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Tenidap enhances P2Z/P2X$_7$ receptor signalling in macrophages

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Abstract

Tenidap is an anti-inflammatory drug whose mechanism of action is not fully understood. It has been shown to block plasma membrane anion transport and to decrease release of interleukin-1β, probably via the inhibition of interleukin-1β converting enzyme. In the present study we showed that: (a) tenidap increases the sensitivity of mouse macrophages to cytotoxic effects mediated by extracellular ATP; (b) tenidap increases lucifer yellow uptake through the macrophage ATP receptor; (c) pretreatment with oxidised ATP, a blocker of the P2Z/P2X$_7$ receptor, inhibits cytotoxicity and lucifer yellow uptake due to the combined effects of ATP and tenidap; (d) macrophages lacking the P2Z/P2X$_7$ receptor are resistant to the synergistic effect of tenidap and ATP. The results suggest that tenidap synergises with extracellular ATP for activation of the P2Z/P2X$_7$ receptor.

Keywords: Tenidap; ATP; P2Z/P2X$_7$ purinoceptor; Macrophage; Inflammation

1. Introduction

The ability to modulate cytokine release is a major goal in the therapy of inflammatory diseases. Attention has been devoted to a novel compound, tenidap, that has shown powerful in vivo anti-inflammatory activity (Blackburn et al., 1991; Sipe et al., 1992; Pelletier et al., 1993; McNiff et al., 1994; Laliberte et al., 1994). While the cellular basis of this anti-inflammatory activity is not well understood, in vitro experiments demonstrated that, in human macrophages, tenidap blocks the release of interleukin-1β caused by several agonists such as bacterial lipopolisaccharide, exogenous ATP and cytotoxic T lymphocytes (Laliberte et al., 1994; Perregaux et al., 1996). The mechanism is not clear, but it may involve the activity of tenidap as inhibitor of plasma membrane anion transport (McNiff et al., 1994).

A novel plasma membrane receptor of immune cells has attracted interest as a potential modulator of interleukin-1β release from mononuclear phagocytes, the P2Z/P2X$_7$ purinoceptor (Perregaux and Gabel, 1994; Ferrari et al., 1996, 1997). This receptor, that selectively binds extracellular ATP, very likely in its fully dissociated form (ATP^{4-}), was originally described in macrophages by Steinberg and Silverstein (1987), extensively characterized in our laboratory (Murgia et al., 1992; Falzoni et al., 1995; Di Virgilio, 1995) and was cloned by Surprenant et al. (1996). P2Z/P2X$_7$ belongs to the subfamily of ligand-gated P2X receptors and is structurally related to a newly identified family of plasma membrane molecules with two transmembrane hydrophobic domains (Surprenant et al., 1995). Other members of this family are Mec-4 of *Caenorhabditis elegans*, mscL of *Escherichia coli*, Kir of β pancreatic acini and amiloride-sensitive epithelial Na^+ channels (North, 1996). Although the physiological function of the P2Z/P2X$_7$ receptor is unknown, some interesting cellular responses are triggered by its activation: apoptotic or necrotic cell death (Murgia et al., 1992; Molloy et al., 1994), release of mature interleukin-1β (Perregaux and Gabel, 1994; Ferrari et al., 1997) and loss of L-selectins (Jamieson et al., 1996). Furthermore, it has also been suggested that this receptor might be involved in the formation of multinucleated giant cells during chronic granulomatous inflammation (Falzoni et al., 1995). These observations point to the P2Z/P2X$_7$ receptor as a potential target for immunomodulation.

We now show that tenidap strongly potentiates the cytotoxic effect of ATP in the J774 mouse macrophage

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cell line and suggest that this is due to synergism at the P2Z/P2X$_7$ receptor.

2. Materials and methods

2.1. Cells

The J774 mouse macrophage cell line was grown in Dulbecco' s modified Eagle medium supplemented with 10% heat-inactivated horse serum, penicillin (100 μg/ml) and streptomycin (100 μg/ml). ATP-resistant J774 cells were selected by repeated rounds of incubation in the presence of 5 mM ATP followed by cloning by limiting dilution. In lucifer yellow uptake experiments, the medium was replaced by a Na^+ saline solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 5.5 mM glucose, 5 mM $NaHCO_3$ and 20 mM Hepes (pH 7.4).

2.2. Measurement of enzymatic activity

Lactate dehydrogenase activity was measured as previously described (Bergmeyer, 1983).

2.3. Microscopy and measurement of changes in plasma membrane permeability

Phase contrast and fluorescence pictures were taken with an inverted microscope (Olympus IMT-2, Olympus Optical, Tokyo, Japan) equipped with a 40× objective. ATP-dependent increases in plasma membrane permeability were measured with the extracellular fluorescent tracer, lucifer yellow (Molecular Probes, Eugene, OR, USA). Cell monolayers were incubated in normal saline for 15 min at 37°C in the presence of various ATP concentrations, 0.25 mM sulfinpyrazone and 1 mg/ml lucifer yellow (Falzoni et al., 1995). After rinsing with complete medium to remove extracellular dye, the cells were examined with the fluorescence microscope.

3. Results

Fig. 1A shows the ATP dose dependence of lactic dehydrogenase release from mouse macrophages in the presence or absence of two different concentrations of tenidap. As documented from previous studies (Steinberg and Silverstein, 1987; Murgia et al., 1992; Laliberte et al., 1994), a 5-h incubation with ATP caused a cytolytic effect that was clearly detectable at concentrations above 2 mM (closed circles). The simultaneous addition of 50 μM tenidap together with ATP caused a dramatic shift to the left of the dose–response curve (closed squares). An increase in the tenidap concentration to 200 μM caused a further shift of the curve to the left, but maximal release was not enhanced (closed triangles). Interestingly, the

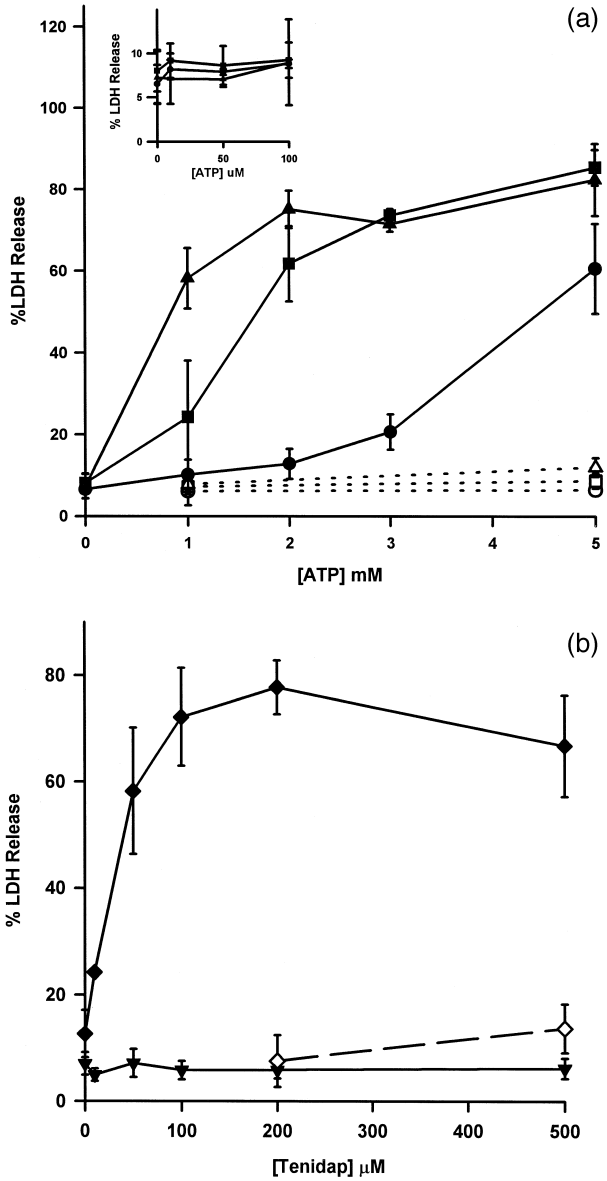

Fig. 1. Dose-dependence of lactic dehydrogenase release in the presence of ATP and tenidap. J774 macrophages were incubated at 37°C in serum-free DMEM medium for 5 h in the presence or absence of the various stimulants. Lactic dehydrogenase release (LDH), expressed as percentage of that in Triton-X100-treated samples, was measured as described in Section 2. Panel A: ATP alone, ●-●; ATP plus tenidap 50 μM, ■-■; ATP plus tenidap 200 μM, ▲-▲; pretreatment with 300 μM oxidized ATP for 2 h followed by ATP, ○-○; pretreatment with 300 μM oxidized ATP for 2 h followed by ATP plus 50 μM tenidap, □-□; pretreatment with 300 μM oxidized ATP for 2 h followed by ATP plus 200 μM tenidap, △-△. Panel B: tenidap, ▼-▼; tenidap plus 2 mM ATP, ◆-◆; pretreatment with 300 μM oxidized ATP followed by tenidap plus 2 mM ATP, ◇-◇. Data are means±S.D. of triplicate determinations of experiments repeated on three different occasions.

maximal cytotoxic effect of 5 mM ATP was very close (about 60% lactic dehydrogenase release) to that observed in the presence of either 50 or 200 μM tenidap (70%–80% release), suggesting that the same macrophage subpopulation was a target for both ATP and tenidap. Pre-treatment

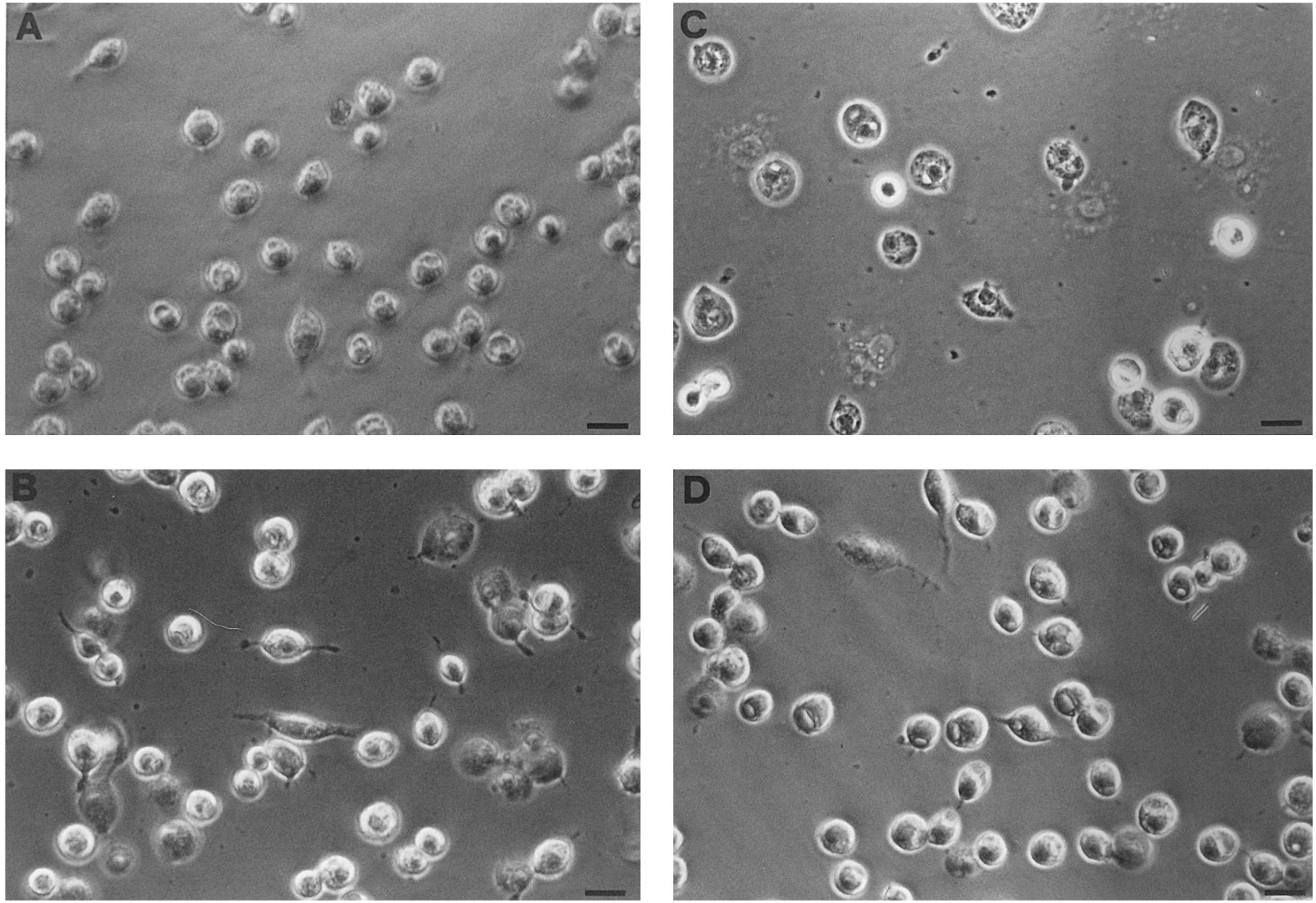

Fig. 2. Oxidized ATP prevents morphological changes associated with cell lysis and due to the combined addition of ATP and tenidap. J774 macrophages were plated in 24 well dishes at a concentration of 20×10^4 in 1 mM Ca^{2+}-containing normal saline and treated for 15 min as follows: panel A, tenidap 200 μM; panel B, 300 μM oxidized ATP followed by tenidap 200 μM; panel C, tenidap 200 μM plus 1 mM ATP; pretreatment with 300 μM oxidized ATP followed by tenidap 200 μM plus 1 mM ATP. Bars = 25 μM.

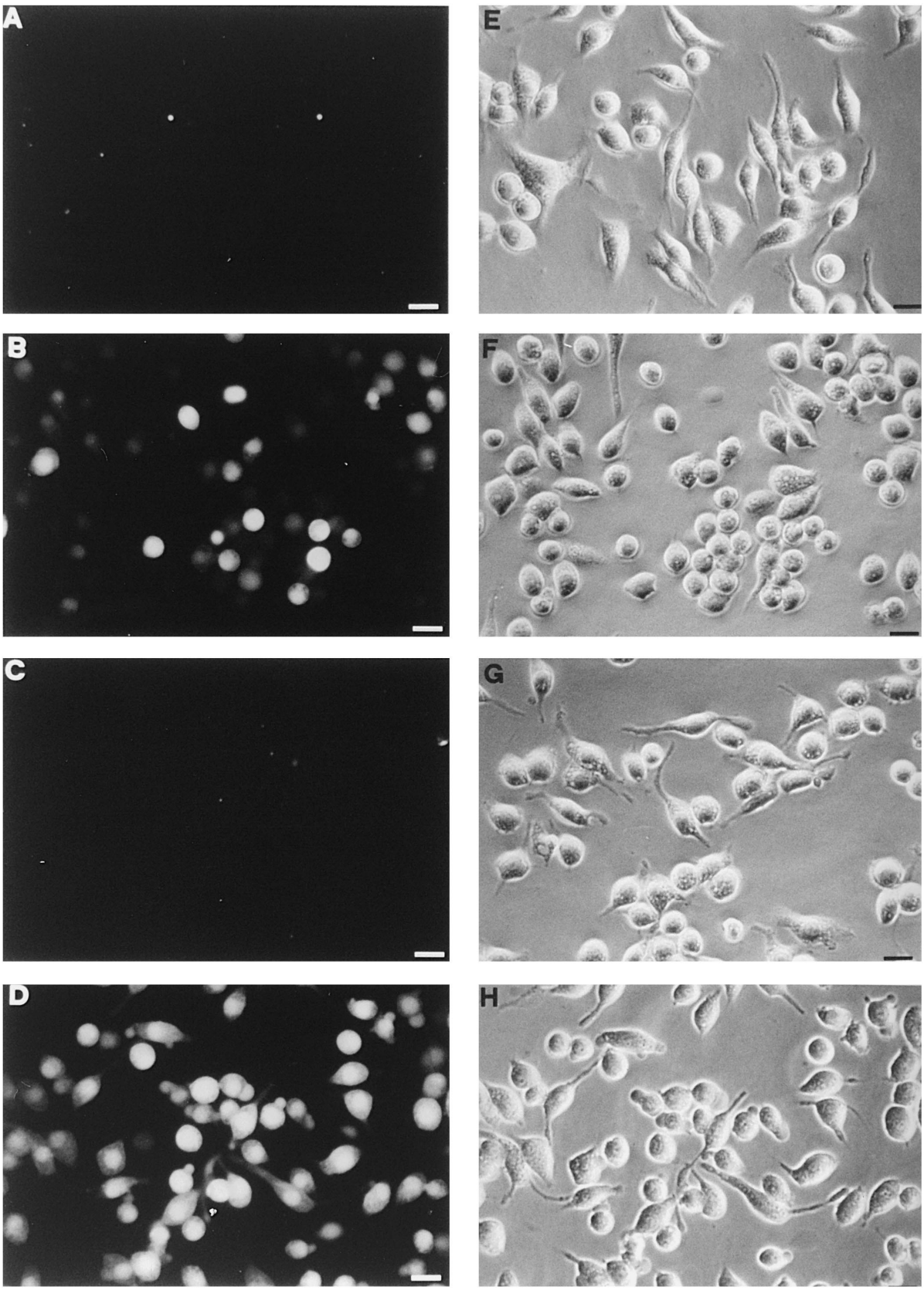

Fig. 3. Effect of the combined addition of ATP plus tenidap on lucifer yellow uptake. Macrophages were incubated as described in Fig. 2. Panels A and B, controls; panels B and E, 3 mM ATP; panels C and G, 50 μM tenidap; panels D and H, 50 μM tenidap plus 3 mM ATP. Bars = 25 μM.

with the P2Z/P2X$_7$ receptor antagonist, oxidised ATP (Murgia et al., 1993) completely blocked the cytotoxic response whether in the absence (open circles) or presence of 50 (open squares) or 200 μM (open triangles) tenidap. At ATP concentrations below 100 μM, i.e., below the threshold for activation of the P2Z/P2X$_7$ receptor (Steinberg and Silverstein, 1987; Murgia et al., 1992; Surprenant et al., 1996), no synergism was observed with either 50 or 200 μM tenidap (insert in Fig. 1A). Although in the presence of 2 mM ATP, synergism was maximal at 100 μM tenidap, a clear cut effect was detectable even at 10 μM (Fig. 1B, closed diamonds). Oxidized ATP prevented the synergism between tenidap and ATP at any tenidap concentration tested (Fig. 1B, open diamonds). Tenidap itself was not toxic at doses up to 500 μM. The effect on cell morphology of the combined application of tenidap plus ATP was dramatic even after a short incubation, 15 min, as shown in Fig. 2, panel C. Oxidized ATP not only prevented the release of lactic dehydrogenase, as shown in Fig. 1, but also prevented morphological changes (Fig. 2, panel D).

The intracellular mechanism of ATP-mediated cytotoxicity has not been extensively investigated, nonetheless it is well established that the first step is binding and activation of the P2Z/P2X$_7$ receptor (Murgia et al., 1992; Surprenant et al., 1996). This receptor/pore renders the plasma membrane permeable to low molecular mass (<900 Da) solutes thus causing a dramatic imbalance of intracellular homeostasis that is the likely cause of cell death. We thus investigated whether macrophages treated with tenidap were more susceptible to ATP-mediated plasma membrane permeabilization. Fig. 3 presents one experiment representative of four others showing that tenidap increased the number of brightly fluorescent macrophages after stimulation with 3 mM ATP, a just-above-threshold concentration for plasma membrane permeabilization in this cell type, in the presence of the fluorescent tracer, lucifer yellow (cf. panels B and D). Furthermore, in the presence of both tenidap and ATP, most macrophages showed the typical rounding and swelling associated with activation of the P2Z/P2X$_7$ receptor, and became loosely adherent to the substrate (panel H). Tenidap itself had no effect on lucifer yellow uptake (panels C and G).

Tenidap is a powerful inhibitor of plasma membrane anion transport, thus potentiation of ATP-dependent cytotoxicity might be due to perturbation of intracellular anion homeostasis. This seems unlikely since 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), a well known blocker of anion transport, increased neither ATP-dependent lactic dehydrogenase release (Fig. 4) nor ATP-dependent lucifer yellow uptake (Fig. 5). In three experiments, each performed in triplicate on different occasions, ATP at the threshold concentration of 1 mM caused lucifer yellow uptake in an average ± S.D. of 10 ± 4% of the macrophages; the addition of 50 μM tenidap increased the number of lucifer yellow-positive cells to 24 ± 0.3%, while

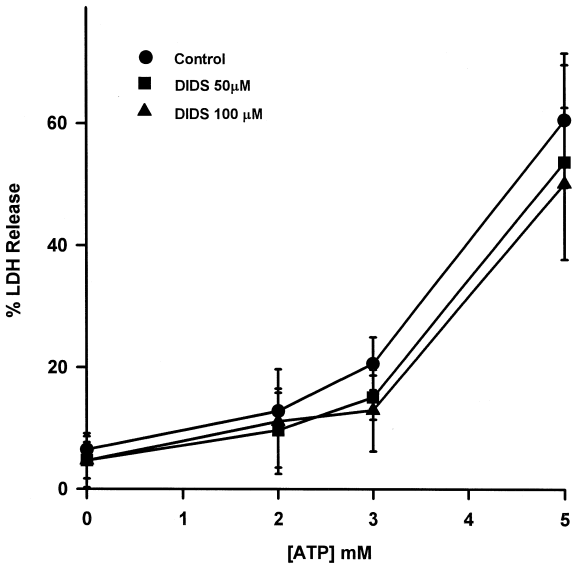

Fig. 4. Lack of synergism of ATP plus DIDS on lactic dehydrogenase release. Macrophages were plated as described in Fig. 1 and treated with ATP (●-●) plus 50 (▲-▲) or 100 (■-■) μM DIDS for 5 h. Data are means ± S.D. of triplicate determinations of experiments repeated on three different occasions.

in the presence of DIDS (100 μM) 12 ± 4% were lucifer yellow-positive (Table 1).

If, as we hypothesize, synergism between tenidap and ATP occurred at the P2Z/P2X$_7$ receptor, then macrophages lacking this receptor should be resistant. This was investigated in J774 macrophage clones selected for lack of expression of the P2Z/P2X$_7$ receptor (Murgia et al., 1992; Chiozzi et al., 1996). As shown in Figs. 6 and 7, the combined addition of tenidap and ATP caused neither release of lactic dehydrogenase nor uptake of lucifer yellow by these cells.

4. Discussion

Immune cells express plasma membrane receptors for extracellular ATP that have been grouped into two broad families, P2Y and P2X purinoceptors (Abbracchio and Burnstock, 1994; Burnstock, 1996). P2Y are typical seven membrane-spanning receptors coupled via G-protein to inositol trisphosphate generation and Ca^{2+} release from intracellular stores. P2X on the contrary are ligand-gated channels that exhibit broad ionic selectivity. So far, three P2X receptors have been described in immune cells: P2X$_1$, P2X$_4$ and P2Z/P2X$_7$ (Di Virgilio, 1995; Surprenant et al., 1996; Buell et al., 1996; Soto et al., 1996). The P2X$_1$ receptor is expressed by HL60 human myelocytic cells, P2X$_4$ has been detected in rat white blood cells, while P2X$_7$ is mainly expressed by mononuclear phagocytes. Compelling evidence suggests that P2X$_7$ is the cytolytic ATP receptor previously named P2Z: transfection of P2X$_7$ cDNA confers sensitivity to ATP-mediated cytotoxicity,

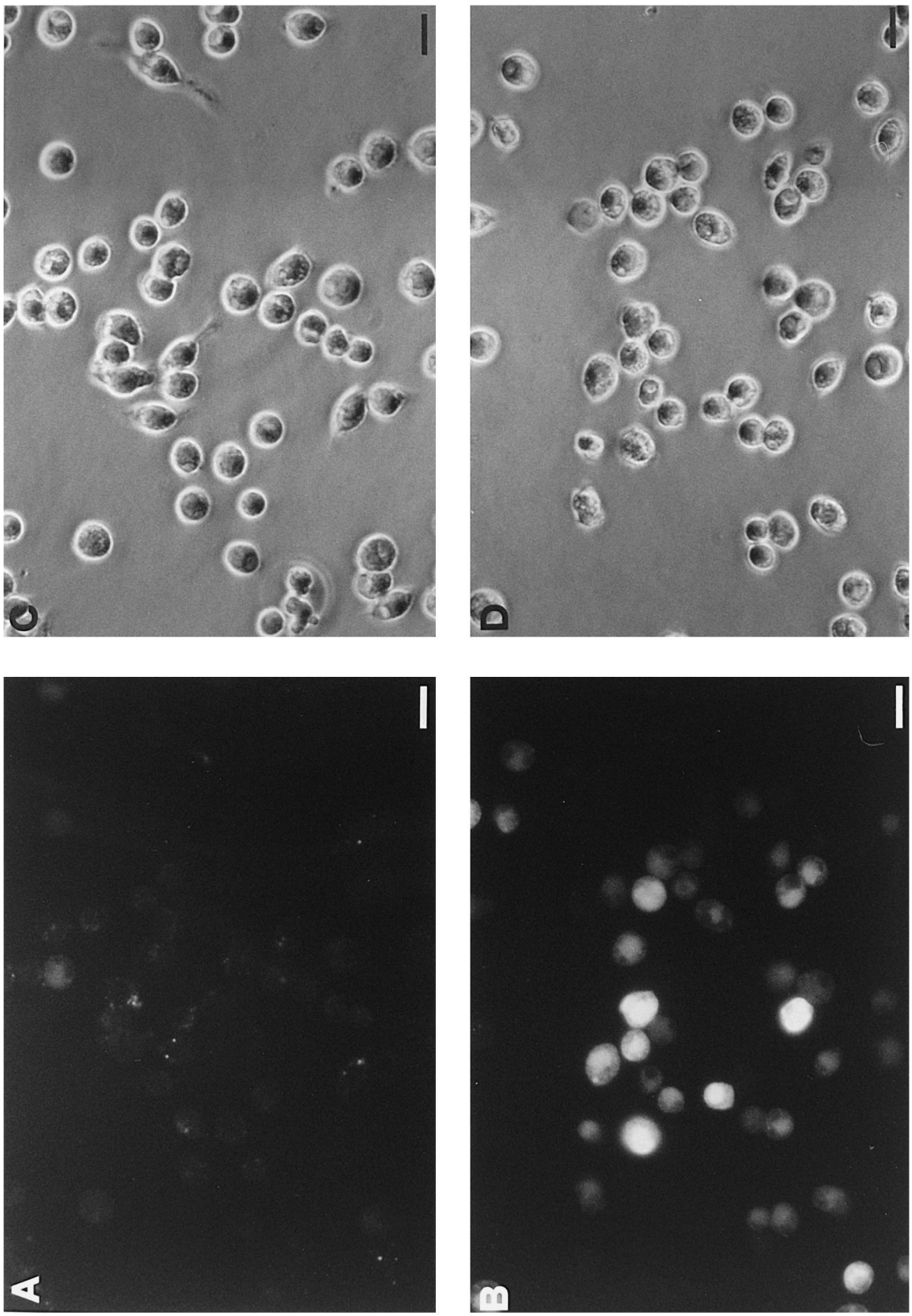

Fig. 5. Lack of synergism of ATP plus DIDS on lucifer yellow uptake. Macrophages were plated at a concentration of 20×10^4 cells/well and incubated in normal Ca^{2+}-containing saline for 15 min as follows: panels A and C, 100 μM DIDS; panels B and D, 100 μM DIDS plus 3 mM ATP.

Table 1
Tenidap increases ATP-induced lucifer yellow uptake

ATP 1 mM	Tenidap 50 μM + ATP 1 mM	DIDS 100 μM + ATP 1 mM
10.4 ± 4.0	24.7 ± 0.3	12.3 ± 4.5

Cells were incubated in the absence or presence of tenidap 50 μM or DIDS 100 μM for 15 min in normal saline containing ATP 1 mM and lucifer yellow 1 mg/ml.
Data are means ± S.D. of three experiments each performed in duplicate, and are expressed as percentages of brightly fluorescent cells vs. total cells.

macrophage cell clones selected for high sensitivity to ATP express very high levels of $P2X_7$ message and protein, macrophage and microglial cell clones resistant to ATP lack both $P2X_7$ message and protein (Surprenant et al., 1996; Ferrari et al., 1997; Chiozzi et al., 1997). Whether $P2X_7$ can also be identified with the permeabilizing ATP receptor described by Cockroft and Gomperts (1979) in mast cells is not yet known.

$P2Z/P2X_7$ receptors appear to be involved in several immune cell responses, among which cytokine secretion and cytotoxicity (Murgia et al., 1992; Perregaux and Gabel, 1994; Di Virgilio, 1995; Ferrari et al., 1996), thus they could be a useful target for immunomodulation. Very few drugs able to modulate these receptors have so far been described. Oxidized ATP and to a more limited extent two aldehyde reagents loosely related to ATP, such as pyridoxal phosphate and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (Murgia et al., 1993; Surprenant et al., 1996; F. Di Virgilio and O.R. Baricordi, unpublished observations), have been shown to act as irreversible inhibitors at this receptor. The mechanism of this effect is not entirely clear. Among agonists, the only known selective stimulator is the ATP analogue benzoylbenzoicATP (BzATP) (Nuttle and Dubyak, 1994; Ferrari et al., 1996).

Tenidap is being evaluated as an anti-inflammatory and anti-arthritic agent. It shares some of the effects of non steroidal anti-inflammatory drugs (NSAIDs), but is more powerful than NSAIDs in clinical studies (Blackburn et al., 1991; Sipe et al., 1992; Pelletier et al., 1993). Tenidap has been shown to inhibit ATP- and cytotoxic T lymphocyte-induced interleukin-1β release from human macrophages (Laliberte et al., 1994; Perregaux et al., 1996) through a mechanism yet to be identified.

Expression of the $P2Z/P2X_7$ receptor confers a clear-cut sensitivity to tenidap. About 20% of wild type J774 macrophages are killed by even 10 μM tenidap in the presence of a threshold concentration of 2 mM ATP. On the contrary, ATP-resistant cells are fully refractory. Furthermore, oxidized ATP prevents all toxic responses due to the combined application of tenidap and ATP.

Tenidap does not increase the cytotoxic effect due to application of a maximal ATP concentration, but instead shifts the dose–dependence curve to the left, an indication that affinity of the $P2Z/P2X_7$ receptor for ATP is increased. On the other hand, the lack of effect on maximal cytotoxic activity suggests that tenidap does not increase the population of ATP-sensitive cells, but rather increase the sensitivity of those already susceptible to ATP, a fraction that ranges from 50% to 80% in the J774 cell line propagated in our laboratory ranges from 50% to 80%.

Some of the cellular effects of tenidap are probably due to its well known inhibitory activity on plasma membrane anion transport systems (McNiff et al., 1994; Laliberte et al., 1994). Nonetheless, we think that this is not the main mechanism underlying its synergism with ATP because DIDS, a well known and widely used blocker of anion transport, lacked any effect either on ATP-dependent lactic dehydrogenase release or on ATP-dependent lucifer yellow uptake.

Tenidap has been increasingly applied in the treatment of chronic inflammatory diseases thanks to its activity as an inhibitor in the arachidonic acid cascade (Moilanen et al., 1988) and of plasma interleukin-6 accumulation. Furthermore, it has been also shown to inhibit the in vitro release of interleukin-1β dependent on several cytotoxic stimuli (Laliberte et al., 1994; Perregaux et al., 1996). The cellular mechanism of tenidap action is unknown, but there is evidence that it causes cytoplasmic acidification, block of anion transport and inhibition of interleukin-1β-converting enzyme. How and whether these effects contribute to the final outcome, i.e., down modulation of the inflammatory response, is an open question.

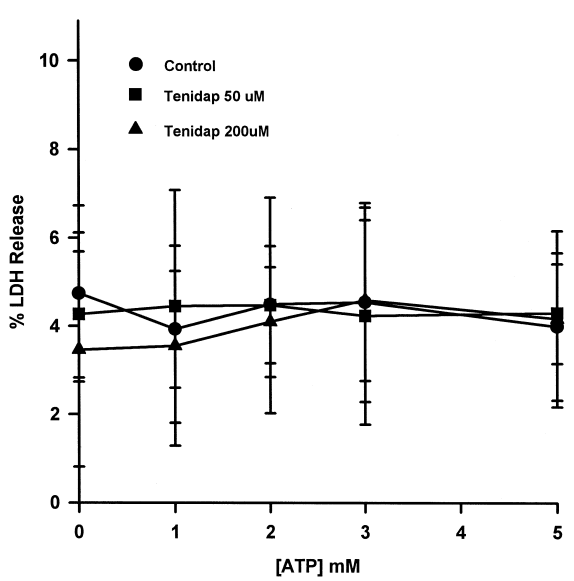

Fig. 6. ATP-resistant macrophages are refractory to the cytotoxic effect of ATP plus tenidap. The ATP-resistant macrophage clone, ATPR12, was incubated as described in Fig. 1 and stimulated for 5 h with increasing ATP concentrations in the absence (●-●) and presence of 50 (■-■) or 200 (▲-▲) μM tenidap. Data are means ± S.D. of triplicate determinations of experiments repeated on three different occasions.

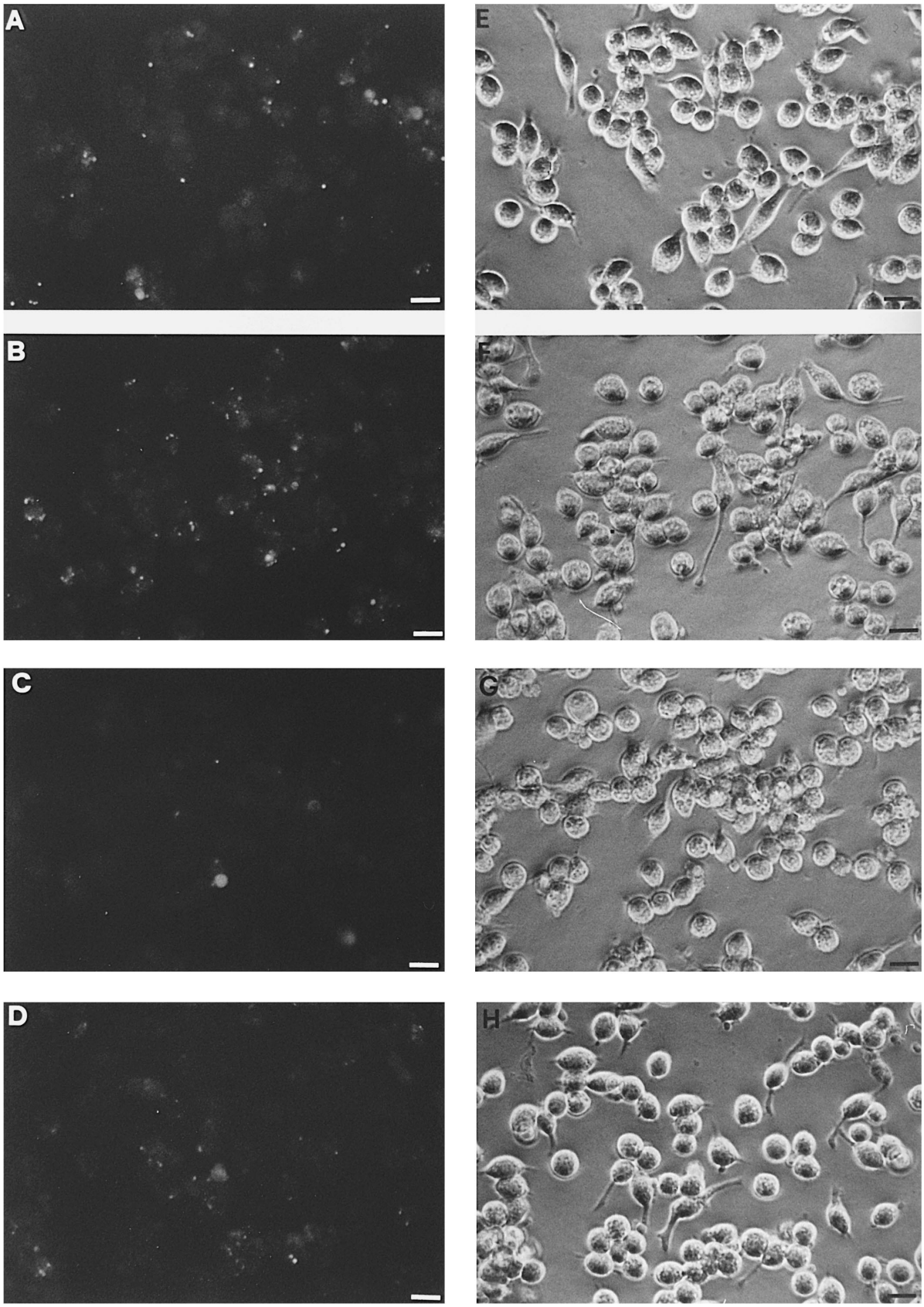

Fig. 7. ATP-resistant macrophages are not permeabilized by the combined addition of ATP plus tenidap. The ATP-resistant macrophage clone, ATPR12, was incubated as described in Fig. 2 and treated as follows: panels A and E, controls; panels B and F, 3 mM ATP; panels C and G, 50 μM tenidap; panels D and H, 3 mM ATP plus 50 μM tenidap.

In the present report we describe a novel possible mechanism of action for tenidap: sensitization of macrophage cells to the cytotoxic effect of extracellular ATP. Although the physiological role of the cytotoxic P2Z/P2X$_7$ receptor is still unknown, it has been speculated that it might be a 'suicide receptor', that may allow elimination of chronically infected macrophages or, more in general, down-modulation of the immune response. Should this apply, it can be hypothesized that the anti-inflammatory effect exerted by tenidap, besides its known inhibitory action on prostaglandin release and cytokine production, might also be due to its ability to sensitize the P2Z/P2X$_7$ receptor to endogenously released ATP or to other as yet unknown endogenous ligands. This sensitizing effect might cause killing of macrophages recruited at the inflammatory site and thus down-modulation of the inflammatory response. Preliminary results obtained in this laboratory (V. Vishwanath and F. Di Virgilio, in preparation) suggest that tenidap has a similar sensitizing activity at the human macrophage P2Z/P2X$_7$ receptor. Our hypothesis regarding the mechanism of action of tenidap is consistent with recent data (Morabito et al., 1998) implicating ATP and purinoceptors in the anti-inflammatory effect of drugs such as sulfasalazine and methotrexate.

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