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Adenine dinucleotide-mediated cytosolic free Ca^{2+} oscillations in single hepatocytes

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Single rat hepatocytes microinjected with aequorin respond to Ca^{2+}-mobilizing agonists, including ADP and ATP, with oscillations in cytosolic free Ca^{2+}. We show here that single rat hepatocytes also respond to the adenine dinucleotides Ap_3A and Ap_4A with Ca^{2+} oscillations which resemble those induced by ADP and ATP.

Ca^{2+} oscillation; Adenine dinucleotide; Purinoceptor; Single hepatocyte

1. INTRODUCTION

Adenine dinucleotides comprise a family of naturally occurring molecules consisting of two adenosine moieties linked by a variable number of phosphates. The adenine dinucleotides Ap_3A and Ap_4A have been identified in a wide variety of mammalian and other cells [1]. Diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A) and diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) comprise up to 5% of all adenine nucleotides stored in the dense bodies of platelets and released into the extracellular environment following platelet activation [2]. Chromaffin cells also store adenine dinucleotides in large amounts [3]. Ap_4A and Ap_5A are co-stored with catecholamines, AMP, ADP and ATP in the secretory granules and, upon release, could reach concentrations of up to 100 μM in the extracellular fluid [2,4]. That adenine dinucleotides could reach physiologically significant concentrations in the extracellular environment, and their relatively long half-lives compared to ATP [2,4,5], have led to investigations of their potential role as novel extracellular effectors. Adenine dinucleotides have thus been found to be active in the platelet activation process [2], in the modulation of smooth muscle tone [5] and in inducing exocytotic secretion from chromaffin cells [6].

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Abbreviations: $[Ca^{2+}]_{free}$, cytosolic concentration of free Ca^{2+}; Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; $Appp_sA$, diadenosine 5′,5‴-P^1,P^3-(P^1-thio)-triphosphate (mixed isomers); Ap_sppp_sA, (S_pS_p)diadenosine 5′,5‴-P^1,P^4-dithiotetraphosphate.

Recent studies have demonstrated the sensitivity of liver to extracellular adenine dinucleotides. In perfused isolated rat liver Ap_3A and Ap_4A stimulate glucose output and a transient net release of Ca^{2+} [7]. In isolated hepatocytes a series of naturally occurring adenine dinucleotides stimulate a dose-dependent activation of glycogen phosphorylase similar to that observed with ATP [8]. The nature of the purinoceptor(s) and transduction mechanism through which the adenine dinucleotides exert these effects remains to be established.

Extracellular ATP and ADP act on rat hepatocytes via P_{2y} purinoceptors (as defined by the classification of Burnstock and Kennedy [9]) to stimulate the hydrolysis of phosphatidylinositol 4,5-biphosphate and subsequent elevation of intracellular calcium [10]. Of interest is the recent finding that Ap_4A and Ap_5A, like ATP [11], evoke an increase in intracellular Ca^{2+} in chromaffin cells [12].

Oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) have been demonstrated in many cell types, including hepatocytes, in response to agonists acting via the phosphoinositide signalling pathway [13,14]. Furthermore, the profile of the $[Ca^{2+}]_{free}$ transients generated by an aequorin-injected hepatocyte is characteristic of the stimulating agonist [15]. Aequorin measurements of $[Ca^{2+}]_{free}$ in single rat hepatocytes have shown that ADP and ATP, thought to act through the same P_{2y} purinoceptor [16], elicit transients of very different profiles in the majority of cells. ADP invariably induces transients of short duration, whereas ATP induces either similar short transients, transients of a longer duration, or a mixture of short and long transients within a single response [17].

The aim of the present studies was to establish

whether the adenine dinucleotides exert their effects on hepatocytes via $[Ca^{2+}]_{free}$ oscillations. The present paper therefore describes the effect of extracellular Ap_3A and Ap_4A on $[Ca^{2+}]_{free}$ in single aequorin-injected hepatocytes.

2. MATERIALS AND METHODS

Hepatocytes were isolated from fed male 150–250 g Wistar rats and prepared for microinjection as described previously [15]. Microdialysis of aequorin, microinjection and data acquisition have been described previously [18]. The experimental medium was Williams Medium E (Gibco) gassed with CO_2/air (1:19) at 37°C. Adenine dinucleotides were added to this medium.

The adenine dinucleotides Ap_3A and Ap_4A were obtained from Sigma. Collagenase was from Boehringer. The phosphorothioate analogues $Appp_sA$ and Ap_sppp_sA were generously provided by G.M. Blackburn, Dept. of Chemistry, University of Sheffield, U.K.

3. RESULTS

Single aequorin-injected hepatocytes responded to extracellular ADP and ATP by the generation of $[Ca^{2+}]_{free}$ oscillations (results not shown) similar in duration and profile to those previously reported [17].

Single hepatocytes, microinjected with aequorin, responded to extracellular Ap_3A and Ap_4A with a series of oscillations in $[Ca^{2+}]_{free}$. The majority of hepatocytes (14 out of 15 cells) responded to Ap_3A, at concentrations above a threshold of approximately 1 μM, with $[Ca^{2+}]_{free}$ transients that were consistent in their duration and profile with a fast falling phase. A typical result is shown in Fig. 1. A single cell was found which failed to respond to Ap_3A at concentrations up to 10 μM but generated $[Ca^{2+}]_{free}$ oscillations in response to 1 μM phenylephrine both before and after stimulation with Ap_3A.

The $[Ca^{2+}]_{free}$ response of hepatocytes to Ap_4A was more variable than that to Ap_3A. In all 17 cells examined Ap_4A (above an approximate threshold concentration range of 1–5 μM) evoked oscillations in $[Ca^{2+}]_{free}$, although the duration of the individual transients varied from cell to cell and, in some cells, between individual transients within a single response. The responses recorded can broadly be classified into three groups, as shown in Fig. 2.

(a) 6 out of 17 cells responded to extracellular Ap_4A with $[Ca^{2+}]_{free}$ oscillations of short duration, consistent in profile (as shown in Fig. 2a) and similar to those recorded in response to Ap_3A (Fig. 1).
(b) 6 out of 17 cells responded to extracellular Ap_4A with $[Ca^{2+}]_{free}$ transients that were consistent in duration, but which had a longer falling phase than those in group (a). A typical result is shown in Fig. 2b. The oscillations within each transient are real and not the result of stochastic noise in the signal.
(c) 5 out of 17 cells responded with $[Ca^{2+}]_{free}$ oscillations whose duration varied from transient to transient. A typical result is shown in Fig. 2c.

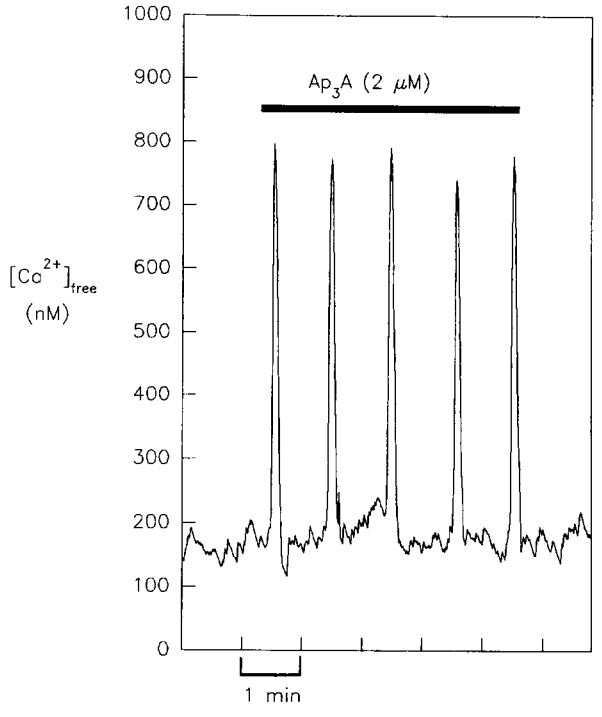

Fig. 1. A single hepatocyte, microinjected with aequorin, was superfused for the period indicated with 2 μM Ap_3A. The time constant for the resting $[Ca^{2+}]_{free}$ was 10 s, and for oscillations 1 s.

The possibility must be addressed that, since the extracellular metabolism of adenine dinucleotides may lead to the liberation of ATP, ADP, AMP and adenosine, the observed effects of extracellular Ap_3A and Ap_4A may be the result of action of these catabolic products rather than of the uncleaved dinucleotides. This appears unlikely since it has previously been demonstrated that, while ATP is completely degraded to adenosine in 30 s, Ap_4A is only marginally degraded after 2 min incubation in a suspension of hepatocytes [8]. Furthermore, in the studies described here, the significant accumulation of ADP and ATP as intermediate catabolites at the cell membrane was less likely, since the single hepatocyte was constantly superfused with medium, thereby providing a continuous supply of fresh adenine dinucleotides and removal of any breakdown products.

The conclusion that the intact adenine dinucleotides are responsible for the observed effects was confirmed by experiments using phosphorothioate analogues of Ap_3A and Ap_4A. These analogues show a much lower rate of cleavage by specific Ap_3A and Ap_4A hydrolases and non-specific phosphodiesterases [19]. Single aequorin-injected hepatocytes responded to extracellular $Appp_sA$, a phosphorothioate analogue of Ap_3A, with a series of $[Ca^{2+}]_{free}$ oscillations similar in time

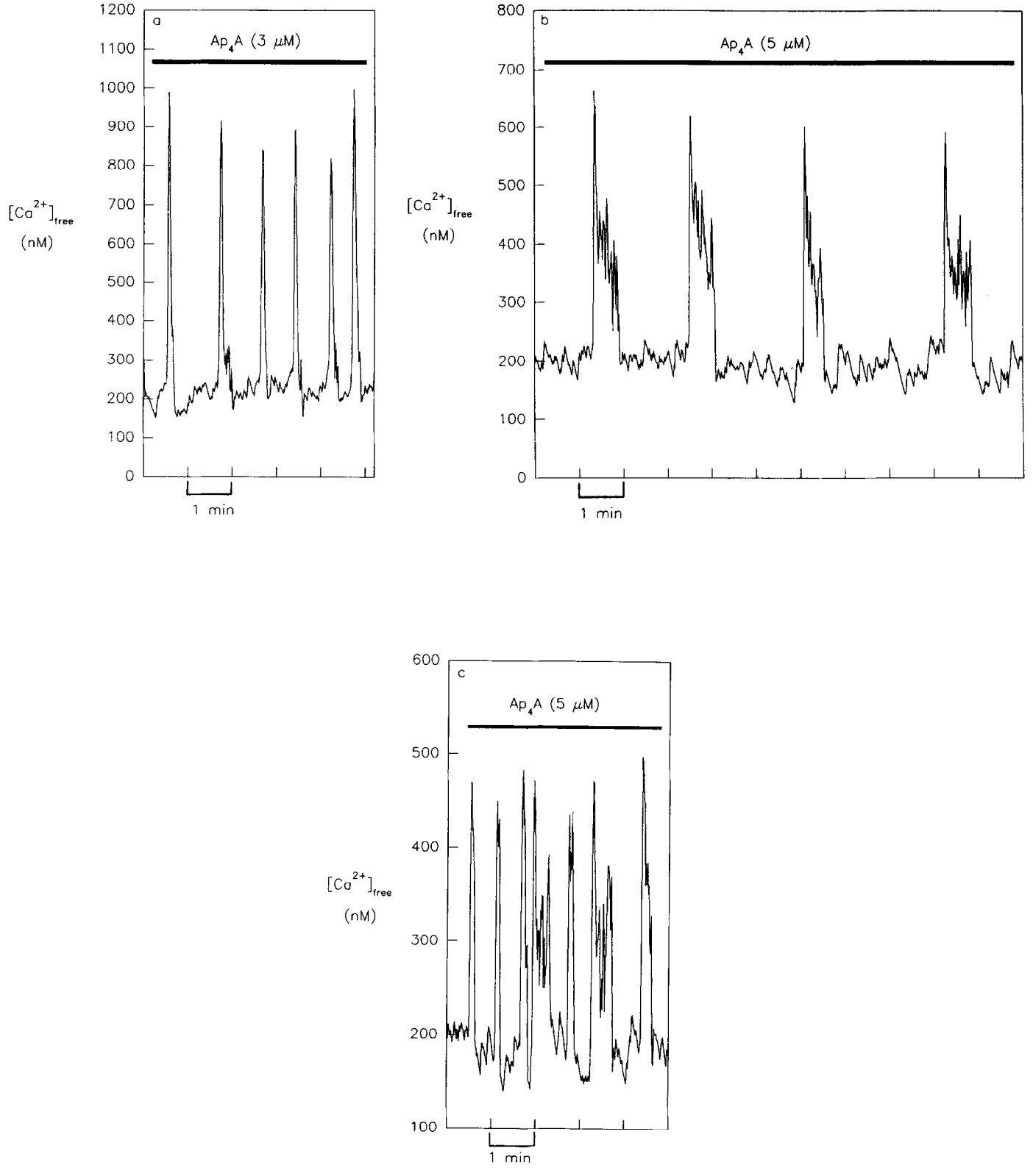

Fig. 2. Variability in the $[Ca^{2+}]_{free}$ response to Ap_4A. Three different hepatocytes, microinjected with aequorin, were superfused with Ap_4A for the periods indicated. The time constants were as in Fig. 1.

course to those evoked by Ap_3A (5 out of 5 cells examined). Extracellular Ap_spppp_sA, a phosphorothioate analogue of Ap_4A, evoked $[Ca^{2+}]_{free}$ oscillations in 3 out of 4 cells examined. It is thus apparent that the intact adenine dinucleotides are biologically active in the stimulation of $[Ca^{2+}]_{free}$ oscillations in single rat hepatocytes.

4. DISCUSSION

The present work is the first demonstration that extracellular adenine dinucleotides can evoke $[Ca^{2+}]_{free}$ oscillations in cells. We have shown here that single aequorin-injected rat hepatocytes respond to extracellular Ap_3A and Ap_4A with a series of $[Ca^{2+}]_{free}$ oscillations. Furthermore, we have shown that the $[Ca^{2+}]_{free}$ oscillations evoked by Ap_3A and Ap_4A are attributable to the actions of the uncleaved dinucleotides. As outlined in the introduction, Ap_3A and Ap_4A are released into the extracellular environment by a variety of cell types and, upon release, may attain physiologically significant concentrations in plasma. Since, compared to mononucleotides, adenine dinucleotides have relatively long half- lives in plasma [2], the results presented in this paper indicate that Ap_3A and Ap_4A, acting directly as uncleaved dinucleotides, may have an important in vivo role as extracellular effectors of Ca^{2+} mobilization in hepatocytes.

It is of interest that the concentrations of extracellular Ap_3A and Ap_4A required to elicit $[Ca^{2+}]_{free}$ oscillations in single hepatocytes are similar to those shown to activate glycogenolysis in hepatocyte populations [8]. It thus appears that the adenine dinucleotide-dependent stimulation of glycogenolysis described previously [8] is mediated via oscillations in $[Ca^{2+}]_{free}$. These observations suggest that extracellular Ap_3A and Ap_4A, like ADP and ATP, act on hepatocytes via the phosphoinositide signalling pathway. This has been shown to be the case in two other cell types. Ap_4A and Ap_5A, like ATP, stimulate an increase in cytosolic $[Ca^{2+}]_{free}$ in a population of resting chromaffin cells [12], apparently acting via a putative P_{2y} purinoceptor [20]. In cultured mesangial cells of rat renal glomeruli, Ap_3A and Ap_4A, like ADP and ATP, appear to induce the release of IP_3 into the cytoplasm [2].

It remains to be determined whether hepatocytes possess unique purinoceptors specific for adenine dinucleotides or whether Ap_3A and Ap_4A share the P_{2y} purinoceptor(s) of ATP and ADP. A unique membrane receptor for Ap_4A has been reported in mouse brain that may also be present in other tissues, including liver [21]. It is of interest that the $[Ca^{2+}]_{free}$ oscillations induced by Ap_3A resemble those induced by ADP [17] in that the transients induced were invariably of short duration. It may prove to be relevant that Ap_3A possesses the same charge as ADP under physiological conditions. It is notable that ATP was previously the only agonist found to induce a variable response [17]; other agonists always elicit consistent transient profiles characteristic of the stimulating agonist [15]. The response to Ap_4A is thus reminiscent of that elicited by ATP in that considerable variation exists in the duration of transients induced. Detailed analysis of transients induced by adenine dinucleotides may provide information pertinent to the characterization of the hepatic purinoceptor(s).

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