Characterization and Functional Significance of Calcium Transients in the 2-Cell Mouse Embryo Induced by an Autocrine Growth Factor

Growth of preimplantation embryos is influenced by autocrine trophic factors that need to act by the 2-cell stage, but their mode of action is not yet described. This report shows that late zygote and 2-cell stage mouse embryos responded to embryo-derived platelet-activating factor (PAF) with transient increases in intracellular calcium concentration ([Ca\(^{2+}\)]), [Ca\(^{2+}\)] transients were single global events and were specifically induced by embryo-derived PAF. They were blocked by inhibition of phospholipase C (U 73122) and an inositol trisphosphate (IP\(_3\)) receptor antagonist (xestospongin C), indicating the release of calcium from IP\(_3\)-sensitive intracellular stores. Transients were also inhibited by the absence of calcium from extracellular medium and partially inhibited by treatment with dihydropyridine (nifedipine, 10 \(\mu\)M), but not pimozide (an inhibitor of an embryonic T-type calcium channel). (±)BAY K8644 (an L-type channel agonist) induced [Ca\(^{2+}\)] transients, yet these were completely inhibited by nifedipine (10 \(\mu\)M). The complete inhibition of BAY K8644, but only partial inhibition of PAF by nifedipine shows that L-type channels were only partly responsible for the calcium influx. Depolarization of 2-cell embryos by 50 \(\mu\)M K\(^{+}\) did not inhibit PAF-induced calcium transients, showing that the influx channels were not voltage-dependent. Depletion of intracellular calcium stores by thapsigargin revealed the presence of store-operated channels. The interdependent requirement for IP\(_3\)-sensitive internal calcium stores and extracellular calcium in the generation of PAF-induced transients may be explained by a requirement for capacitative calcium entry via store-operated channels. A functionally important role for the PAF-induced transients is supported by the observation that inhibition of IP\(_3\) transients by a PAF-antagonist (WEB 2066) or an intracellular calcium chelator (1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester; BAPTA-AM) caused marked inhibition of early embryo development. Growth inhibition by BAPTA-AM was relieved by addition of exogenous PAF.

The cells of the mammalian preimplantation embryo (from the time of fertilization until the implantation of the blastocyst into the uterus) form the progenitor cells for all other cell lineages. The regulation of the growth and survival of the cells of the early embryo is, however, poorly understood. Mammalian preimplantation embryos develop in vitro with simple medium requirements and have no absolute requirement for exogenous vitamins, hormones, or growth factors. This contrasts with the absolute requirement of normal somatic cells for exogenous mitogens and survival factors. The continued mitoses of preimplantation embryo cells in the absence of exogenous growth factors implicates a role for endogenous, autocrine trophic factors, or the constitutive activation of signaling pathways in the early embryo. Several lines of evidence support a role for the former: (i) the rate of embryo development in vitro is density-dependent, with embryos growing in relatively small volumes (in large groups) developing more successfully than those grown in large volumes (or individually) (1, 2); (ii) the synthesis by the preimplantation embryo of a number of growth factor ligands and their receptors (3–8); and (iii) the capacity of some exogenous growth factors to enhance embryo metabolism in vitro and to compensate for the adverse effects of culture in large medium volumes (1, 2, 9).

Experimental partial deprivation of released autocrine trophic factors did not arrest the cell-cycle at given checkpoints (9). Rather, there was progressive loss of viability with increased cell death as embryos progressed past the 8-cell stage. This finding suggests that the autocrine factors may act as survival factors rather than classical growth factors (triggering progression through specific cell-cycle checkpoints). While several autocrine factors have been implicated, platelet-activating factor (PAF)\(^1\) seems to be one of the first produced, being synthesized de novo by the embryo soon after fertilization (10, 11). Its actions are required by the mid-2-cell stage for normal rates of embryo survival (9).

Despite this range of supportive data, there is limited direct evidence for the action of autocrine trophic factors in early embryo development. Transgenic and recombinant knock-out models have not generally been informative of the growth requirements of the early embryo prior to implantation. This may be due to extensive redundancy of regulatory pathways.

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\(^1\) The abbreviations used are: PAF, platelet-activating factor; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester; [Ca\(^{2+}\)]), intracellular calcium concentration; modHTF, modified human tubal fluid; Hepes-modHTF, Hepes-buffered modified human tubal fluid; IP\(_3\), inositol trisphosphate; PLC, phospholipase C; BSA, bovine serum albumin; EGF, epidermal growth factor; CCE, capacitative calcium entry; SOC, store-operated calcium channels.
Calcium Transients in Mouse Embryos

Growth/survival factors exert their effects by generating secondary messengers that then exert downstream actions. The generation of transient increases in intracellular free calcium concentrations ([Ca\(^2+\)]\(_i\)) is a universally important secondary messenger (12). ([Ca\(^2+\)]\(_i\)), transients are known to be important regulators of the early embryo’s developmental program. The fertilizing sperm induces a series of calcium transients in oocytes that cause oocyte activation and have longer term consequences for normal embryo development (13–16). A ([Ca\(^2+\)]\(_i\)), transient also occurs at the time of nuclear envelope breakdown in the mouse zygote (17). The post-fertilization calcium oscillations and the transients associated with nuclear envelope breakdown require factor(s) derived from the sperm that become associated with the nuclear membrane. There is no direct evidence of a developmental requirement for calcium transients in the mammalian early embryo outside these defined periods, although evidence that induction of artificial transients in morulae enhanced the rate of blastocyst formation (18, 19) and the inhibition of embryo development by a calmodulin antagonist (W-7) suggests a role (20). It was recently shown (21) that exogenous PAF may induce calcium transients in the 2-cell mouse embryo although a mechanism for this action was not defined, nor was it established whether embryo-derived PAF also induced such responses.

The aims of the study were, therefore, to screen preimplantation stage mouse embryos for evidence of [Ca\(^2+\)]\(_i\), transients; to define the causative agent of any transient observed; to provide characterization of the [Ca\(^2+\)]\(_i\), transients; and to assess whether their induction had developmental consequences for the early embryo. We found that embryo-derived PAF caused transients in late zygotes and 2-cell embryos during interphase and that they were necessary for normal rates of embryo development in vitro.

MATERIALS AND METHODS

Animals—Female Swiss albino mice (Laboratory Animal Services, University of Sydney, NSW, Australia), 6–9 weeks old, were superovulated by intraperitoneal injection of 10 IU of equine chorionic gonadotropin (eCG; Collectivite, Roden, Germany) followed 48 h later by 10 IU of human chorionic gonadotropin (Chorulon, Intervet). Females were then left unattended or paired with males of proven fertility. Fertilization occurred by 13 h after human chorionic gonadotropin. Day 1 of pregnancy was confirmed by the presence of a copulation plug. The use of animals was in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and was approved by the Institutional Animal Care and Ethics Committee.

Collection Medium and Embryo Collection—All components of medium were tissue culture grade from Sigma. Unless otherwise stated all media were supplemented with 3 mg of BSA/ml (Fraction V, CSL Ltd., Melbourne, Victoria, Australia).

Mice were killed by cervical dislocation. Embryos or cumulus masses were flushed from the reproductive tract using Hapes-buffered modified human tubal fluid medium (Hapes-modHTF (1)) with 3 mg of BSA/ml. Zygotes and oocytes were freed from their cumulus cells by brief exposure to 300 IU of hyaluronidase (Sigma). Embryos fertilized in the reproductive tract were collected by dissection from the ampulla region of the oviduct. Zygotes and embryos that were not immediately prepared for calcium imaging were cultured in bicarbonate-buffered modified HTF medium (modHTF (1)) containing 3 mg of BSA/ml at 37 °C, 5% CO\(_2\) in air.

Calcium Imaging—Calcium imaging was performed with embryos mounted in a chamber on a microscope coverslip. The chamber contained ~0.5 ml of medium and was perfused with medium at 37 °C at a rate of 1 ml/min. Perfusion medium was the same composition as Hapes-modHTF (1) with 3 mg of BSA/ml, but without ppt BSA, in a ratio of 1:4 with 0.15 M NaCl in BSA-free medium.

Embryos were washed 3 times in Hapes-modHTF and incubated with Fura-2 AM (2 μM; Molecular Probes, Eugene, OR) in BSA-free perfusion medium for 15 min and washed in BSA-free perfusion medium before calcium imaging.

The perfusion chamber was constructed from perspex (40 × 70 × 5 mm, width × length × depth) into which an elliptical slot was created. The slot was sealed at the base with a glass coverslip (with high vacuum grease, Ajax Labs, Sydney, NSW, Australia). The slot was held approximately 0.5 ml of medium. The top of the slot was open and medium was added at the rate of 1 ml/min, at one end of the slot and removed at the far end. Fluid was pumped through the chamber by a variable speed peristaltic pump.

Embryos in BSA-free perfusion medium were placed onto a Cell-Tek (In Vitro Scientific, Bedford, MA)-treated glass coverslip that was attached to the perfusion chamber. After establishing baseline readings and subtracting background readings, perfusion was initiated. Relative changes in intracellular calcium concentration were measured using fluorescence ratiometric imaging of Fura-2 at excitation wavelengths of 340 and 380 nm. One frame (0.04 s) was captured at each wavelength every 1 s. The [Ca\(^2+\)]\(_i\), is the average over an entire cell (zygote-8-cell stage) or an entire embryo (morulae) and thus may not reflect the peak calcium concentrations achieved within regions of a cell. Results were recorded with a Panasonic video camera (model WV-BP 310/A) linked to a Macintosh computer via a PixeLink frame grabber. Images were captured and analyzed with ImageJ software (Improvision, Coventry, UK). All imaging was performed on a Nikon Diaphot microscope using 100 W Xenon illumination, and a ×20 Olympus DPlan Apo UV lens. Up to 10 embryos were within the field of view of this objective and were imaged simultaneously.

Calibration Procedure for Measuring Intracellular Calcium Concentration—Intracellular calcium concentrations were calculated using the equations of Grynkiewicz et al. (23). 

\[
R_{\text{max}} / R = 1 + K_{\text{diss}} [Ca^{2+}] / 1 + K_{\text{max}} [Ca^{2+}] + [Ca^{2+}]^2
\]

was initiated. Relative changes in intracellular calcium concentration were measured using fluorescence ratiometric imaging of Fura-2 at excitation wavelengths of 340 and 380 nm. One frame (0.04 s) was captured at each wavelength every 1 s. The [Ca\(^2+\)]\(_i\), is the average over an entire cell (zygote-8-cell stage) or an entire embryo (morulae) and thus may not reflect the peak calcium concentrations achieved within regions of a cell. Results were recorded with a Panasonic video camera (model WV-BP 310/A) linked to a Macintosh computer via a PixeLink frame grabber. Images were captured and analyzed with ImageJ software (Improvision, Coventry, UK). All imaging was performed on a Nikon Diaphot microscope using 100 W Xenon illumination, and a ×20 Olympus DPlan Apo UV lens. Up to 10 embryos were within the field of view of this objective and were imaged simultaneously.

Treatments—The effect of the following exogenous growth factors was tested: EGF (human recombinant EGF, Sigma, lot 62H04135), insulin-like growth factor 1 (human recombinant insulin-like growth factor 1, Sigma, lot 83H09655), transforming growth factor-β (porcine platelet transforming growth factor-β, R&D Systems Inc., MN, lot 1095); platelet-derived growth factor (human platelet-derived growth factor, R&D Systems Inc., lot D711); and PAF (Sigma, equal mixture of 1-O-acetyl-2,3-bis(acylamino)propanoyl-acetyl-sn-glycerol-3-phosphocholine). EGF, insulin-like growth factor-1, transforming growth factor-β, and platelet-derived growth factor were first dissolved in 0.1% acetic acid, freeze dried, and then brought to working concentrations in perfusion medium. PAF was prepared as a 10 mg/ml stock solution in chloroform. Aliquots were removed to a siliconized glass test tube, reduced to dryness under a stream of N\(_2\), and dissolved in perfusion medium to the desired concentration.

Inhibitors and antagonists were also used. WEB 2086 (a competitive PAF-receptor antagonist, a gift of Boehringer Ingelheim AG, Ingelheim, Germany) was dissolved in perfusion medium. A stock solution of BNI (PAF antagonist, a gift of Institute Henri Beaufour, Paris, France) was prepared in MeSO\(_4\). Working solutions were prepared by dilution in perfusion medium to give a final MeSO\(_4\) concentration less than 0.2% (w/v). BAPTA-AM (0.1 and 0.5 μM; Sigma) was prepared in Hapes-modHTF. Embryos were exposed to BAPTA-AM for 20 min washed and then added to the perfusion chamber. In some cases, BAPTA-AM-treated embryos (0.5 or 1 μM; for 20 min at 27 h post-fertilization; early 2-cell stage) were set up in culture in modHTF, either untreated or supplemented with 20 ng of PAF/ml, and development monitored each 24 h for 96 h.

To determine the dependence of transients on extracellular calcium, responses to embryo-derived PAF or exogenous PAF were examined in perfusion medium free of calcium (with osmolality adjusted with NaCl). The role of calcium channels was assessed by the inhibitory action of nifedipine (Calbiochem, L-type channel blocker) or pimozide (Sigma, T-type channel blocker). The presence of a functional L-type calcium channel was confirmed by challenging 2-cell embryos with mixed enantio-meric solution of (+) and (-) nifedipine (porcine cardiac, Sigma) which has a net agonistic action on calcium channels. Internal stores of calcium were depleted by use of the calcium-ATPase inhibitor, thapsigargin (1 μM, Sigma). It was prepared in BSA-free medium and embryos were treated with thapsigargin for 15 min prior to PAF challenge. The action of phospholipase C (PLC) in generating the intracellular [Ca\(^2+\)], transient was measured by treating 2-cell embryos with U73122 (5 μM, Calbiochem) for 5 min prior to

[21906]
calcium transients, a range of peptide growth factors was collected and prepared in protein-free conditions and then exposed to embryoid cultures. The response of individual 2-cell embryos pretreated with rPAF acetylhydrolase and then challenged with exogenous PAF (372 nM) for 5 min. This was followed by washout with perfusion medium and then challenged with a second PAP treatment at 75 min. Arrows show the times of PAP challenge.

**Fig. 1.** The change of [Ca\(^{2+}\)]i, was assessed in 2-cell embryos, a, when the embryos were in medium containing albumin (●, n = 50) or in protein-free medium (■, n = 50), b, pretreated with rPAF acetylhydrolase (174 µg/ml, ■, n = 55) or vehicle control (●, n = 53), before perfusion of the chamber with albumin containing medium. The results are the mean and S.E. of (n) embryos per treatment.

**RESULTS**

**Spontaneous [Ca\(^{2+}\)]i Transients Occurred in 2-Cell Embryos but Required Albumin in the Extracellular Medium**—A surprising initial observation was that 2-cell embryos often displayed a spontaneous [Ca\(^{2+}\)]i transient soon after imaging commenced without other treatment. This only occurred if embryos were collected and prepared in protein-free conditions and then exposed to medium containing albumin during imaging; in the absence of albumin from perfusion medium, [Ca\(^{2+}\)]i transients were not seen (Fig. 1a). This calcium response was consistently observed as a single global calcium transient throughout the entire cell (zygote), or in both cells of the 2-cell embryo. Repetitive oscillations over the time frame of observation (30 min) were not detected. The transients were not artifactual responses to changes in temperature or osmolality since these potential variables were carefully controlled.

The loading of embryos with Fura-2 AM, their subsequent washing and set-up on the imaging slide were all performed in protein-free medium. The response to medium containing albumin may indicate that albumin itself acted as a signaling molecule, or that it acted as an acceptor for a released embryoid-derived trophic molecule. Albumin is required for the release of PAF from cells (25), including the 2-cell embryo (26, 27), so we tested the hypothesis that PAF accumulates on the embryo’s outer plasma membrane during processing in protein-free medium and is released upon exposure to albumin. This was done by determining whether the embryo response to albumin was inhibited by treating the embryos with exogenous rPAF acetylhydrolase (degrading PAF to an inactive form) prior to exposure to albumin. After Fura-2 AM loading, embryos were incubated with 174 µg of rPAF acetylhydrolase or vehicle for 15 min. They were then washed and challenged with perfusion medium containing BSA (Fig. 1b). The rPAF acetylhydrolase treatment inhibited (p < 0.001) calcium transients in response to perfusion.

**Spontaneous Calcium Transients Are Caused by Embryo-Derived PAF**—The inhibition of spontaneous transients after rPAF acetylhydrolase treatment suggests that PAF was the only agent responsible for inducing these transients. To determine whether other embryonic growth factors might also cause calcium transients, a range of peptide growth factors was added as exogenous components of medium: EGF (1–1000 ng/ml), insulin-like growth factor-1 (1–1000 ng/ml), platelet-derived growth factor (0.1–2.5 ng/ml), and transforming growth factor-β (0.1–10 ng/ml). None of these induced any observable change in [Ca\(^{2+}\)]i, (results not shown).

Following embryo-derived PAF-induced [Ca\(^{2+}\)]i transients in 2-cell embryos, the same embryos failed to show any response to an immediate further challenge with exogenous PAF (results not shown) suggesting desensitization/dow-regulation of the response. Down-regulation of an initial response to exogenous PAF (372 nM) also occurred; rPAF acetylhydrolase-treated embryos were exposed to exogenous PAF for 5 min and then at various intervals a second PAF challenge was applied. No embryos showed a [Ca\(^{2+}\)]i response to the second PAF challenge at 15 or 25 min after the first challenge. By 35 min a small number (17%) responded and the proportion responding further increased at 40 min (75%) and 75 min (79%) (75 min results are shown in Fig. 2).

**The Response of Embryos to PAF Was Specific and Receptor-dependent**—To enable the characterization of the action of PAF-antagonists on PAF-induced [Ca\(^{2+}\)]i, transients in 2-cell embryos, it was necessary to standardize the concentration of the PAF challenge. This was achieved by degrading externalized embryo-derived PAF with rPAF acetylhydrolase followed by exposure of embryos to exogenous PAF. Embryos (collected 29–31 h post-fertilization) responded to PAF (0.037–372 nM) in a dose-dependent manner (Fig. 3). At the lowest concentrations (0.037 nM) responses were not common and were of low amplitude when they did occur. At a concentration of 0.37 nM, low amplitude (p < 0.01) transients were consistently observed. At a concentration of 3.7, 37.2 (results not shown), and 372 nM responses were of a consistently high amplitude. At 0.37 nM,
the time taken for the peak amplitude of the response to be achieved was longer ($p < 0.01$) compared with higher doses. Transients in response to exogenous PAF were typically single peaks; repetitive oscillations of short duration were rarely observed. The new baseline established after the [Ca$^{2+}$]i peak was generally higher than the original baseline, perhaps indicating residual capacitative calcium entry (CCE).

WEB 2086 (0.44–44 μM), a competitive PAF-receptor antagonist (28), reduced the mean amplitude of [Ca$^{2+}$]i transients in response to exogenous PAF (37 nM) compared with control responses. Responses were reduced ($p < 0.0001$) at 0.4–4.4 μM (Table I) and essentially abolished at 44 μM. BN 50730 also caused partial inhibition ($p < 0.001$) of [Ca$^{2+}$]i transients induced by PAF but was not as effective as WEB 2086 (Table I).

The specificity of PAF’s action was further confirmed by testing the response of embryos to the enantiomeric isomer of PAF (3-O-alkyl-2-acetyl-sn-glycero-1-phosphocholine). Over a concentration range of 40–400 nM, enantiomeric PAF was without effect on the [Ca$^{2+}$]i of 2-cell embryos (35–37 h) that had been pretreated with rPAF acetylhydrolase (results not shown).

The Ontogeny of Responsiveness to PAF—Unfertilized oocytes and young zygotes (7–9 h after fertilization) failed to show any [Ca$^{2+}$]i transients in response to medium containing albumin. By 10–13 h after fertilization, 8 of 32 zygotes showed detectable [Ca$^{2+}$]i transients, and these were of modest amplitude (87.8 ± 24.5 nM above baseline values).

For 2-cell embryos collected fresh from the reproductive tract, transients in response to embryo-derived PAF varied with the age of the embryo (Fig. 4a). At 27–29 h after fertilization there were modest responses to embryo-derived PAF. By 31–33 h after fertilization the average [Ca$^{2+}$]i transient was significantly ($p < 0.01$) greater in peak amplitude above baseline and reached its peak earlier ($p < 0.01$) than observed at 27–29 h. At 35–37 h embryo responses to embryo-derived PAF had declined in average amplitude ($p < 0.01$) and took longer ($p < 0.05$) to achieve peak amplitude above baseline than observed at 31–33 h. By 41–43 h the responses were significantly ($p < 0.05$) attenuated compared with each other time point tested for 2-cell embryos. No [Ca$^{2+}$]i transients were observed in 4-cell, 8-cell, and morulae stage embryos (not shown).

To determine whether these changes in responsiveness during development were due to changes in the availability of embryo-derived PAF or to changes in the capability of embryos to respond to PAF, we compared the embryo-derived PAF induced [Ca$^{2+}$]i responses to those induced by exogenous PAF (372 nM) (in rPAF acetylhydrolase treated embryos). Oocytes and early embryos (7–9 h after fertilization) did not respond to exogenous PAF, but by 10–13 h after fertilization embryos showed a [Ca$^{2+}$]i response. The peak amplitude of the response to exogenous PAF was significantly ($p < 0.0001$) larger (157.1 ± 16.4 nM above baseline) than the embryo-derived PAF-induced responses.

In 2-cell embryos the [Ca$^{2+}$]i response to exogenous PAF was greater than the spontaneous responses at each of the times studied (Fig. 4b). In contrast to spontaneous [Ca$^{2+}$]i transients, the amplitude of the response to exogenous PAF was similar ($p > 0.05$) at 27–29 h and 31–33 h after fertilization. Transients were significantly attenuated at 35–37 and 41–43 h after fertilization ($p < 0.01$) compared with younger 2-cell embryos. At each of these times, the average amplitude of response to exogenous PAF was greater than the response to embryo-derived PAF in corresponding embryos. Only 4 of 38 4-cell embryos showed modest but variable [Ca$^{2+}$]i transients (47.3 ± 22.9 nM [Ca$^{2+}$]i above baseline; $n = 4$), and no 8-cell embryos displayed a response to exogenous PAF ($n = 37$).

The greater amplitude of responses to exogenous PAF in late zygotes and 27–29 h-old 2-cells shows that embryo-derived PAF may be limiting at those times. The failure of early zygotes to respond to either source of PAF shows that they were not capable of responding at that time. Furthermore, the similar

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### Table I

| Antagonist concentration | Maximum rise in calcium concentration above baseline (nM) | Mean ± S.E. |
|-------------------------|--------------------------------------------------------|-------------|
| WEB 2086                |                                                        |             |
| 0 μM                    | 415 ± 100.2                                            | 270 ± 42.0  |
| 0.04                    | 159.0 ± 22.9                                          | ND*         |
| 0.44                    | 203.0 ± 29.3                                          | ND          |
| 4.40                    | 131.4 ± 30.1                                          | 104.0 ± 14.0|
| 44.0                    | 19.8 ± 3.6                                            | 136.0 ± 19.0|

* ND, not determined.

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**Fig. 3.** The change in [Ca$^{2+}$]i in 2-cell embryos (29–31 h post-human chorionic gonadotropin) in response to increasing concentration of exogenous PAF: 0.037 nM (●, n = 32), 0.37 nM (●, n = 37), 3.7 nM (▲, n = 50), and 372 nM (▼, n = 54).

**Fig. 4.** a, the [Ca$^{2+}$]i response of 2-cell embryos to embryo-derived PAF. Embryos were collected and tested at 27–29 h ($n = 48$), 31–33 h ($n = 57$), 35–37 h ($n = 48$), and 41–43 h ($n = 27$) after fertilization. The results are the mean ± S.E. of the number ($n$) of embryos shown. b, calcium transients induced by exogenous PAF (372 nM). 2-Cell embryos were collected fresh from the reproductive tract, treated with rPAF acetylhydrolase to degrade embryo-derived PAF and then challenged with exogenous PAF. The results were from cohorts of embryos collected at different times during the 2-cell stage of development as indicated: 27–29 h ($n = 34$), 31–33 h ($n = 61$), 35–37 h ($n = 54$), and 41–43 h ($n = 37$) after fertilization. The results are the mean ± S.E. of the number ($n$) of embryos shown.
loss of responsiveness to both forms of PAF in the late 2- and 4-cell embryo shows that this was also not primarily due to a lack of embryo-derived PAF.

The Onset of Responses to PAF Requires Transcription from the Zygotic Genome—To define the time of onset of responsiveness more precisely, zygotes were collected from the reproductive tract approximately 5 h after fertilization and cultured in vitro. They were treated with rPAF acetylhydrolase and then challenged with exogenous PAF at 10, 11, and 12 h after fertilization and the [Ca\(^{2+}\)] response monitored. At 10 h, consistent but relatively low amplitude responses were observed (Fig. 5). The average amplitude of the responses increased (p < 0.01) with each subsequent hour of culture (11 and 12 h post-fertilization). When zygotes were cultured in α-amanitin for the same periods, the [Ca\(^{2+}\)] response to PAF was significantly inhibited (p < 0.0001) at all time points (the inhibition of the response by α-amanitin at 12 h after fertilization is shown in Fig. 5). Therefore, the onset of [Ca\(^{2+}\)] transients in response to exogenous PAF first occurred from 10 h after fertilization and required a transcriptional event.

PAF-induced Calcium Transients Required Extracellular and Intracellular Calcium—A role for internal calcium stores was implicated by inhibition of PAF-induced responses by the inhibition of PLC by U73122 (5 μM, 5 min) while its inactive analogue (U73343, 5 μM, 5 min) was without effect (Fig. 6). It seems likely that the action of PLC was to produce IP3 since the calcium transients (Fig. 8), suggesting the involvement of a phospholipase C inhibitor U73343, being an inactive analogue of U73122, and the effect of xestospongin C (10 μM). Each panel shows a typical response. Each treatment was performed 3 times with 6–8 embryos per replicate. Embryos were treated with U73122 and U73343 for 5 min and xestospongin for 30 min prior to PAF challenge. The arrow shows the time of PAF challenge.

A role for extracellular calcium in generating the response was also implicated by the failure of 2-cell embryos to respond to PAF in calcium-free medium (Fig. 7). This was the case for both embryo-derived and exogenous PAF. The addition of EGTA (Fig. 7) to calcium-free medium caused a [Ca\(^{2+}\)] transient even in the absence of PAF, illustrating an unexpected aspect of calcium homeostasis in the early embryo that requires further investigation.

Treatment of 2-cell embryos with a dihydropyridine (nifedipine, 10 μM) significantly (p < 0.001) inhibited PAF-induced calcium transients (Fig. 8), suggesting the involvement of a dihydropyridine-sensitive calcium channel in generating the rise in [Ca\(^{2+}\)]. BAY K8644 induced calcium transients in a dose-dependent manner confirming the presence of a functional L-type calcium channel in the 2-cell embryo (Fig. 9). However, the [Ca\(^{2+}\)] transient induced by BAY K8644 was entirely inhibited by 10 μM nifedipine (Fig. 9), indicating that the calcium influx induced by PAF was not entirely accounted for by the L-type channel. The 2-cell embryo also possesses a T-type (low voltage-activated) channel that is inhibited by pimozide (29). However, pimozide had no influence (p > 0.05) on PAF-induced [Ca\(^{2+}\)] transients (results not shown) suggesting that the T-channel was not involved in this response. Depolarization of 2-cell embryos by perfusing with 25 or 50 mM K\(^{+}\) for 5 min (perfusion media had NaCl isomotically replaced with KCl) did not induce [Ca\(^{2+}\)] transients or changes in the baseline calcium levels (not shown). In the presence of 50 mM K\(^{+}\) 2-cell embryos still showed characteristic transients to embryo-derived PAF, however, the amplitude of the responses was consistently lower than responses in control media (Fig. 10). This result shows that the PAF-induced calcium influx was not voltage-gated, but as expected the rate and extent of calcium influx was influenced by the membrane potential.

The activation of store-operated calcium channels (SOC) in...
non-excitable cells is induced by release of calcium from IP_{3}-sensitive stores (30). Experimental activation of SOCs can be achieved by depletion of internal calcium stores by thapsigargin. Treatment of 2-cell embryos with thapsigargin (1 \text{mM}) caused a characteristic transient of similar magnitude as that caused by PAF (Fig. 11). Continued treatment with thapsigargin resulted in a new baseline being established that was consistently higher than the pretreatment baseline. In the absence of extracellular calcium, thapsigargin treatment still caused a characteristic transient but the \([\text{Ca}^{2+}]_i\) then declined to below original baseline (Fig. 11). The establishment of a new higher baseline in medium containing calcium after thapsigargin treatment is indicative of SOC activity, and consequent CCE.

**PAF-induced Calcium Transients in the Early Embryo Influence Their Subsequent Developmental Potential—Buffering intracellular calcium by treatment of embryos for 20 min with 1 \text{mM} BAPTA-AM caused significant inhibition \((p < 0.001)\) of embryo-derived PAF-induced-[Ca\(^{2+}\)]_i transients (Fig. 12). Following BAPTA-AM treatment, however, the addition of exogenous PAF was capable of inducing transients with an amplitude that was significantly greater \((p < 0.001)\) than those in...
response to embryo-derived PAF in the presence of BAPTA-AM, and similar to those induced by embryo-derived PAF in control embryos (in the absence of BAPTA-AM) (Fig. 12). Exposure of 2-cell embryos to 1.0 μM BAPTA-AM for 20 min followed by their culture in vitro, caused a significant (p < 0.01) reduction in the proportion of embryos developing to the 4-cell stage and then on to the blastocyst stage (Table II). Increasing the culture time to 120 h did not increase the number of embryos developing to the blastocyst stage (not shown). The addition of exogenous PAF to medium partially ameliorated the actions of BAPTA-AM. At the 4-cell and 8-cell stage, the presence of exogenous PAF prevented the inhibition of development below control rates (p > 0.05). By the morulae and blastocyst stages, however, some inhibitory effects were also manifested by BAPTA-AM-treated embryos exposed to PAF. The rate of cell-cycle progression throughout the preimplantation phase as assessed by the number of cells/blastocyst was markedly retarded (p < 0.01) by the brief exposure to BAPTA-AM at the two-cell stage. This was significantly reversed by the presence of exogenous PAF. The results showed that short-term buffering of intracellular calcium with BAPTA-AM caused long-term adverse effects on normal embryo development. A significant component of the adverse effects of BAPTA-AM was due to the inhibition of PAF-induced transients, since supplementation of medium with PAF could partially alleviate the consequences of BAPTA-AM treatment.

The effect of the PAF-antagonists WEB 2086 and BN 50730 on the development of zygotes in vitro was assessed (Table III). Zygotes were cultured singly in 10-μl drops of modHTF under oil for 96 h and the proportion that developed to the blastocyst stage was assessed. WEB 2086 (44 μM) significantly (p < 0.001) reduced the proportion of zygotes that developed to the blastocyst stage and the number of cells within each of the resulting blastocysts. The antagonist did not block zygote to 2-cell progression but caused progressive loss of viability with development, much as results from PAF deprivation (9). BN 50730 (4.4 and 44 μM) had no adverse effect on the development of zygotes to the blastocyst stage, consistent with its lower effectiveness at blocking PAF-induced [Ca^{2+}], transients. The development of embryos collected at the late 2-cell stage (34 h after fertilization) to the blastocyst stage was unaffected by WEB 2086 (44 μM).

**DISCUSSION**

This study showed that embryo-derived PAF induces transient increases in the [Ca^{2+}], in the late zygote and 2-cell mouse embryo in vitro. These [Ca^{2+}], transients were not apparently associated with specific cell-cycle checkpoints but occurred during interphase of the cell-cycle. The induction of these [Ca^{2+}], transients was important for normal embryo development since buffering intracellular calcium concentration with BAPTA-AM inhibited development and this could be partially alleviated by exogenous PAF. A concentration of a PAF-antagonist (WEB 2086) that inhibited [Ca^{2+}], transients also limited embryo growth.

The specificity of the action of PAF in inducing [Ca^{2+}], transients was shown by: (a) its concentration dependence; (b) the homologous desensitization of the response by repeated exposure to PAF; (c) its inhibition by a selective PAF-receptor antagonist; (d) the failure of the enantiomeric isomer of PAF to elicit [Ca^{2+}], responses; (e) the inhibition of spontaneous transients by exposure of embryos to rPAF acetylhydrolase; and (f) the failure of a range of other growth factors to elicit [Ca^{2+}], transients.

The onset of responsiveness of zygotes to PAF required a transcriptional event that was inhibited by α-amanitin. It will be of interest to determine whether this transcription is of a PAF-receptor or an essential component of the transduction pathway. We believe these [Ca^{2+}], transients to be the earliest description of a functional response by the embryo that is dependent upon transcription from the zygotic genome. It will therefore be an important tool for investigating the regulatory processes controlling the onset of transcription from the zygotic genome.

By comparing the embryo’s responses to embryo-derived and exogenous PAF, it was possible to define whether [Ca^{2+}], transients were limited by the availability of embryo-derived PAF or the capability of the embryo to respond to the available PAF. From the time of onset of the embryo’s responsiveness to PAF until 31–33 h after fertilization the magnitude of transients was limited primarily by the availability of embryo-derived PAF. After this time the response became progressively attenuated due to limitations in the capability of embryos to respond to the available PAF. By the 4- and 8-cell stages, embryos were generally not capable of responding with [Ca^{2+}], transients to either embryo-derived PAF or exogenous PAF. This ontogeny of responsiveness to PAF is interesting, since it was previously shown (9) that exposure of embryos to PAF was required by the mid-2-cell stage. If PAF was limited up to that time (by culturing at low embryo densities), increased rates of embryonic lethality resulted, but embryos could be rescued by addition of PAF to medium by the 2-cell stage. Exposure to PAF after the 2-cell stage was unable to rescue PAF-deprived embryos (9).

The results of the current study provide evidence that the [Ca^{2+}], transients required both extracellular calcium and release of intracellular stores of calcium. The need for extracellular calcium was supported by the requirement for extracellular calcium and the partial dihydropyridine (nifedipine) sensitivity of the PAF-induced transients. The presence of a functional dihydropyridine-sensitive L-type channel was confirmed by the action of BAY K8644 in inducing [Ca^{2+}], transients. We believe this is the first functional evidence for this.
Calcium Transients in Mouse Embryos

Table II
The effect of buffering intracellular calcium with BAPTA-AM on embryo development

| Treatment          | n  | 24 h 4-cells | 48 h 8-cells | 72 h Morulae | 96 h Blastocyst |
|--------------------|----|--------------|--------------|--------------|----------------|
| Control            | 60 | 90           | 85           | 80           | 80             |
| BAPTA-AM           | 60 | 62           | 52           | 47           | 40             |
| BAPTA-AM + PAF     | 50 | 78           | 70           | 56           | 56             |

Significantly different (p < 0.01) from the development rate (Chi square) or cell numbers (analysis of variance) in control embryos.

Table III
The effect of WEB 2086 and BN 50730 on embryo development through to the blastocyst stage

| Embryo type | Proportion of embryos (%) developing to the blastocyst stage (total number of embryos cultured, antagonist concentration (μM)) |
|-------------|-------------------------------------------------------------------------------------------------------------------|
|             | 0 | 4.4 | 44 |
| WEB 2086    | Zygotes 60.0 (120) | 55.0 (120) | 38.3 (120)² |
|             | 2-cells 90.0 (40)  | ND²       | 86.1 (36)   |
| BN 50730    | Zygotes 57.5 (80)  | 57.8 (90)  | 51.1 (90)   |

² p < 0.001 by Chi square analysis. All comparisons are to control treatment.

The observations of a requirement for extracellular calcium and the involvement of IP₃-sensitive stores in intracellular calcium suggest an interdependence of these two sources of calcium. Mobilization of intracellular calcium stores by the PLC/IP₃ pathway is widely implicated in the opening of store-operated channels to initiate CCE (30). Rises in intracellular calcium resulting from this mechanism are more sustained, resulting in prolonged elevation compared to the transients observed in excitable cells, and this is the pattern observed in PAF-induced embryos. These sustained elevations of intracellular calcium are important in non-excitable cells, and are known to regulate many cellular processes including gene transcription (31, 32). The observation that PAF-induced calcium transient caused a new, higher baseline calcium concentration to be detected if prior treatment with rPAF acetylhydrolase was not served if prior treatment with rPAF acetylhydrolase was not available to interact with PAF receptors, as demonstrated by the inhibitory action of a PAF-receptor antagonist, WEB 2086. This hypothetical mechanism provides the basis for an autocrine loop.

The homologous desensitization of the embryos response to a second PAF challenge by both embryo-derived and exogenous PAF may ensure that the persistent presence of PAF in the embryos vicinity does not cause chronic elevation of [Ca²⁺]. The incidence of [Ca²⁺], transients may therefore be regulated by both the rate of PAF synthesis/release by the embryo and the rate at which the embryo becomes resensitized after a PAF-induced response. This model provides a mechanism whereby intermittent [Ca²⁺], transients could occur during the late zygote and 2-cell stage. Such intermittent calcium transients could provide an information-rich mechanism for signal generation. The observation that exogenous PAF also induced [Ca²⁺], transients confirms an earlier observation (21). In our hands, however, responses to exogenous PAF were not regularly observed if prior treatment with rPAF acetylhydrolase was not used. This seems to be due to embryo responses to PAF being down-regulated by homologous desensitization for long periods.
The preparation of embryos in protein-free medium or with rPAF acetylhydrolase was required to allow them to escape this down-regulation, and to thus consistently detect PAF-induced transients.

Inhibition of long-term embryo development by exposure of 2-cell embryos during early interphase to BAPTA-AM for a brief period (20 min) is consistent with a hypothesis that PAF-induced \([\text{Ca}^{2+}]\) transients at the 2-cell stage act to initiate a developmental program. However, buffering cell cycle-dependent \([\text{Ca}^{2+}]\) transients can block mitosis (17). In this study the limited exposure to BAPTA-AM model and the ability of exogenous PAF to reverse its effects argue that a major cause of the growth restriction caused by BAPTA-AM treatment was a consequence of inhibiting embryo-derived PAF calcium transients. A functionally significant role for PAF-induced calcium transients is further supported by the reduction of embryo development induced by the PAF-antagonist WEB 2086 at concentrations that inhibited calcium transients. Another antagonist, BN 50730 was relatively ineffective in blocking PAF-induced development induced by the PAF-antagonist WEB 2086 at concentrations that inhibited calcium transients. Another antagonist, BN 50730 was relatively ineffective in blocking PAF-induced \([\text{Ca}^{2+}]\), and it had no apparent affect on development.

The findings of: (a) the occurrence of PAF-induced calcium transients in the early embryo; (b) their inhibition by buffering intracellular calcium with BAPTA-AM, leading to retarded embryo development; (c) the partial relief of this inhibition by excess exogenous PAF; and (d) the correlation between the inhibition of \([\text{Ca}^{2+}]\), transients by a PAF antagonist and its inhibition of embryo development; together argue that mobilization of calcium transients by embryo-derived PAF form an important autocrine signaling pathway in the zygote and 2-cell embryo that is required for the subsequent normal rates of development of the early preimplantation embryo in vitro.

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