Involvement of Inositol 1,4,5-Trisphosphate and Calcium in the Action of Adenine Nucleotides on Aortic Endothelial Cells*

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ADP and ATP, in the 1–100 μM range of concentrations, increased the formation of inositol phosphates in bovine aortic endothelial cells. The accumulation of inositol trisphosphate in response to adenine nucleotides was rapid (maximum at 15 s) and transient. This material was identified as the biologically active iso-mer inositol 1,4,5-trisphosphate on the basis of its retention time by high-performance liquid chromatography on an anion-exchange resin. AMP and adenosine have no effect on inositol phosphates. The action of ATP and ADP was mimicked with an equal potency and activity by their phosphorothioate analogs, ATPγS and ADPβS, and with a lower potency by adenosine 5′-(β,γ-methylene)triphosphate, whereas adenosine 5′-(α,β-methylene)triphosphate was inactive. In the same range of concentrations, ADP and ATP induced an efflux of 45Ca2+ from prelabeled bovine aortic endothelial cells and increased the fluorescence emission by cells loaded with quin-2. Here, too, AMP and adenosine were completely inactive. The outflow of 45Ca2+ induced by ADP was partially maintained in a calcium-free medium. These data suggest that in aortic endothelial cells, P2-purinergic receptors, of the P2x subtype, are coupled to the hydrolysis of phosphatidylinositol bisphosphate by a phospholipase C. It is likely that the release of prostacyclin and endothelium-derived relaxing factor in response to ADP and ATP is a consequence of this initial event.

EXTRACELLULAR ADP AND ATP EXERT AT LEAST TWO ACTIONS ON ARTERIAL ENDOTHELIAL CELLS (1, 2): THEY INDUCE THE RELEASE OF "ENDOTHELIUM-DERIVED RELAXING FACTOR" (EDRF), A STILL-UNIDENTIFIED VASODILATOR (2), AND OF PROSTACYCLIN (PGI2), A POTENT INHIBITOR OF PLATELET AGGREGATION (3–5). BECAUSE ADENINE NUCLEOTIDES ARE RELEASED FROM ACTIVATED PLATELETS, THEIR STIMULATORY EFFECT ON PGI2 RELEASE MIGHT HAVE THE PHYSIOLOGICAL FUNCTION OF LIMITING THE EXTENT OF PLATELET AGGREGATION AND HELPING TO LOCALIZE THROMBUS FORMATION TO AREAS OF ENDOTHELIAL DAMAGE. STUDIES OF AGONIST SPECIFICITY HAVE INDICATED THAT BOTH EFFECTS OF ADENINE NUCLEOTIDES ARE MEDIATED BY P2-PURINERGIC RECEPTORS, ACCORDING TO BURNSTOCK’S CLASSIFICATION (1, 2): ADP AND ATP ARE ALMOST EQUIPOTENT, WHEREAS AMP AND ADENOSINE ARE COMPLETELY INACTIVE. UNTIL RECENTLY, THE MOLECULAR PROPERTIES OF THESE UBQUITOUS RECEPTORS AND, IN PARTICULAR, THE NATURE OF THEIR TRANSDUCTION MECHANISMS WERE COMPLETELY UNKNOWN. WE HAVE RECENTLY OBSERVED THAT THE RELEASE OF PGI2 FROM BOVINE AORTIC ENDOTHELIAL CELLS IS STIMULATED SYNERGISTICALLY BY PHORBOL 12-MYRISTATE 13-ACETATE AND IONOPHORE A23187, INDICATING A COOPERATION BETWEEN CYTOSOLIC CALCIUM AND PROTEIN KINASE C (6). THIS LED US TO MAKE THE HYPOTHESIS THAT THE P2-RECEPTORS IN ENDOTHELIAL CELLS, LIKE MANY OTHER RECEPTORS (7, 8), ARE COUPLED TO A PHOSPHOLIPASE C HYDROLYZING PHOSPHATIDYLINOSITOL BISPHOSPHATE TO INOSITOL TRISPHOSPHATE, A CALCIUM-MOBILIZING MEDIATOR, AND DIACYLGLYCEROL, AN ACTIVATOR OF PROTEIN KINASE C. IT HAS BEEN SHOWN RECENTLY THAT ATP AND ADP ACTUALLY INCREASE IP3 FORMATION IN ISOLATED RAT HEPATOCYTES (9) AND H-35 HEPATOMA CELLS (10). IN THIS ARTICLE, WE SHOW THAT ADP AND ATP PROVOKE A RAPID AND TRANSIENT INCREASE OF INS-1,4,5-P3 AND OF THE CYTOSOLIC Ca2+ CONCENTRATION IN BOVINE ENDOTHELIAL CELLS.

EXPERIMENTAL PROCEDURES

CULTURE OF ENDOTHELIAL CELLS—BOVINE AORTIC ENDOTHELIAL CELLS WERE OBTAINED BY COLLAGENASE DIGESTION OF THE AORTA EXCISED FROM A FRESHLY SLAUGHTERED COW, AS DESCRIBED PREVIOUSLY (4, 11). THEY WERE CULTURED IN A MEDIUM OF THE FOLLOWING COMPOSITION: DMEM (70%, v/v), Ham’s F-12 (20%, v/v), fetal calf serum (10%, v/v), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B. The cells were seeded on 100-mm Petri dishes and incubated at 37°C under an atmosphere of 5% CO2, 95% air. The medium was changed the following day and later on every 3 days. After 4 or 5 days, the primary cultures formed confluent monolayers and could be subcultured. The cells were detached by a 5-min incubation in a Ca2+- and Mg2+-free Hanks’ buffer containing trypsin (10 mg/dl) and EDTA (1 mM). Thereafter, they were seeded in 35-mm Petri dishes, and the culture was pursued in the same conditions.

CELL LABELING AND INOSITOL PHOSPHATE EXTRACTION— TO LABEL THE PHOSPHOINOSITIDES, THE ENDOTHELIAL CELLS WERE INCUBATED FOR 48 h, IN THE SAME CONDITIONS AND CULTURE MEDIUM AS DESCRIBED ABOVE, WITH [3H]inositol (19 μCi/ml). THEN THE MEDIUM WAS REMOVED, AND THE CELLS WERE WASHED TWICE WITH DMEM AND PRECUBATED IN DMEM FOR 30 min; THE MEDIUM WAS THEN REPLACED, AND THE TESTED AGENTS WERE ADDED. THE INCUBATION WAS STOPPED BY RAPID REMOVAL OF THE MEDIUM, FOLLOWED BY 2 ML OF PERCHLORIC ACID (3%) ADDED TO THE CELLS. THE CELLS WERE SCRAPED, AND THE DISHES WERE RINSED WITH 2 ML OF PERCHLORIC ACID (1%). PROTEINS WERE REMOVED BY CENTRIFUGATION (1000 × g, 10 min,

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4°C), and the supernatant containing the inositol phosphates was neutralized with 1 ml of HEPES (0.375 M) and about 2 ml of KOH (0.765 M) to a final pH of 7.7–7.8. After 30 min at 0°C, KClO₄ was eliminated by centrifugation (1000 × g, 10 min, 4°C). The supernatant was collected, and the pellet was washed with a mixture of Na₂B₄O₇ (5 mM) and Na₂HPO₄ (12 ml of 60 mM ammonium formate, 5 mM Na₂B₄O₇) or 28 ml of 150 mM ammonium formate, 5 mM Na₂B₄O₇; 24 ml of 0.4 M ammonium formate, 0.1 M formic acid; and 24 ml of 0.8 M ammonium formate, 0.1 M formic acid, respectively. A 4-ml aliquot of each fraction was counted in liquid scintillation. In each experiment, the effectiveness of the separation was checked using a mixture of [°H]IP₃ and [°3P]Ins-1,4,5-P₃, prepared as described (12).

IP₃ Characterization by Anion-exchange HPLC—The IP₃-containing fraction, eluted from the Dowex AG 1-X8 column with 1 M ammonium formate, was desalted by chromatography on a Sephadex G-10 column and eluted with water/isopropl alcohol (98:2, v/v). The IP₃-containing fraction was lyophilized and redissolved in 0.5 ml of water containing a standard of [°3P]Ins-1,4,5-P₃ (±1000 cpm), prepared from human erythrocytes as described (13). That mixture was injected onto a Partisil SAX column (ø 25 mm, 10-µm particles; Whatman), using a U6K injector (Waters Associates). The column was washed with water for 8 min and then eluted by three successive convex gradients (10 min each) of increasing 1.7 M ammonium formate buffer adjusted to pH 3.7 with phosphoric acid, as described (13). The flow rate was 1.2 ml/min; 0.6-ml fractions were collected and counted in liquid scintillation for °H and °3P radioactivity. The pumps (Model 501) and the solvent gradient controller were from Waters Associates.

Measurement of "Ca²⁺ Efflux—Bovine aortic endothelial cells were seeded on plastic coverslips (Thermanox, Lux) and cultured in the same medium as described above, until they reached confluency. Then they were incubated, for at least 4 h, in the complete culture medium containing 100 µM CaCl₂ (100 µCi/ml). The coverslips were rapidly rinsed with HEPES buffer containing 0.1 mM EGTA and then rinsed a second time and incubated in a medium of the following composition: NaCl (120 mM), KCl (5 mM), MgCl₂ (1 mM), Na₂HPO₄ (5 mM), glucose (10 mM), CaCl₂ (1 mM), HEPES (20 mM), pH 7.4. The time course of "Ca²⁺ release was followed by transferring the coverslips every 4 min to a new test tube containing 2 ml of the same medium prewarmed at 37°C and, where indicated, the agonist under investigation. At the end of the experiment, the cells were lysed overnight in an hypotonic solution of Pronase (20 µg/ml). The radioactivity in the cell lysate and the various fractions was determined by liquid scintillation. Results represent the "Ca²⁺ radioactivity in each fraction as the percentage of the radioactivity remaining associated with the cells.

Monitoring of Cytosolic "Ca²⁺ Concentration with Quin-2—Bovine aortic endothelial cells were cultured on glass coverslips, in the same culture medium as described above (except for the omission of amphotericin B), until confluency was reached. Then the cells were incubated for 10 min in 1 ml of culture medium containing 50 µM quin-2 AM. After washing, they were allowed to equilibrate for 20 min at 37°C in the same incubation medium as described above (but without Na₂HPO₄). Measurements were performed in a Perkin-Elmer LS-3 spectrofluorometer, in a plastic cuvette maintained at 37°C. The coverslips were aligned at 30° to the incident beam. The excitation and emission wavelengths were, respectively, 340 and 432 nm. The cytoplasmic concentration of "Ca²⁺ was determined by comparing the fluorescence signal of the cells with the fluorescence of the "Ca²⁺-saturated indicator and to the autofluorescence of the cells. The maximal fluorescence was obtained by permeabilization of the cells with digitonin (30 µM); after that, the extracellular calcium concentration was increased to 10 mM. The autofluorescence was obtained after addition of MnCl₂ (1 mM) which quenches the fluorescence of quin-2. The cytoplasmic concentration of "Ca²⁺ was then computed according to the formula described by Moelenaar et al. (14). The values of fluorescence were corrected before calculation to take into account the steady decrease in the cell fluorescence, probably due to indicator loss. This loss was assumed to be linear, provided the cells had been equilibrated for a sufficient time, and was extrapolated from the baseline behavior. To check whether agonist addition affected the cell autofluorescence, the signal was also recorded at an excitation wave-
IP$_3$ formation was rapid in onset and transient (Fig. 1): a maximum stimulation was reached around 15 s, and the level of IP$_3$ had almost returned to the base line after 1 min. IP$_2$ accumulation was slightly delayed as compared with IP$_3$, and more sustained; this time shift was even more striking for IP$_1$ (Fig. 1). The stimulatory effects of ADP and ATP on inositol phosphates accumulation had a similar magnitude, whereas AMP and adenosine were completely inactive (Fig. 2). ADP and ATP induced a dose-dependent increase of inositol phosphates formation in the 1–100 μM range of concentrations (Fig. 3). The effects of ATP and ADP were mimicked by the phosphorythioate analogs ATPγS (three experiments, Fig. 4A) and ADPβS (four experiments, Table I). ATPγS had the same potency and the same maximal activity as ATP (Fig. 4B). At 100 μM, APPNP induced an inositol phosphate accumulation of similar magnitude as 10 μM ADP or ATP (Table I), whereas it was barely effective at 10 μM. Substitution of a methylene linkage for a bridging oxygen to give APCPP abolished the capacity to stimulate inositol phosphate accumulation (Fig. 4A). The identity of $[3^H]$$IP_1$ extracted from the endothelial cells 15 s following the addition of ADP was further characterized by HPLC on an anion-exchange column. It coeluted perfectly with an authentic standard of Ins-1,4,5-P$_3$ prepared from human erythrocytes (Fig. 5). Neither Ins-1,3,4-P$_3$ nor inositol tetrakisphosphate, which are separated from Ins-1,4,5-P$_3$ on this column, could be detected in these conditions.

Because Ins-1,4,5-P$_3$ is known to release Ca$^{2+}$ from the endoplasmic reticulum into the cytoplasm (7, 16), we investigated the action of adenine nucleotides on Ca$^{2+}$ distribution in endothelial cells by using two different methods: measurement of Ca$^{2+}$ efflux and direct monitoring of the cytoplasmic Ca$^{2+}$ concentration by means of the fluorescent indicator quin-2. As shown on Fig. 6A, ADP and ATP increased, with a comparable potency, the outflow of 45Ca$^{2+}$ from prelabeled bovine endothelial cells. Unstimulated cells released 13–15% of the remaining 45Ca$^{2+}$ radioactivity between 20 and 24 min (five experiments). If ATP or ADP (100 μM) was present during that period, the release of 45Ca$^{2+}$ reached values ranging from 30 to 60% (ATP) and 30 to 50% (ADP) (four experiments). Neither AMP nor adenosine, at a 100 μM concentration, had a significant effect on 45Ca$^{2+}$ release (three experiments) (Fig. 6B). The increase of 45Ca$^{2+}$ outflow induced by ADP or ATP was rapid in onset and transient (Fig. 6C); however, the progressive decrease of 45Ca$^{2+}$ specific activity during the experiment did not allow us to determine the exact time course of the purinergic response. Extracellular calcium depletion (no CaCl$_2$, 0.1 mM EGTA) diminished but did not abolish the effect of adenine nucleotides (three experiments) (Fig. 6D). ATP and ADP, but neither AMP nor adenosine, increased the fluorescence of bovine aortic endothelial cells loaded for 10 min with quin-2AM (50 μM) (Fig. 7). This increase was rapid in onset and reached a maximum after 10–20 s. Thereafter, the fluorescence rapidly decreased to a sustained level that was still higher than the base-line value after 10 min. The resting cytosolic concentration of free Ca$^{2+}$ was 72 ± 12 nM (mean ± S.D., 12 experiments). It raised to values typically between 200 and 600 nM following the addition of 10 μM ADP (280 ± 71 nM, n = 5) or 50 μM ATP (231 ± 69 nM, n = 3). If the loading period was prolonged up to 1 h, the time course of the response to ADP or ATP was altered (slower onset and decay) by the well-known phenomenon of Ca$^{2+}$ buffering (17). When the extracellular calcium (1 mM) was complexed by 1 mM EGTA, the transient rise in cytosolic Ca$^{2+}$ was maintained, whereas the more sustained increase was abolished (Fig. 8).
Experimental Procedures. Results are expressed as counts/min (mean ± S.D. of triplicate determinations in one representative experiment). These various phenomena were characterized by the same agonist specificity, indicating the involvement of P₂-purinergic receptors, and similar concentration-action relationships. Furthermore, the effect of adenine nucleotides on "Ca²⁺ efflux and the cytosolic Ca²⁺ concentration was maintained in a calcium-free medium, indicating the involvement of an intracellular store, possibly the endoplasmic reticulum, which is the target of Ins-1,4,5-P₃ (7, 16). Significant increases of IP₃ formation and "Ca²⁺ efflux were observed in response to micromolar concentrations of ATP or ADP (Fig. 3 and 6A). The plasma concentration of ATP + ADP following platelet activation by thrombin in uitro is ±20 μM; even higher concentrations could be obtained locally when aggregated platelets release their granule contents (1). It is therefore likely that the responses to adenine nucleotides that we have described here could occur in uitro when platelets aggregate on the site of an endothelial injury.

It was recently shown that bradykinin, which stimulates PG₁₂ and EDRF release like adenine nucleotides, increases the formation of inositol phosphates (18, 19) and the cytoplasmic concentration of Ca²⁺ (20) in aortic endothelial cells. The time course of the bradykinin effect on inositol phosphates is quite similar to that of adenine nucleotides action observed in our study. However, it was not determined which IP₃ isomer was formed in response to bradykinin: the biologically active Ins-1,4,5-P₃ or Ins-1,3,4-P₃, the role of which is still undetermined (13, 15, 21). At an early time following the ADP challenge, we found that Ins-1,4,5-P₃ is present in the bovine aortic endothelial cells. This does not rule out the possibility that Inositol 1,3,4,5-tetrakisphosphate and Ins-1,3,4-P₃ could be formed in endothelial cells, because in other cell types these compounds accumulate at a slower rate than Ins-1,4,5-P₃ (13).

Our study confirms the recent findings of Luckoff and Busse (22) and Hallam and Pearson (23) who have shown that ATP increases the cytoplasmic concentration of free Ca²⁺ in endothelial cells from bovine and piglet aorta, respectively. However, Luckoff and Busse (22) reported that ADP was only a partial agonist, slightly more active than AMP, while Hallam and Pearson (23) did not compare the effects of the various adenine nucleotides; this observation was not consistent with the involvement of P₂-purinergic receptors. In human umbilical vein endothelial cells, ATP stimulates

![Figure 4: Action of ATP analogs on inositol phosphate accumulation in bovine aortic endothelial cells. A, the labeled cells (10⁶ cells/dish) were challenged during 15 s by ATP (10 μM), ATP₇S (10 μM), APCPP (10 μM), or the combination ATP (10 μM) + APCPP (10 μM). The inositol phosphates were extracted and separated on Dowex formate (see "Experimental Procedures"). Results are expressed as mean ± S.D. of triplicate determinations in one representative experiment. B, the labeled cells were stimulated for 15 s by ATP (10 μM), ATP₇S (10 μM), APCPP (10 μM), or the combination ATP (10 μM) + APCPP (10 μM). The inositol phosphates were extracted and separated on Dowex formate (see "Experimental Procedures"). Results are expressed as mean ± S.D. of triplicate determinations in one representative experiment.](image-url)

![Figure 5: Ins-1,4,5-P₃ characterization by HPLC. Bovine aortic endothelial cells.](image-url)
ATP Increases $IP_3$, $Ca^{2+}$ in Bovine Aortic Endothelial Cells

FIG. 6. Effect of adenine nucleotides on $^{45}Ca^{2+}$ outflow from prelabeled bovine aortic endothelial cells. The $^{45}Ca^{2+}$ released during each 4-min period is expressed as the percentage of the radioactivity remaining associated with the cells. C, ADP was present from 20 min until the end of the experiment. A, B, and D represent the fractional release of $^{45}Ca^{2+}$ in the period 20–24 min, during which the tested agents were added. D, some of the coverslips were incubated, from the beginning, in a medium containing no $CaCl_2$ and 0.1 mM EGTA. Results represent the mean ± range of duplicate measurements in one representative experiment. Cont., control.

PGI$_2$ release, whereas ADP is completely inactive, suggesting the existence of more than one type of receptor for adenine nucleotides on endothelial cells (24). We have shown that ADP and ATP are almost equally active in increasing the level of IP$_3$ and the cytosolic concentration of free $Ca^{2+}$, whereas AMP is completely ineffective. The reason for the difference between our data and those of Luckoff and Busse (22) is unclear. The stimulating effect of ATP and ADP on inositol phosphate accumulation was mimicked by the analogs ATP$_7$S, ADP$_7$S, and APPNP, indicating that a phosphorylation reaction is unlikely to play a role in this action of adenine nucleotides. The equipotency and equiactivity of ATP$_7$S with ATP, the lower potency of APPNP, and the inactivity of APCPP in stimulating inositol phosphate formation are in complete agreement with the results of Needham et al. (25), who compared the effects of these various nucleotides on the release of PGI$_2$ from porcine aortic endothelial cells. In particular, the inactivity of APCPP is consistent with the conclusions of these authors that P$_y$ receptors in endothelial cells belong to the P$_y$ subtype. The various responses elicited by adenine nucleotides in aortic endothelial cells—IP$_3$ formation, modification of $Ca^{2+}$ distribution measured with both tracer fluxes and fluorescent indicator, release of PGI$_2$ and EDRF—are thus characterized by the same agonist specificity, suggesting that they all derive from the activation of P$_y$-purinergic receptors. The only inconsistency is that ATP$_7$S and ADP$_7$S, which are equiactive with ATP and ADP in stimulating PGI$_2$ release (25), behave as partial
ATP Increases IP₃, Ca²⁺, in Bovine Aortic Endothelial Cells

Our data on aortic endothelial cells are consistent with the recent reports that ADP and ATP increase IP₃ formation in isolated rat hepatocytes (9) and H-35 hepatoma cells (10). Cells of various origin are not equally sensitive to the adenine nucleotides; whereas in isolated rat hepatocytes half-maximal effects were obtained at submicromolar concentrations (9), an increase of IP₃ level in H-35 hepatoma cells could not be detected below 10 μM ATP. In bovine aortic endothelial cells, the concentration-action curve characterizing the effects of ATP and ADP on inositol phosphates spans the 1–100 μM range and is almost perfectly superimposable on the curves relating nucleotide concentrations to the release of PGI₂ (4, 25) or EDRF (26). The coupling with the hydrolysis of phosphatidylinositol bisphosphate by a phospholipase C seems thus to be a general feature of P₂Y-purinergic receptors. Extracellular ATP can also increase the cytoplasmic concentration of free Ca²⁺ by completely different mechanisms. In Ehrlich ascites tumor cells, ATP at micromolar concentrations induces a rapid mobilization of intracellular Ca²⁺ and a slow influx of extracellular Ca²⁺: these effects are not mimicked by ADP (27). ATP at millimolar concentrations induces the depolarization of mouse macrophages and an increase of cytosolic Ca²⁺ level, possibly via the phosphorylation of membrane proteins (28). In snail neurones, nanomolar concentrations of ATP activate Ca²⁺ channels, an effect partially mimicked by ADP (29). Finally, the sustained increase of cytosolic Ca²⁺ induced by adenine nucleotides in aortic endothelial cells is dependent on extracellular Ca²⁺ (Fig. 8) and might thus involve the activation of Ca²⁺ channels, which need to be characterized. Cultured aortic endothelial cells, in which adenine nucleotides produce the physiologically relevant effect of increasing PGI₂ synthesis, constitute a good model for further biochemical studies of P₂-purinergic receptors.

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REFERENCES