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By Releasing ADP, *Acanthamoeba castellanii* Causes an Increase in the Cytosolic Free Calcium Concentration and Apoptosis in Wish Cells

A. MATTANA,[1]* M. G. TOZZI,[1] M. COSTA,[1] G. DELOGU,[2] P. L. FIORI,[2] AND P. CAPPUCCINELLI[2]

Department of Pharmacological Sciences[1] and Department of Biomedical Sciences, Division of Experimental and Clinical Microbiology,[2] University of Sassari, Sassari, Italy

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The role played by soluble molecules that may participate in acanthamoebal cytopathogenicity has yet to be fully characterized. We demonstrate here that *Acanthamoeba castellanii* trophozoites constitutively release ADP in the medium. Cell-free supernatants prepared from *A. castellanii*, by interaction with specific P_{2y2} purinoceptors expressed on the Wish cell membrane, caused a biphasic rise in $[Ca^{2+}]_i$, extensive cell membrane blebbing, cytoskeletal disorganization, and the breakdown of nuclei. Cell damage induced by amoebic supernatants was blocked by the P_{2y2} inhibitor Suramin. The same results were found in Wish cells exposed to purified ADP. These findings suggest that pathogenic free-living *A. castellanii* may have a cytopathic effect on human epithelial cells through ADP release, by a process that begins with a rise of cytosolic free-calcium concentration, and culminates in apoptosis.

Acanthamoeba is a genus of small free-living amoebas characterized by a life cycle of active trophozoites and dormant cysts (21, 28). Human infection due to *Acanthamoeba* spp. involving the brain, eyes, lungs, and skin has increased significantly during the last 10 years (10, 13, 16). Numerous in vitro studies, carried out to elucidate the virulence factors responsible for *Acanthamoeba* infections, have shown both contact-dependent cell killing and cell-free cytopathogenicity due to metabolites released by trophozoites (14, 15, 22), which confirms that amoebic exotoxins, enzymes, or other unidentified molecules may be involved in the interactions between the amoebas and host cells. The role played by soluble molecules that may participate in acanthamoebal cytopathogenicity has yet to be completely elucidated.

Alizadeh et al. (2, 22) have reported that corneal epithelial cells, melanoma cells, and murine neuroblastoma cells exposed to aqueous extracts of *Acanthamoeba* trophozoites undergo lysis by a process involving apoptosis.

We have previously shown (17) that heat-resistant and nonproteinic molecules with a low molecular weight (<10 kDa), released by viable *A. castellanii* trophozoites, produce a serious cytopathic effect in human epithelial Wish cells in vitro, causing a cytosolic-free-calcium ($[Ca^{2+}]_i$) increase, morphological changes, cytoskeletal alterations, a decrease in cell viability, and cell death. Since the increase in cytosolic free-calcium concentration was immediate in Wish cells, in this connection we also hypothesized that the $[Ca^{2+}]_i$ rise may be the prime cause of cell death, but the identity of the molecules inducing calcium overload and the mechanisms of cell death were not characterized.

The present investigation was undertaken to identify the chemical nature of these amoebic toxic compounds and to further characterize their cytotoxic action on human epithelial Wish cells.

Our study was performed using trophozoites of *A. castellanii*, isolated from a case of amoebic keratitis (in Ancona, Italy), axenically grown at 25°C in PYG medium (11). The species identification of this isolate was based on cyst morphology and indirect immunofluorescence microscopy. Our previous observations have shown that this *A. castellanii* isolate, at 37°C, exerted contact-dependent cell killing on Wish cells.

Preparation of amoebic cell-free supernatants. Amoebic cell-free supernatants were prepared as described previously (17). Amoebas were washed twice in phosphate-buffered saline solution (PBS) without Ca^{2+} and Mg^{2+} at pH 7.4 were resuspended (6×10^6 cells/ml) in the same buffer or in RPMI medium containing 20 mM HEPES and incubated for 2 h at 25°C. Supernatants of these cultures, obtained by centrifugation at $500 \times g$ for 15 min, were treated at 95°C for 10 min, ultrafiltered through Centriprep-10 microconcentrators (Amicon), which have a molecular cutoff of 10 kDa, and used immediately after processing as cPBS and cRPMI, respectively. In particular, cPBS was used only in $[Ca^{2+}]_i$ measurements, since some aromatic RPMI medium components could interfere with this assay.

Characterization of heat-resistant compounds. To characterize the chemical nature of the heat-resistant compounds with low molecular weight released by *Acanthamoeba* trophozoites we used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and capillary electrophoresis techniques. Several peptides, such as staphylococcal aplha-toxin and delta-toxin (5), dissolving in the lipid bilayer have been shown to increase the membrane permeability to specific inorganic ions and to produce cytolysis. Tricine SDS-PAGE experiments, performed according to the method of Schagger and von Jagow (26), nevertheless excluded the presence of small peptides in both cPBS and cRPMI (data not shown). Capillary electrophoresis analysis of amoebic-cell-free super-

* Corresponding author. Mailing address: Dipartimento di Scienze del Farmaco, Via Muroni 23/A, 07100 Sassari, Italy. Phone: 0039-79-228744/228711. Fax: 0039-79-228712. E-mail: dsfanto@ssmain.uniss.it.

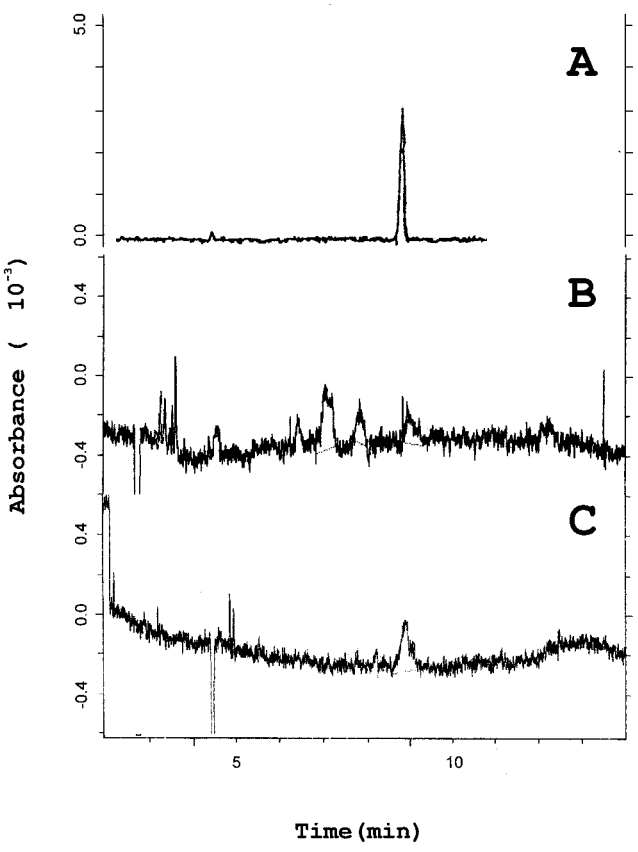

FIG. 1. Electropherograms of 100 μM purified ADP solution (A), heat-treated filtered cell-free RPMI (cRPMI) conditioned for 2 h by *A. castellanii* (6×10^6 trophozoites/ml) (B), or heat-treated filtered cell-free PBS (cPBS) conditioned for 2 h by *A. castellanii* (6×10^6 trophozoites/ml) (C).

natants was performed according to the method of Banditelli et al. (4), with a P/ACE 2100 System (Beckman Instruments) at 20 kV, using capillary tubing 50 μm in diameter and 50 cm long and a buffer of 40 mM glycine and 50 mM sodium dihydrogen phosphate (pH 9). These experiments have shown the presence of ADP in significant micromolar concentrations (Fig. 1), and the ADP concentration in both cPBS and cRPMI was calculated to be about 20 μM. Similar results were obtained when we incubated amoebae for 2 h at 37°C. Extracellular purine nucleosides and nucleotides are ubiquitous, phylogenetically ancient intercellular signals that can interact with specific cell surface receptors to mediate a variety of biological responses (20, 23). In the past decade, the cytotoxic properties of extracellular nucleotides have received a lot of attention. It has been shown, in fact, that ATP and ADP can affect the plasma membrane permeability of cultured cells (9, 27) and cause elevation of the $[Ca^{2+}]_i$ concentration and apoptosis in certain types of cell (1, 7, 19, 25, 30). In order to establish the role of amoebic released-ADP in *A. castellanii* cell-free cytopathogenicity, therefore, we have compared the cytopathic effect exerted on human epithelial Wish cells, by both ultrafiltered *Acanthamoeba* cell-free supernatants and external ADP (ADP_0).

$[Ca^{2+}]_i$ measurement. The Wish cell line was routinely maintained in RPMI 1640 medium (GIBCO BRL/Life Technologies Italy) supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL/Life Technologies Italy), 100 U of penicillin G, and 100 μg of streptomycin sulfate per ml and grown in 25-cm^2 plastic flasks at 37°C in a 5% CO_2 atmosphere. Intracellular calcium in Wish cells was monitored by using the fluorescent calcium probe FURA 2-AM (Sigma-Aldrich S.r.l., Milano, Italy). Dye loading was standardized by incubating cells, suspended in HEPES buffer (137 mM NaCl, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM KCl, 15 mM glucose; pH 7.4); with 3 μM Fura 2-AM for 10 min at room temperature. Loaded cells were washed twice with the same buffer, and the assay was performed on a stirred aliquot (0.5 ml), at 37°C, with the use of a Hitachi F-2000 spectrophotometer. The excitation and emission wavelengths were 340 to 380 nm and 510 nm, respectively, detected every 500 ms, and stored in separate memories of the F-2000 spectrophotometer. A data Menager was used to monitor the fluorescence signal of Fura-2 AM-loaded cells. Basal and stimulated cytosolic calcium were quantified according to the method of Grynkiewicz et al. (12) by using the ratio technique and a K_d of 224 nM as the dissociation constant of Fura-2 AM; maximal and minimal values of fluorescence were evaluated after the addition of of 0.006% Triton X-100 and 10 mM EGTA, respectively. Hitachi F-2000 software was used for calculation.

In single isolated Wish cells, the resting level of the cytosolic-free-calcium was calculated to be 196.38 ± 12.72 nM (n = 42), and the addition of PBS alone did not cause variation ($[Ca^{2+}]_i$ = 191.96 ± 18.44; n = 12). Stimulation with 80 μl of 20 μM ADP_0 (Sigma-Aldrich S.r.l.) or with 80 μl of cPBS (containing approximately the same concentration of amoebic released ADP) led to a rapid biphasic increase of $[Ca^{2+}]_i$, which consisted of an initial transient elevation, maintained for up to 100 s, followed by a sustained elevation at levels higher than the basal value (Fig. 2 A and B). These experiments resulted in a peak increase in $[Ca^{2+}]_i$ of 165.82 ± 20.26 nM (n = 6) or 217.82 ± 31.95 nM (n = 6), respectively, and between these conditions any statistical difference was shown by a two-tailed Student's t test calculation. We further investigated the mechanism of ADP_0 and cPBS to induce $[Ca^{2+}]_i$ increase on Wish cells. Upon stimulation of the Wish cells, with ADP_0, with external ATP (ATP_0; Sigma-Aldrich S.r.l.) or with adenosine (Sigma-Aldrich S.r.l.) at micromolar concentrations (3.2, 6.4, and 12.8 μM), the potency of the different purine nucleotides in elevating the $[Ca^{2+}]_i$ was ATP > ADP, whereas adenosine was inefficient. Both 12.8 μM ADP_0 and 12.8 μM ATP_0 resulted in peak increases of $[Ca^{2+}]_i$ of 375.14 ± 20.7 nM (n = 6) and 624.70 ± 24.6 nM (n = 6), respectively. These results indicated that the response to either nucleotide was mediated by functional P_2 purinoceptors, expressed on the Wish cell membrane. The classification of P_2-purinergic receptors distinguishes two major classes: ionotropic P_2 purinoceptors (P_{2x}), which are ligand-gated receptors containing an intrinsic ion channel, and metabotropic P_2 purinoceptors (P_{2y}), which belong to the superfamily of G protein-coupled receptors (9). Multiple subclasses of P_{2y} purinoceptors are well known ($P_{2y1-2y5}$). Upon testing the effect of several specific antagonists, we identified and characterized the nucleotide receptor expressed in Wish cells on which ADP_0 acted.

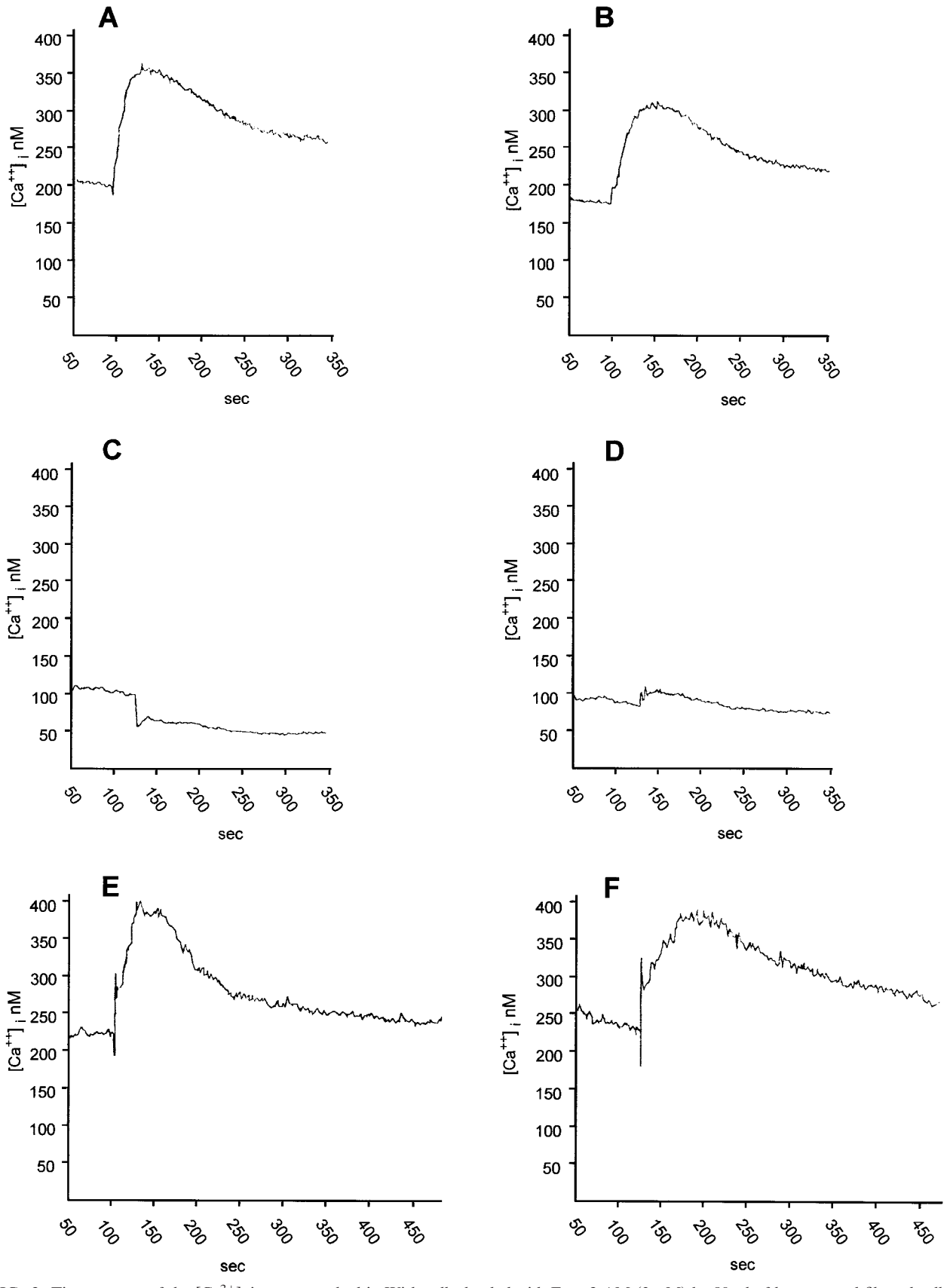

FIG. 2. Time courses of the $[Ca^{2+}]_i$ increase evoked in Wish cells, loaded with Fura 2-AM (3 μM) by 80 μl of heat-treated filtered cell-free PBS (cPBS), conditioned for 2 h by (6×10^6/ml) *A. castellanii* trophozoites (A) by the same volume of PBS containing 20 μM ADP_0 (B); by cPBS (C) or 20 μM ADP_0 (D) after chelation of extracellular Ca^{2+} with 5 mM EGTA; and by cPBS (E) and 20 μM ADP_0 (F) after 20 min of loaded Wish cell exposure to 10 μM ryanodine.

TABLE 1. Effect of two purinergic antagonists on the cytosolic free-calcium peak increase ($\Delta[Ca^{2+}]_i$) induced on Wish cells stimulated with heat-treated filtered cell-free PBS–20 mM HEPES conditioned for 2 h by *A. castellanii* trophozoites (6×10^6/ml) (cPBS) or PBS–20 mM HEPES containing 20 μM ADP_0[a]

Sample	Mean $\Delta[Ca^{2+}]_i$ (nM) ± SE with:					
	No treatment	Suramin (P_{2x}–P_{2y2} antagonist) at:			PPADS (P_{2x} antagonist) at:	
		10 μM	15 μM	20 μM	5 μM	10 μM
cPBS	217.82 ± 31.95	58.28 ± 3.12*	38.70 ± 2.76*	0 ± 0*	236.91 ± 29.54†	241.27 ± 34.18†
PBS-ADP (20 μM)	165.82 ± 20.26	24.84 ± 1.16*	20.02 ± 1.10*	0 ± 0*	218.49 ± 15.80†	236.44 ± 36.04†

[a] Cells stimulation was obtained with 80 μl of the sample indicated. Values are means ± the standard error of at least six experiments. *, $P < 0.01$ versus the respective controls; †, not significant. The basal value of the $[Ca^{2+}]_i$ in Wish cells is 196 ± 12.72 nM ($n = 42$).

Suramin (Sigma-Aldrich S.r.l.), a compound known to compete with ATP and ADP for their binding sites (P_{2x} and P_{2y2} receptor antagonists), nearly abolished the effect of both ADP_0 and cPBS on a $[Ca^{2+}]_i$ increase, while the purinergic P_{2x} receptor antagonist pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; Sigma-Aldrich S.r.l.) was ineffective (Table 1). This clearly indicated that cPBS, like external ADP, acted on P_{2y2} purinergic receptors expressed in Wish cell membranes to induce calcium overload. It is known that, by means of this nucleotide receptor, micromolar concentrations of ADP are sufficient to activate Ca^{2+} mobilization, inositol-1,4,5-triphosphate accumulation and Ca^{2+} influx in most cells (24).

To further investigate the Ca^{2+} signaling pathway coupled to the action exerted by ADP_0 and cPBS on Wish P_{2y2} receptors, we removed extracellular Ca^{2+} by chelation with 5 mM EGTA. In this condition ADP_0 and cPBS evoked solely the initial phase of $[Ca^{2+}]_i$ peak, but the response was now transient, and no sustained plateau phase could be observed (Fig. 2C and D). To further explain whether the rise of $[Ca^{2+}]_i$ could be dependent on ion release from intracellular calcium stores, target cells were exposed to ryanodine (Sigma-Aldrich S.r.l.) that blocks release of calcium ions from endoplasmic reticulum (18). Ryanodine treatment of Wish cells for 20 min did not abolish the $[Ca^{2+}]_i$ increase induced by ADP_0 or cPBS; however, the initial phases of the response were significantly reduced (Fig. 2E and F). These results suggested that, in response to both ADP_0 and cPBS, the initial phase of the cytosolic-free-calcium increase was caused by depletion of the intracellular calcium stores, whereas the peak and the plateau phase depended on the transmembraneous influx of Ca^{2+}. Amoebic soluble metabolites which could act directly on Wish cell calcium channels were not present in cPBS; in fact, the calcium channel blocker Diltiazem (Sigma-Aldrich S.r.l.) did not abolish the cPBS-induced $[Ca^{2+}]_i$ increase (data not shown).

Phase-contrast microscopy, actin microfilament analysis, and assessment of apoptosis. It is known that $[Ca^{2+}]_i$ deregulation is an important link and signaling event in cells following a variety of injuries. The $[Ca^{2+}]_i$ increase, by activation of proteases, endonucleases and phospholipase, can cause cellular prelethal changes which can occur by two principal patterns: oncosis or apoptosis (29). It has also been demonstrated that changes in cytoskeletal organization can be relevant for the cell destiny (6). Hence, to characterize the cytopathic effect induced by amoebic-cell-free supernatant and ADP_0, Wish cell monolayers growing on glass coverslips, in a CO_2 incubator at 37°C, were exposed for 1, 3, 6, 9, and 24 h to 0.5 ml of cRPMI (obtained as described above), 0.5 ml of RPMI containing 20 μM ADP_0, or the same volume of fresh medium (cell controls). At the selected time intervals, the cell morphology was observed with a Zeiss (Tilaval 31) microscope equipped with a 40 × lens. After incubation with cRPMI or RPMI containing 20 μM ADP_0, Wish cells underwent morphological changes which are typical of classical apoptosis. In both cases they developed extensive cell membrane blebs, nuclear condensation, and overall cell shrinkage (Fig. 3A, C, and E). Cell damage, on the contrary, was not observed in samples incubated for up to 9 h, with cRPMI or RPMI containing 20 μM ADP_0, in the presence of 20 μM suramin (Fig. 3B, D, and F).

Actin microfilaments, at the same selected time intervals, were visualized by using rhodamine-conjugated phalloidin (Sigma-Aldrich S.r.l.) as we had previously described (17). In control cells, F-actin organized in stress fibers was mainly distributed at the cell periphery as a continuous thin circumferential band (Fig. 4A). The exposure to cRPMI or to RPMI containing 20 μM ADP_0, in a time-dependent way, caused the cells to become round and led to the breakdown of the actin network (Fig. 4C, D, F, and G). In addition, Wish cells exposed to ADP_0 or cRPMI showed the same fluorescence pattern on actin microfilaments.

Cell monolayers growing on coverslips under the same experimental conditions as those described above were also examined by light microscopy after DAPI (4′,6′-diamidino-2-phenylindole) staining in order to visualize the nuclei. After incubation, the cells were washed with PBS, fixed in 70% ethanol at 0°C, and stained with 2× SSC buffer (1× is 0.15 M NaCl plus 0.015 M sodium citrate) containing 200 ng of DAPI(Sigma-Aldrich S.r.l.) per ml. Coverslips, repeatedly washed with 2× SSC buffer–0.05% Tween 20, were mounted in Gelvatol (Monsanto Corp.) and then examined with a Nikon Optiphot microscope. The fluorescence microscopy of nuclei showed different forms of chromatin aggregation and nuclear fragmentation that increased in proportion to the exposure time to both cRPMI and RPMI containing 20 μM ADP_0. After 3 of incubation all control cells showed a normal nuclear morphology (Fig. 4B), while about 11% of nuclei were apoptotic in cells exposed to cRPMI or ADP_0 (Fig. 4E and H).

Conclusions. The present findings show that *A. castellanii* trophozoites constitutively release ADP in the medium and suggest that this compound might play an important role in cell-free cytopathogenicity due to *A. castellanii*. Our data, albeit indirectly, demonstrate that ADP is very probably the

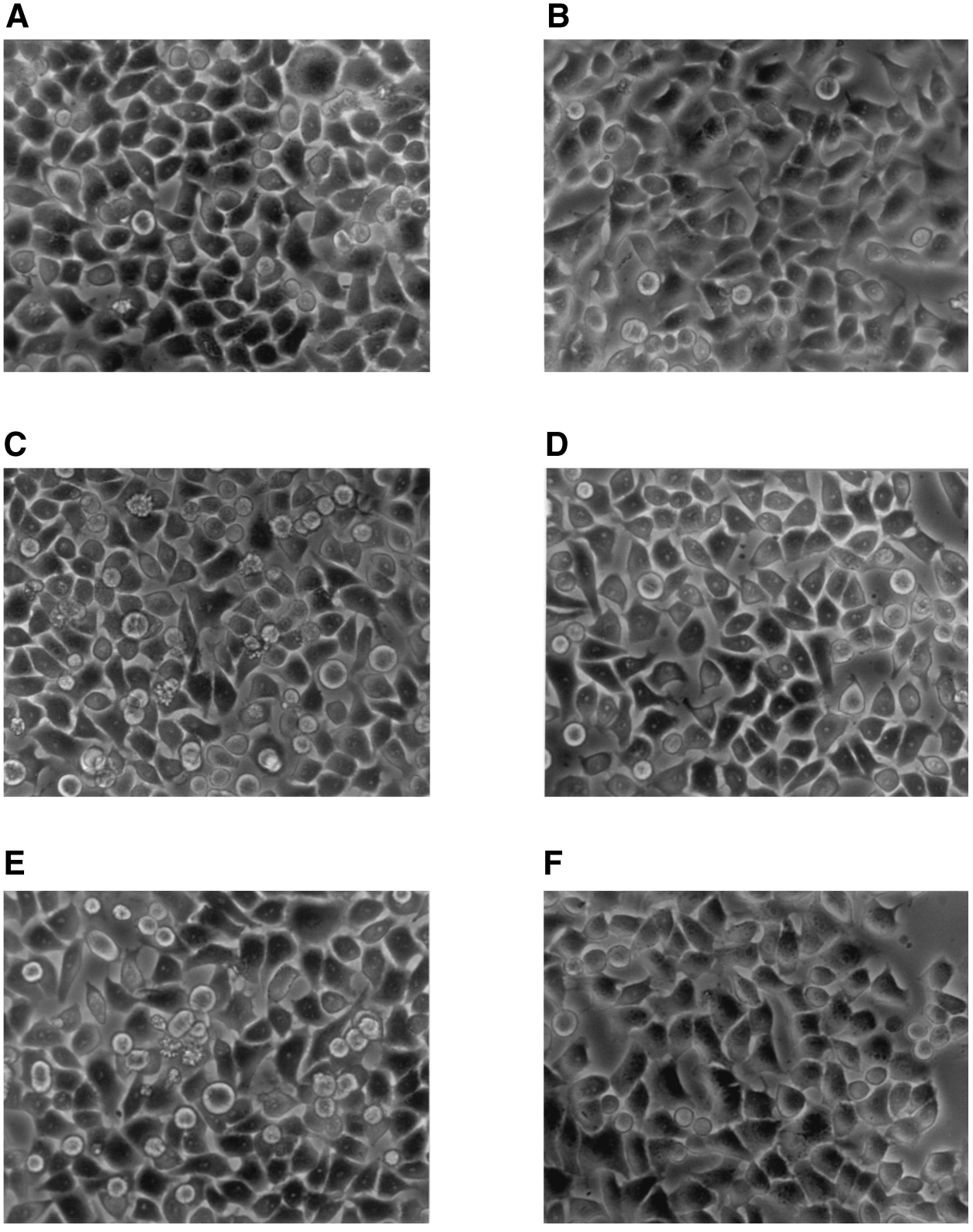

FIG. 3. Phase-contrast microscopy of Wish cells incubated for 3 h at 37°C in 5% CO_2 atmosphere with RPMI medium in the absence (A) or in the presence (B) of 20 μM suramin; with heat-treated filtered cRPMI, conditioned for 2 h by 6×10^6 *A. castellanii* trophozoites, in the absence (C) or in the presence (D) of 20 μM suramin; or with RPMI containing 20 μM ADP_0 in the absence (E) or in the presence (F) of 20 μM suramin. Magnification, × 400.

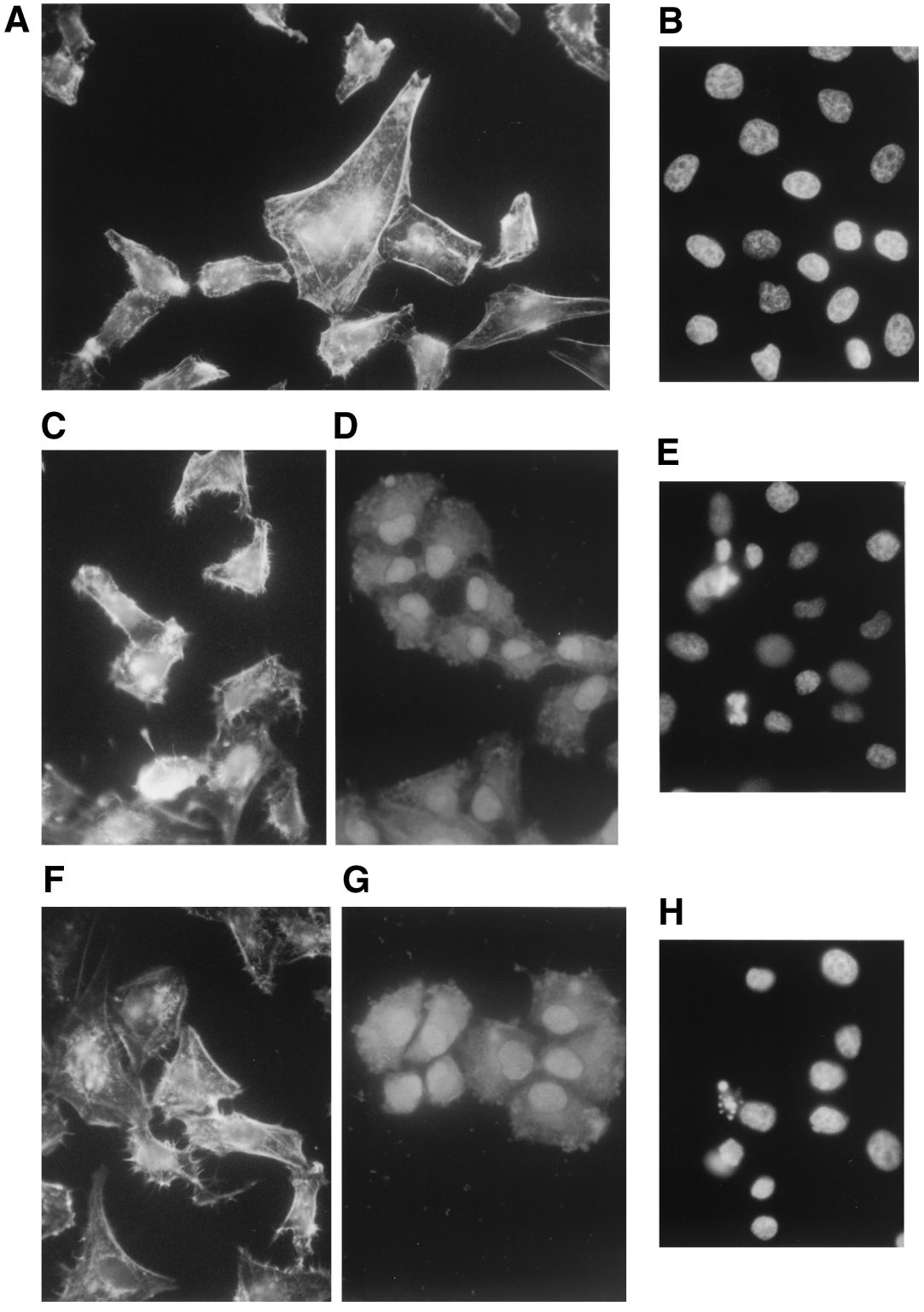

FIG. 4. Actin microfilaments of Wish cells incubated for 3 h at 37°C in 5% CO_2 atmosphere with RPMI medium (A), with cRPMI for 3 h (C) and 6 h (D), or with RPMI containing 20 μM ADP_0 for 3 h (F) and 6 h (G) and nuclei of Wish cells incubated for 3 h at 37°C in a 5% CO_2 atmosphere with RPMI medium (B), with cRPMI (E), or with RPMI containing 20 μM ADP_0 (H). Magnifications: A, C, D, F, and G, ×420; B, H, and F, ×350.

heat-resistant low-molecular-weight component of the amoebic-cell-free supernatant that causes increase in cytosolic free calcium, morphological changes, cytoskeletal damage, and death in Wish cells. In fact, purified ADP and *A. castellanii* culture supernatants cause a similar pattern of calcium fluxes and apoptotic cell death that are blocked by the P_{2y2} inhibitor suramin. It has been shown that ATP can also interact with P_{2y2} cell surface receptors to induce elevation of cytosolic free calcium and apoptosis in mammalian cells. In our experimental conditions, however, only ADP has been detected in the conditioned medium. We still do not know whether apoptotic cell death caused by both cRPMI and purified ADP depends directly on ADP molecules or also on ADP-metabolite generates, for example, by cleavage of the phosphate groups. Further studies are necessary to conclusively demonstrate this and to determine whether the ADP released from *A. castellanii* trophozoites is derived from exocytotic granules and/or vesicles or from the cytosolic ADP pool via intrinsic plasma membrane channels or pores.

Extracellular purine nucleotides are a universal and primitive system of intercellular signals that are capable of modulating several cellular functions. Some amoebae, e.g., *Dictyostelium discoideum*, use purines as intracellular messenger as well as for intercellular signaling (8). There is now significant evidence that extracellular ATP acts as an additional lytic mediator involved in cell-mediated cytotoxicity (24). In fact, it is released from activated cytotoxic T lymphocytes and, by interacting with cell surface purinoreceptors, induces cell death through both colloido-osmotic lysis and apoptosis (3).

Therefore, it is not surprising that *Acanthamoeba* may use the release of ADP as a system to kill the target cells. It is possible that, in *Acanthamoeba*-mediated cytolysis, released ADP may act as an extracellular messenger molecule that works together with other known or unknown secreted agents. To our knowledge, however, this is the first time that results have been presented that show purinic nucleotides as cytotoxic mediators involved in the interactions that occur between pathogenic free-living amoebae and the host cells.

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