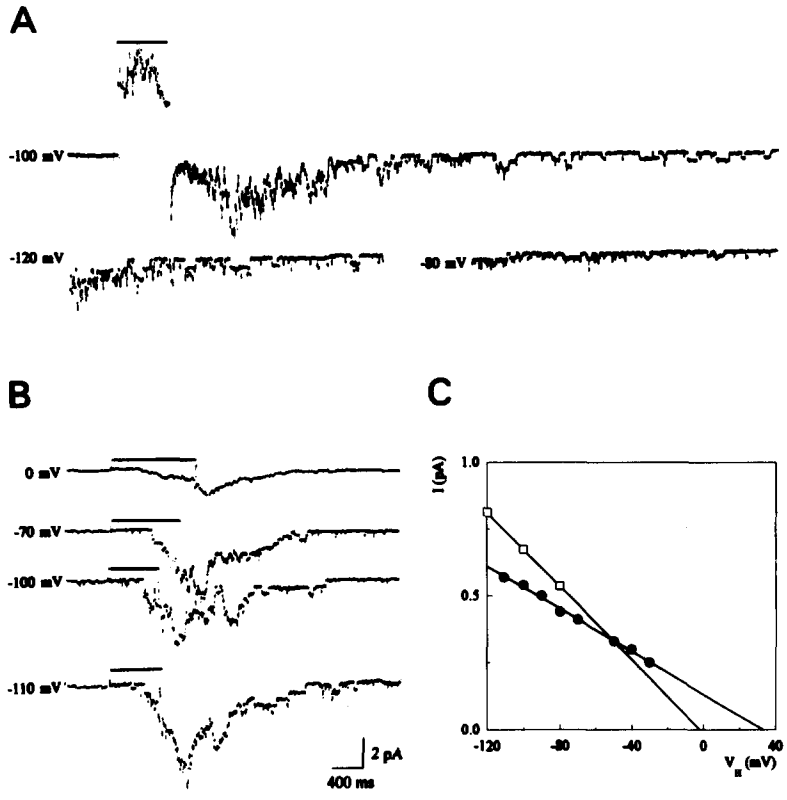

Fig. 2. ATP_o-induced single channels in macrophages. (A) A typical outside-out records showing single channel activity after ATP application using normal extracellular and intrapipette solutions. The same membrane patch is shown after three different stimulus at −100 mV, −120 mV, and −80 mV. (B) Another membrane patch using normal extracellular solution and low-potassium intrapipette solution at four different holding potentials as indicated. Note that an inward current is still present at O mV (upper trace). (C) *I–V* plot of the channels shown in A (open squares) and B (filled circles). Iontophoretic applications of ATP are indicated by horizontal bars. The traces shown here were filtered at 300 Hz during replay.

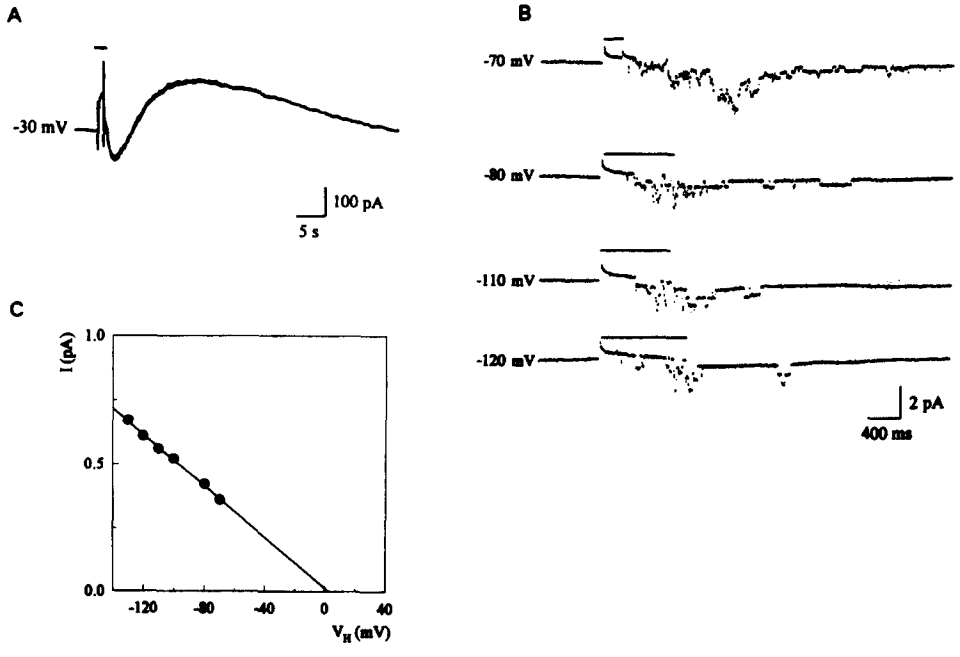

Fig. 3. ATP_o-induced currents in phagocytic cells of the thymic reticulum. (A) A typical biphasic ATP_o-induced current in a whole-cell patch, using normal extracellular solution and an intrapipette solution containing 0.1 mM EGTA. (B) Single channel activity of an outside-out patch of the same cell at four different holding potentials, using the same extracellular and intrapipette solutions. (C) *I–V* plot of the single channel shown in (B). Iontophoretic applications of ATP are indicated by horizontal bars. The traces shown here were filtered at 300 Hz during replay.

3.3. Single-channel currents associated with the ATP_o-induced cation currents of phagocytic cells of the thymic reticulum (P-TR)

The existence of P_2 receptors in several cells of the immune-inflammatory system raises the question of whether they also express the same single channel we have here described for macrophages. Thymus derived cells are specially interesting to be studied due to the important neuro-immune modulatory pathways present in this organ [25,26]. We have recently observed that P-TR also display ATP_o-induced cation current and plasma membrane permeabilization typical of P_{2Z} receptors (unpublished results). We have then chosen these cells to obtain more information about the single channels associated with P_{2Z} receptors (Fig. 3). ATP_o applications can promptly induce biphasic currents observed in whole-cell records similar to what has already been described for macrophages (Fig. 3A) [13]. Outside-out patches excised from ATP_o-responding P-TR displayed only inward currents as in the case of macrophages but cleaner single channel records could be obtained (Fig. 3B). The ATP_o-induced single channel conductance of two patches were 5.0 and 5.4 pS and the reversal potentials were +2.5 and +0.2 mV, respectively (Fig. 3C). No other ATP_o-activated channels were observed in outside-out patches of these cells ($n = 4$) under our experimental conditions.

4. Discussion

We and others have previously described fast-activating ATP_o-induced non-selective cation currents associated with P_2 purinergic receptors in macrophages [12–15], mast cells [16], phagocytic cells of the thymic reticulum (unpublished results), and other cell types at the whole-cell level. This current has been usually associated with P_{2Z} receptors but its relationship with membrane permeabilization, one of the hallmarks of this sub-type of purinergic receptor, has never been established. Attempts to resolve these cation currents at the single channel level has not been successful. Noise analysis has been used in outside-out of mast cells [16], leading to an estimated conductance in the range 1–10 pS. However, at least two other ATP_o-induced single channels have been detected in macrophages: the typical Ca^{2+}-activated K^+ channel was observed in cell-attached experiments using mice peritoneal macrophages [27], and a small divalent cation channel (11 pS) was described in cell-attached and outside-out patches of rat peritoneal macrophages [28]. The later channel was detected using divalent cations as charge carriers and its activity in outside-out membrane patches was dependent on the presence of GTP-γ-S.

The difficulties in obtaining single channel records associated with ATP_o-induced cation currents in cells that display P_{2Z} receptors and the lack of electrophysiological evidence for a large conductance pore, has lead to different hypothesis regarding the relationship between cation currents and membrane permeabilization to solutes with a M_r below 1000, the two hallmarks of these purinergic receptors. In mast cells, it has been suggested that larger pores of variable size could result from fusion of smaller channels [16]. This hypothesis has also been considered for the case of macrophages [12]. However, it is also possible that these are two different phenomena triggered by one or more receptors [1,29]. In macrophages, it has been proposed that the gap-junction protein connexin-43 would be involved in membrane permeabilization [30], but no electrophysiological data supporting the functional expression of connexin-43 pores have so far been described. The recent finding that differential activation of the cation current and the non-selective pores can be achieved in *Xenopus* oocytes expressing macrophage mRNA favor the two-phenomena hypothesis and suggests that some of the specific experimental conditions that has been used in patch-clamp experiments do not favor the formation of large pores [29]. The elucidation of these questions requires the resolution at the single channel level of the currents associated with P_{2Z} receptors.

Here, we report for the first time the existence of a small (5–8 pS) cation channel associated with the inward current induced by application of ATP_o in two phagocytic cell types. In macrophages, it was usually observed in tail currents of outside-out patches but in P-TR, cleaner records were obtained. Ion replacement experiments using macrophages showed that its reversal potential shifted from +1 mV in normal solutions to +34 mV in the low-potassium intrapipette solution, a value close to +36 mV, the calculated value for the equilibrium potential of monovalent cations in this solution. Our results suggest that the channel we have described displays a non-selective monovalent cation conductance. These values are similar to what have already been described for the whole-cell currents [13], leading us to conclude that this 5–8 pS channel is the main carrier of the cation current associated with the binding of ATP^{4-} to P_2 purinergic receptors in whole-cell and outside-out patch-clamp records of macrophages and P-TR. Although this current is triggered by ATP^{4-} and, therefore, has been associated with P_{2Z} receptor, additional experiments using more specific agonists are required to clarify this point.

The conductance of a whole macrophage and of a macrophage patch stimulated by ATP_o could reach up to 8 nS and 300 pS respectively, corresponding to at least 1025 channels per cell and 38 channels per patch. These data indicate that the noisy records frequently observed in macrophages are at least in part due to the large number of channels usually present in membrane patches, but the presence of other channels not triggered by ATP in some patches (data not shown) may also be involved.

The results here described for two different cell types are indicative that other cells that also express P_{2Z} recep-

tors may have channels with similar conductance. In accordance to this hypothesis, it has been proposed on the bases of noise analysis performed in outside-out membrane patches that the single channel conductance associated with the cation current of mast cells is in the range of 1 to 10 pS [16].

The fact that large cation currents and single channels can be obtained in outside-out membrane patches using pipettes filled with high EGTA solution without GTP analogs, is consistent with the current view that P_{2Z} receptors are associated with intrinsic ion channel [3].

Although some of the effects of ATP_o has been identified for more than two decades, the physiological role of P_2 purinergic receptors and the endogenous sources of agonists are not well established. The expression of P_{2Z} and other purinergic receptors in several cells of the immune-inflammatory system make them potential targets for pharmacological intervention in several immune-pathologic situations. The possibility of performing a more specific, receptor-based intervention improves with the increasing comprehension of the several receptor subtypes and the availability of new purinergic analogs. Two recent examples were obtained in the literature: P_{2U} receptors of airway epithelia is being considered as a target to help control fluid transport in the lungs of cystic fibrosis patients [6] and 2-methylthio-L-ATP, a weak agonist of P_{2Y} receptors, has been used to block endotoxic syndrome in LPS-injected mice [8]. Electrophysiological analysis of receptor–ligand interaction can be a powerful tool to support pharmacological progress in this field.

Acknowledgements

The authors dedicate this paper in honor of Prof. Carlos Chagas Filho, founder of the Institute of Biophysics, on the occasion of its 50th anniversary. We are grateful to Mariana P. Silveira and Vandir da Costa for continuous technical support, and to Drs. Roberto Saraiva and David C. Spray for critical reviews of the manuscript and helpful discussions. This work was financed by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Financiadora de Estudos e Projetos (FINEP), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

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