response (Fig. 4*a*). Elevation of external Ca^{2+} from the normal level of 2 mM to 10 mM significantly reduced the rate of failure in evoking ACh release. The onset times of the current events following depolarizing steps were also significantly reduced in high Ca^{2+} medium (Fig. 4*b*, upper graph). When the myocyte cytosolic Ca^{2+} was buffered at a low level (10^{-7} M) by loading a Ca^{2+} buffer, BAPTA, the depolarization-evoked secretion events were abolished (Fig. 4*b*, lower graphs), leaving random spontaneous events occurring at a reduced frequency. These results are consistent with a Ca^{2+}-regulated evoked secretion, triggered by the Ca^{2+} influx through voltage-dependent calcium channels in the myocyte membrane. It is also clear that the excitation-secretion coupling in the myocyte was significantly weaker than that of the neuromuscular junction, where the delay of onset of evoked responses is less than 1 ms and the failure occurs only at much lower level of external Ca^{2+}.

In summary, we have shown that myocytes loaded with exogenous ACh exhibited spontaneous quantal ACh secretion and depolarization-evoked ACh release, with a Ca^{2+}-dependence reminiscent of ACh secretion from presynaptic nerve terminals. The ACh appeared to accumulate in cytoplasmic compartments before secretion. Thus, with sufficient ACh supply in the cytosol, Ca^{2+}-regulated exocytotic secretion of ACh can occur in a non-neuronal cell. In comparison with neuronal secretion, however, excitation-secretion coupling in the myocyte is weak. Future work of introducing synapse-specific components[22-25] into ACh-loaded myocytes should reveal their individual contribution to the assembly and the regulation of an efficient mechanism for transmitter secretion. □

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Patterns of elevated free calcium and calmodulin activation in living cells

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THE temporal and spatial dynamics of intracellular signals and protein effectors are being defined as a result of imaging using fluorescent reagents within living cells[1-5]. We have described a new class of fluorescent analogues[2] termed optical biosensors[6], which sense chemical or molecular events through their effects on protein transducers[7]. One example of this new class of indicators is MeroCaM, an environmentally sensitive fluorophore which when it is attached to calmodulin reflects the activation of calmodulin by calcium *in vitro*[2]. We report here that the rise in free calcium and MeroCaM activation occur in the same period during serum stimulation of quiescent fibroblasts. MeroCaM activation also correlates with the spatial pattern of increased free calcium and the contraction of transverse fibres during wound healing[1,8-10]. Finally, migrating fibroblasts in the later stages of wound-healing exhibit an increasing gradient of free calcium and MeroCaM activation from the front to the rear.

We investigated two cellular events to test prevailing hypotheses, derived from solution biochemistry[11,12], concerning calmodulin's role in calcium-linked activation of cell functions. First, we correlated calcium transients and MeroCaM activation during serum stimulation of quiescent cells to test whether calmodulin activation is coupled to calcium elevation. Second, we mapped the spatial variation of free calcium concentration and MeroCaM activation in polarized, motile cells during wound-healing to explore the potential role of calcium–calmodulin signalling in regulating myosin II-based contraction of transverse fibres[1,8-10].

MeroCaM undergoes a calcium-dependent, threefold change in excitation ratio *in vitro* (Fig. 1, inset) at long wavelengths (608/532 nm) that minimize cell damage and interference from cellular autofluorescence[2] (Fig. 1). The biosensor's suitability for ratio imaging is valuable for quantitative interpretation of fluorescence in living cells[13]. Ratio imaging eliminates effects of variable intracellular biosensor concentration, cellular pathlength and other factors[13]. Since MeroCaM was characterized *in vitro*[2], an optimized preparative procedure has produced MeroCaM containing 80–90% of a single fluorescent product and less than 5% unlabelled calmodulin. Titration of this preparation with myosin light-chain kinase produced a slight change in excitation ratio (0.15-fold), indicative of binding to this target protein with $K_d < 10$ nM, and demonstrating that the fluorescence response of MeroCaM is not greatly affected by binding to a target protein.

Investigation of MeroCaM behaviour in live cells directly tests the hypothesis that calmodulin is activated by increased cytoplasmic free calcium. MeroCaM response was studied during serum stimulation of quiescent Swiss 3T3 fibroblasts. Such stimulation results in a rapid increase of intracellular free calcium followed by a return to almost initial values within several minutes[14-16]. Figure 1 shows experiments in which quiescent fibroblasts were injected with MeroCaM or the fluorescent calcium indicator, Calcium Green. The results verified the previously reported calcium response to stimulation[14-17] and the large cell-to-cell heterogeneity of response[5,14,15,18], and demonstrated the responsiveness of MeroCaM to calcium *in vivo*. Serum stimulation produced a transient increase in the average MeroCaM excitation ratio, indicating calmodulin activation in these cells.

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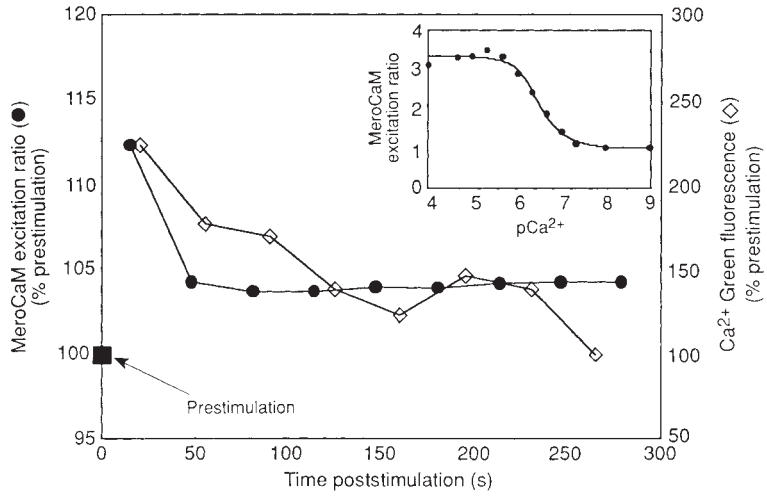

FIG. 1 Responses of MeroCaM and the fluorescent calcium indicator Calcium Green during serum stimulation of quiescent Swiss 3T3 fibroblasts. Calcium Green was microinjected into quiescent Swiss 3T3 fibroblasts and the average cellular fluorescence determined periodically for each cell during stimulation with serum (excitation, 470 nm emission, 530 nm). A representative trace from one cell is shown. In all 19 cells tested, serum induced an initial increase of 25–180% in Calcium Green fluorescence. Fluorescence then dropped towards prestimulation values, with additional smaller fluorescence maxima observed in some cases. These results demonstrated the time course of the calcium transients as a reference for MeroCaM studies. Cells were also microinjected with MeroCaM and the average fluorescence excitation ratio was determined periodically for each cell during stimulation (605/530 nm; emission, 635 nm). In 4 out of 10 cells examined, the average cellular fluorescence ratio increased 5–20% at the first time point after stimulation, indicative of activation of MeroCaM. Four cells showed an increase of less than 5%, and in one the fluorescence only decreased. After the initial poststimulation time point, the ratio always decreased sharply, then reached a plateau above prestimulation levels or continued to drop. The fluorescence ratio of MeroCaM varied by at most 5% over 100 seconds following 'stimulation with low-serum control medium. We cannot explain the drop in fluorescence of a subpopulation of cells. Controls rule out simple photobelaching artefacts. Cells were prepared and stimulated according to published methods[18]. All images used were acquired analysed and displayed with the Multimode microscope (Biological Detection Systems, PA). A cooled CCD camera was employed with 2–3 second exposure. Inset, the calcium-dependence of MeroCaM's excitation ratio, from ref. 2.

Imaging of individual cells revealed spatial aspects of calmodulin activation not accessible using other approaches. Figure 2 demonstrates the temporal and spatial heterogeneity of calmodulin activation in stimulated cells. The ratio-imaging technique normalizes local probe concentration[5,13], so that the signals shown represent MeroCaM activation. In a number of cells, calmodulin activation occurred at the cell periphery immediately after stimulation, and then propagated towards the nucleus. The level of calmodulin activity in these areas fell and rose during movement to the perinuclear region. These spatially heterogeneous responses could result from subcellular variations in calcium concentration, or from effects of target proteins on calmodulin's calcium affinity[12]. It has previously been demonstrated that serum stimulation of quiescent fibroblasts causes stress fibres to contract and condense in the perinuclear region[18]. Calmodulin target enzymes such as myosin light-chain kinase are thought to regulate myosin II (refs 19–21) and so may condense with the stress fibres.

We also analysed MeroCaM in highly polarized cells undergoing wound-healing to test whether myosin-II based contractions[1,8–10] *in vivo* involve calmodulin activation. A wound was introduced in a monolayer of Swiss 3T3 fibroblasts and cells along the wound's edge were microinjected with MeroCaM alone or in combination with the calcium indicator (legends to Figs 3 and 4). Figure 3 shows the temporal and spatial dynamics of calmodulin activation monitored early in the wound-healing process. Calmodulin activation was greatest in the lamellae where contraction of transverse fibres made the anterior of the cell narrower and induced detachment from neighbouring cells[1,8–10]. Figure 4*a* and *b* shows that elevated free calcium correlates with MeroCaM activation in the lamellum, where transverse fibres contract while transporting to the perinuclear region[1]. The localized rise in free calcium and activation of calmodulin in this region indicated that contraction of the transverse fibres is regulated spatially by calcium and calmodulin[19]. Figure 4*c* and *d* shows the existence of an increasing gradient

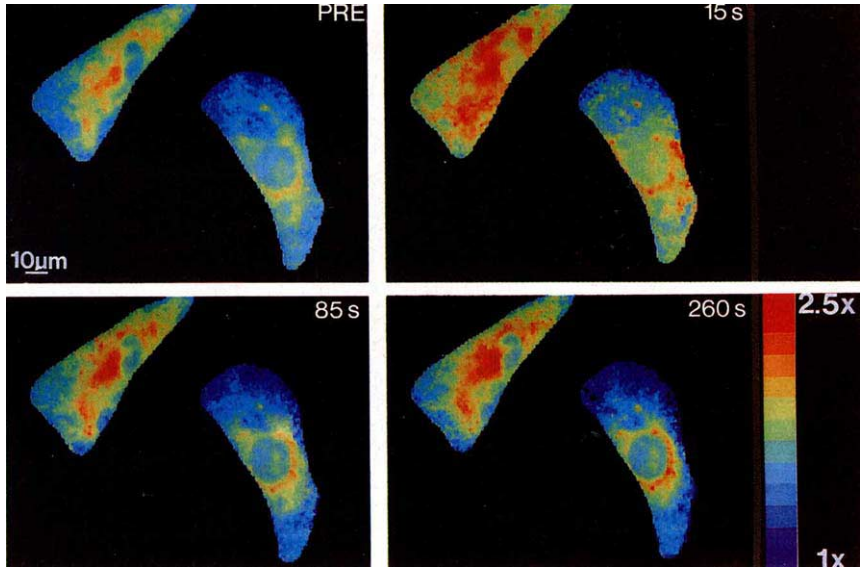

FIG. 2 Spatial distribution of calmodulin activation in fibroblasts during serum stimulation. Quiescent Swiss 3T3 fibroblasts were microinjected with MeroCaM according to ref. 25 and the biosensor's excitation ratio was imaged during serum stimulation. The spatial distribution of calmodulin activation is shown. Higher excitation ratios, and hence greater calmodulin activation, correspond to warmer colours (red maximal). The difference between the highest and lowest values corresponds to a 2.5-fold difference in excitation ratio. Free calcium concentration of quiescent fibroblasts is below 0.1 μM and stimulation with serum induces heterogeneous responses of up to several micromolar at peak values[14–16]. This representative sequence of images illustrates movement of activated regions towards the nucleus over time, most clearly visible in the cell on the upper left. Estimated intracellular concentrations were Calcium Green, 10 μM; MeroCaM, 10–30 μM.

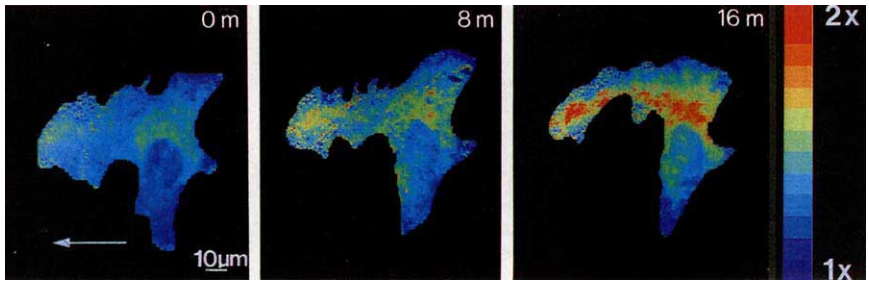

FIG. 3 Subcellular localization of calmodulin activation in fibroblasts during the lamellar contraction phase of wound-healing. A 'wound' was introduced in a monolayer of Swiss 3T3 fibroblasts[8-10] and cells at the edge of the wound were microinjected with MeroCaM. The excitation ratio of MeroCaM was imaged in time-lapse in individual cells as they contracted during the separation from neighbouring cells. In all actively contracting cells (7 of 14 cells examined), calmodulin activity was greatest in the leading lamellae, regions undergoing strong contraction of transverse fibres that contain actin and myosin II[8-10]. Note the increased activation of MeroCaM in the time series as the leading lamellum is constricted by the contraction. As in Fig. 2, warmer colours correspond to greater MeroCaM ratios, and hence to greater calmodulin activation. There was a 2.5-fold difference between regions of lowest activity (blue) and highest activity (red) (see Fig. 1). The arrow indicates the direction of orientation and movement. Cells were microinjected 5 h after wounding and images were taken 30 min later at 8-min intervals. A control experiment to determine the distribution of MeroCaM was done by co-injecting MeroCaM with the volume indicator fluorescein–dextran (M_r 10K). The ratio image (MeroCaM/fluorescein–dextran) using the isosbestic point of MeroCaM demonstrated a relatively uniform distribution of MeroCaM, except where there was extensive contraction which is consistent with earlier studies with fluorescent analogues of calmodulin[25,26].

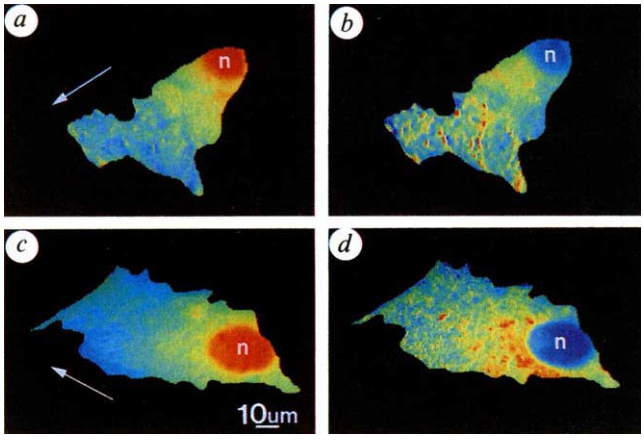

FIG. 4 Subcellular localization of increases in free calcium ions and MeroCaM activation at single time points in fibroblasts during two stages of wound-healing[1,8-10]. Cells were co-injected with a mixture of Calcium Green/Dextran 10K, the calcium-insensitive volume indicator Cascade Blue/Dextran 10K (both from Molecular Probes), and MeroCaM to produce ratio images mapping regions of elevated free calcium (Calcium Green/Cascade Blue) and MeroCaM activation (excitation 605/530 nm, emission 635 nm). *a* and *b*, One time point during the contraction of transverse fibres demonstrates the similar pattern of elevated free calcium and MeroCaM activation in the lamellum during the lamellar contractile phase of wound-healing. The pattern of elevated free calcium (*a*) and MeroCaM activation (*b*) were very similar in 12 cells investigated. *c* and *d*, Increasing gradient of free calcium (*c*) and MeroCaM activation (*d*) from the front of the cell to the rear of the lamellum of a migrating cell. This gradient was detected in all 12 polarized cells. Warmer colours represent both elevated free calcium and MeroCaM activation. Arrows indicate the direction of orientation and movement; *n*, nucleus. The relatively high and sustained free calcium concentration in the nucleus is detected in many activated cells and is not understood, but may reflect nuclear activation[15]. MeroCaM activation is not always correlated with elevated free calcium in the nucleus. A quantitative ratio method is currently being developed to take advantage of the relatively high fluorescence signal from Calcium Green even at low free-calcium-ion concentrations. Neutral dextrans will be used as optimal carriers of ion indicators to maintain the indicator in the free cytoplasmic compartment[5,13].

of free calcium and calmodulin activation from the front to the rear of migrating cells during later stages of wound-healing. The elevated free calcium in the rear of locomoting fibroblasts is comparable to that originally demonstrated in amoebae[22] and then in eosinophils[23]. The gradient of free calcium may be involved in regulating the polarity of actin and myosin II assembly at the front of the migrating cell[8,10], as well as in the rearward transport and contraction of the transverse fibres. The myosin II-based transverse fibres contract maximally and then disappear as they transport into the perinuclear region[1,3,8-10]. The mottled appearance of the MeroCaM activation (Fig. 4*d*), especially in the perinuclear region, may indicate a concentration of calmodulin during the maximal contraction of transverse fibres.

The ability to study the temporal-spatial dynamics of chemical and molecular processes in individual cells with a variety of reagents and modes of light microscopy will be important in defining the mechanisms of cell functions such as motility[24]. □

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