

Exogenous ATP raises cytoplasmic free calcium in fura-2 loaded piglet aortic endothelial cells

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Cultured piglet endothelial cells were grown to confluence on glass coverslips and loaded with the fluorescent Ca^{2+} indicator, fura-2. Using a dual-wavelength excitation fluorescence spectrophotometer it was found that ATP caused a rapid transient elevation in $[\text{Ca}^{2+}]_i$ in the presence of extracellular calcium which decreased to a maintained elevated level. With no extracellular calcium ATP evoked a similar transient increase which returned to the basal level. Addition of 50 mM K^+ had no effect on $[\text{Ca}^{2+}]_i$ or on the effect of ATP on $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} . The data suggest that ATP causes both discharge of calcium from an intracellular pool and influx across the plasma membrane although this is unlikely to be via a voltage-operated channel. ATP stimulated simultaneously the production of PGI_2 to a similar extent in the presence or absence of extracellular calcium. Elevated $[\text{Ca}^{2+}]_i$ may be an important activation pathway in the endothelial cell.

ATP Fura-2 Ca^{2+} (Piglet aorta, Endothelial cell)

1. INTRODUCTION

Endothelial cells line the inner surface of the blood vessels and can modulate the activity of surrounding hormonally-sensitive cells such as smooth muscle and platelets. These inhibitory actions are caused by the formation and release of two active constituents from the endothelial cell. PGI_2 , a potent inhibitor of platelet activation, is formed on stimulation with ATP at P_2 -receptors [1], and with other agonists such as bradykinin and thrombin [2,3] and a yet unidentified factor, but termed endothelial-derived relaxing factor because it can cause the relaxation of pre-constricted smooth muscle, is also released from endothelial cells on stimulation by several agonists including ATP [4–6]. The intracellular mechanism(s) con-

trolling the formation and release of PGI_2 and EDRF has not yet been identified. Indeed little is known of the P_2 -receptor transduction couple although in liver adenine nucleotides cause increased inositol lipid hydrolysis [7]. However, calcium ionophores can stimulate the production of PGI_2 from cultured endothelial cells [3] and can stimulate the relaxation of pre-constricted aortic rings which have an intact endothelial cell layer [6]. Furthermore the action of agonists that can cause relaxation in an endothelial cell-dependent manner can be inhibited by removing the extracellular bathing Ca^{2+} . These observations suggest that an important activation pathway is the elevation of endothelial cell $[\text{Ca}^{2+}]_i$. A recent report has shown that adenine nucleotides can raise cytoplasmic free calcium in monolayers of cells loaded with the fluorescent Ca^{2+} indicator quin2 [8]. These authors found transient increases in $[\text{Ca}^{2+}]_i$ on stimulation with ATP that were similar in the presence or absence of extracellular Ca^{2+} and so they concluded that ATP raises $[\text{Ca}^{2+}]_i$ mainly by

Abbreviations: fura-2AM, fura-2 penta-acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, cytoplasmic free calcium concentration; PGI_2 , prostacyclin; 6-keto- $\text{PGF}_{1\alpha}$, 6-keto-prostaglandin $\text{F}_{1\alpha}$; EDRF, endothelium-derived relaxing factor

discharging Ca^{2+} from an intracellular pool. However, using quin2 in monolayers of cells only 1 μm thick is technically limiting because of the inherent poor fluorescent properties and consequent large buffering problems with the dye resulting in poor signal-to-noise and time resolution.

In this study piglet aortic endothelial cells were loaded with the highly fluorescent Ca^{2+} indicator, fura-2 [9], and the effects of ATP and bradykinin on $[\text{Ca}^{2+}]_i$ and PGI_2 production measured simultaneously. Fura-2 is approx. 30-fold more fluorescent than quin2 (mole per mole) and detailed analysis of the stimulated $[\text{Ca}^{2+}]_i$ transients could be made using a dual-wavelength excitation fluorimeter. The data suggest that although the initial peak transient is caused by the discharge of Ca^{2+} from an intracellular store, there is also a stimulated influx component.

2. MATERIALS AND METHODS

Fura-2AM was purchased from Molecular Probes, Oregon, and ionomycin was from Calbiochem.

Thoracic aortic endothelial cells were prepared and placed in culture as described [10]. When confluent the cells were replated on glass coverslips. After 24 h the coverslips were removed to Dulbecco's modified Eagles' medium containing 2 μM fura-2AM and incubated for 45 min at 37°C giving a final cytoplasmic fura-2 concentration of 30–50 μM . The coverslips were then placed in 145 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 10 mM Hepes, 10 mM glucose, 0.5 mM CaCl_2 at pH 7.4 and 37°C until ready for use.

The coverslips were positioned across the diagonal of a quartz cuvette containing the above Hepes medium with either 1 mM CaCl_2 or 1 mM EGTA and the cuvette was placed in a 37°C thermostatted holder in a Spex dual-wavelength excitation fluorescence spectrophotometer. Fluorescence was normally recorded at 0.5 s intervals with excitation at 340 nm and emission at 500 nm, and calibrated exactly analogously to quin2 except $F_{\min} = 0.39 (F_{\max} - F_{\text{Mn}})$ [11]. In some experiments two alternating excitation wavelengths were used, 340 nm and 380 nm; 500 nm emission. While using an excitation of 340 nm gives an increase in the emitted light at 500 nm with increasing $[\text{Ca}^{2+}]_i$, 380 nm excitation gives a decrease. A ratio of the

two outputs gives greater sensitivity and $[\text{Ca}^{2+}]_i$ can be determined directly. A full discussion of calibration procedures is given elsewhere [12,13]. However the method for calibration of the fluorescence differs from that previously reported because the component of fluorescence caused by the endothelial cell and the coverslip is significant. To reveal the size of this autofluorescence, the divalent cation ionophore ionomycin was added to each experimental run followed by 1 mM Mn^{2+} (see fig.3). Mn^{2+} is thus translocated across the plasma membrane by the ionophore resulting in the quenching of the fluorescence caused by the intracellular fura-2 as the Mn^{2+} binds to the dye. Once these values for autofluorescence have been measured at both 340 and 380 nm excitation, they can be subtracted from the raw data before calculating the ratio. It should be noted that values for R_{\max} and R_{\min} obtained for fura-2 standards in free solution should also be corrected for Mn^{2+} quenching (cf. [12,13]). The values calculated and used for calibration with our Spex fluorimeter were: R_{\max} , 20; R_{\min} , 0.89; S_{f2}/S_{b2} , 10.3.

PGI_2 formation and release were estimated by determining the total amounts of its stable metabolite, 6-keto- $\text{PGF}_{1\alpha}$. Aliquots of the bathing medium were taken at fixed time points after stimulation and analysed by radioimmunoassay [14].

3. RESULTS AND DISCUSSION

In the presence of 1 mM extracellular Ca^{2+} , an optimal concentration of ATP, 20 μM , causes a rapid elevation of $[\text{Ca}^{2+}]_i$ from the resting level of 120 ± 10 nM to a peak within 10 s of 680 ± 15 nM ($n = 6$; fig.1A). $[\text{Ca}^{2+}]_i$ then falls rapidly back towards the basal level over the next 60 s. The minimally effective dose of ATP was 0.1 μM . The rapidity with which $[\text{Ca}^{2+}]_i$ declines may be caused by ectonucleotidases present on the cell surface which quickly lower the ATP concentration [15]. Alternatively the P_2 -purinoceptor may be rapidly desensitised. As a second dose of ATP has no further effect on $[\text{Ca}^{2+}]_i$ the latter seems more likely (fig.1A). Indeed a subsequent dose of bradykinin does increase $[\text{Ca}^{2+}]_i$ again (data not shown in the presence of extracellular calcium but see fig.2A with 0-extracellular calcium). Addition of 50 mM KCl has no effect on either the resting concentra-

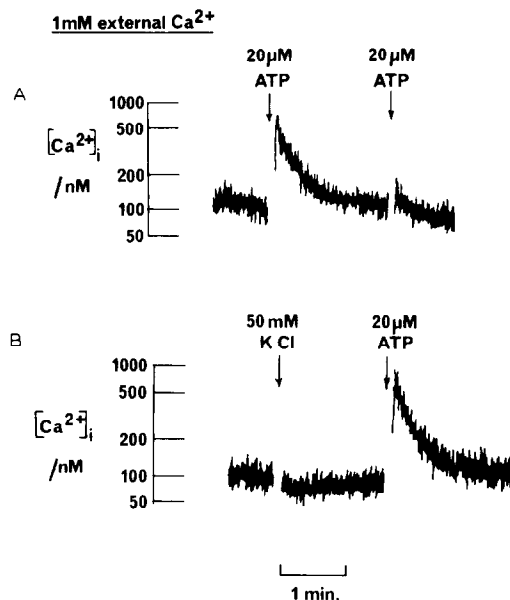


Fig.1. In 1 mM extracellular $CaCl_2$. (A) Effect of ATP, 20 μ M, on fluorescence from fura-2-loaded piglet endothelial cells at 340 nm excitation and 500 nm emission reporting $[Ca^{2+}]_i$. (B) Effect of 50 mM KCl on $[Ca^{2+}]_i$ and on the transient elevation in $[Ca^{2+}]_i$ in response to ATP.

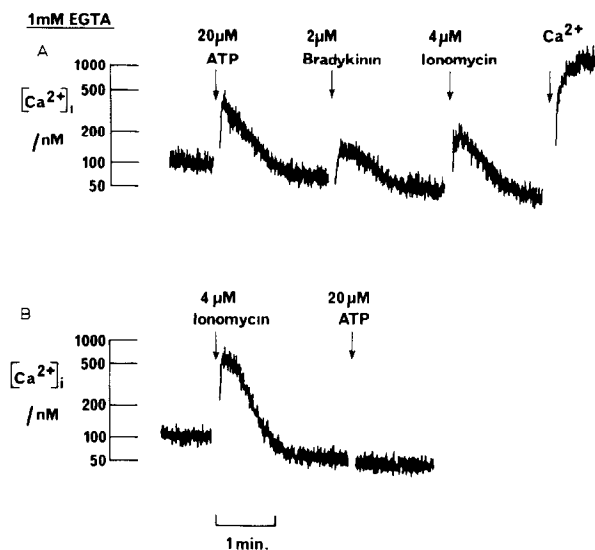


Fig.2. In 0, Ca^{2+} media, with no added $CaCl_2$ and 1 mM EGTA. (A) Effects of ATP, bradykinin followed by ionomycin on $[Ca^{2+}]_i$; (B) the effect of ionomycin followed by ATP on $[Ca^{2+}]_i$.

tion of $[Ca^{2+}]_i$ or on the stimulated transient elevation in $[Ca^{2+}]_i$ (fig.1B). Furthermore ATP responses are not affected by preincubation for several minutes with the organic Ca^{2+} channel blockers nifedipine, diltiazem, or D600 (at concentrations of each up to 20 μ M; not shown).

Fig.2 shows the results from a similar experiment except that now the cells are in Ca^{2+} -free medium with 1 mM extracellular EGTA. ATP causes an equally rapid elevation in $[Ca^{2+}]_i$ from 85 ± 10 nM to 520 ± 20 nM ($n = 6$). $[Ca^{2+}]_i$ then rapidly decreases to basal levels. As removing extracellular calcium has only a small effect on the peak $[Ca^{2+}]_i$ it appears that ATP initially elevates $[Ca^{2+}]_i$ mainly by causing a discharge from an intracellular store. Further additions of bradykinin and then the calcium ionophore ionomycin in Ca^{2+} -free medium produce further transient elevations in $[Ca^{2+}]_i$ suggesting that the intracellular store is not completely discharged by stimulation with ATP even though a further addition of an optimal dose of ATP is without further effect.

In fig.2B the order of addition is reversed; ionomycin, 4 μ M, produces a large increase in $[Ca^{2+}]_i$ and ATP now has no further effect on $[Ca^{2+}]_i$. This result suggests that ATP can cause the release of Ca^{2+} from an intracellular pool that is also available in its entirety to the ionophore, and is consistent with the presently held view in many other cell types that the internal store is membrane-bounded, and possibly an endoplasmic reticular membrane system.

Fig.3 shows the raw data from an experiment using a dual-wavelength excitation fluorimeter. The continuous trace shows the fluorescence emitted at 500 nm with an excitation of 340 nm. The discontinuous trace shows the output with a 380 nm excitation beam. Stimulation with 20 μ M ATP causes a rapid increase in fluorescence with 340 nm excitation while the fluorescence with 380 nm excitation simultaneously decreases reflecting an increase in the Ca^{2+} concentration. Ionomycin causes a further large and more prolonged change in the emitted light at the two wavelengths as would be expected in the presence of extracellular calcium. (Subsequent addition of 1 mM Mn^{2+} produces a decrease in both wavelength intensities as it quenches the fluorescence attributable to fura-2 to reveal autofluorescence, see section 2). Fig.4A shows the calculated trace of $[Ca^{2+}]_i$ from fig.3;

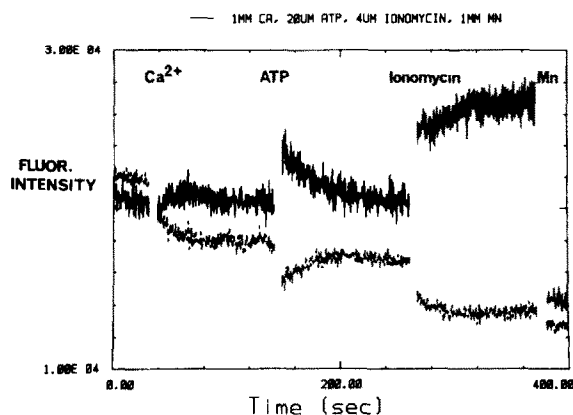


Fig.3. Dual-excitation wavelength experiment showing the effect of ATP and ionomycin in the presence of 1 mM CaCl_2 . The solid line shows fluorescence collected at 500 nm with 340 nm excitation; the intermittent line, data collected at 500 nm with 380 nm excitation. The addition of 1 mM MnCl_2 was made at the end of the experiment to determine the level of cell auto-fluorescence prior to calibration (see section 2 and [11]).

fig.4B shows the calculated trace of a similar experiment with 1 mM EGTA. It is apparent that in 1 mM Ca^{2+} , $[\text{Ca}^{2+}]_i$ declines rapidly after the peak stimulated ATP response and reaches a steady-state level approximately double the normal resting level, whereas in Ca^{2+} -free medium $[\text{Ca}^{2+}]_i$ returns back to the resting level. These data suggest there is an influx component and may explain why transient increases in $[\text{Ca}^{2+}]_i$ in the presence of extracellular calcium are consistently larger than in its absence. Although there may be an influx component across the plasma membrane, as with the ADP receptor on human platelets [16] it appears unlikely to be via a voltage-operated channel (cf. fig.1B). Organic voltage-operated channel blockers had no effect on the maintained elevated $[\text{Ca}^{2+}]_i$.

With 1 mM Ca^{2+} , resting cells accumulated 102 ± 20 pg/ 10^6 cells of the PGI_2 metabolite, 6-keto- $\text{PGF}_{1\alpha}$, over 10 min. ATP, 20 μM , for 3 min caused an increase to 1200 ± 45 pg/ 10^6 cells ($n = 4$). In 1 mM EGTA the level of 6-keto- $\text{PGF}_{1\alpha}$ rose to 1240 ± 90 pg/ 10^6 cells with 20 μM ATP.

The results presented here show that endothelial cell $[\text{Ca}^{2+}]_i$ is elevated on stimulation with the P_2 -purinoceptor agonist ATP with the simultaneous formation and release of PGI_2 . ATP

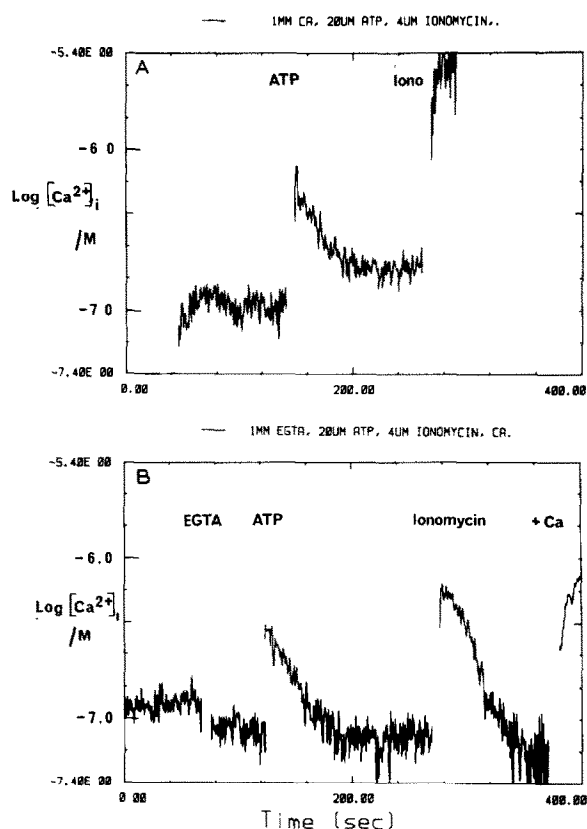


Fig.4. Calibrated $[\text{Ca}^{2+}]_i$ measurements from dual-excitation wavelength experiments performed (A) in the presence of 1 mM CaCl_2 (from same data as in fig.3) and (B) in the absence of extracellular calcium with 1 mM EGTA. 20 μM ATP, 4 μM ionomycin and 2 mM CaCl_2 were added as indicated.

produces the rise in $[\text{Ca}^{2+}]_i$ mainly by discharge of Ca^{2+} from an internal store but there also appears to be an influx component. Agonist-stimulated increases in the permeability of the blood platelet plasma membrane to divalent cations have been more definitively demonstrated using extracellular Mn^{2+} [17]. Stimulation with ADP, PAF or thrombin resulted in the rapid quenching of the cytoplasmic dye indicating that Mn^{2+} had fluxed into the cytoplasm. Similar experiments using Mn^{2+} with fura-2-loaded endothelial cells have so far proved impossible because the relatively low cytoplasmic concentrations of the dye, 30–50 μM , together with the basal leak of Mn^{2+} into the cell, result in a rate of quench of the dye in excess of

20%/min; too great to detect a further agonist-stimulated rate of Mn^{2+} influx and dye quench. The relative importance of these fluxes and of elevated $[Ca^{2+}]_i$ in causing release of arachidonic acid and consequent formation and release of PGI_2 and/or EDRF is still to be determined. Although the presence or absence of extracellular calcium appears to have little influence on the production of PGI_2 on stimulation with ATP in this system, the prolonged influx of Ca^{2+} may be important for EDRF release because the action of agonists that cause relaxation of intact arteries by stimulating endothelial cells can be inhibited by removing the extracellular bathing Ca^{2+} .

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