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Elementary Ca2+ Signals Through Endothelial TRPV4 Channels Regulate Vascular Function

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Major features of the transcellular signaling mechanism responsible for endothelium-dependent regulation of vascular smooth muscle tone are unresolved. We identified local calcium (Ca2+) signals (“sparklets”) in the vascular endothelium of resistance arteries that represent Ca2+- influx through single TRPV4 cation channels. Gating of individual TRPV4 channels within a four-channel cluster was cooperative, with activation of as few as three channels per cell causing maximal dilation through activation of endothelial cell intermediate (IK)- and small (SK)-conductance, Ca2+-sensitive potassium (K+) channels. Endothelial-dependent muscarinic receptor signaling also acted largely through TRPV4 sparklet-mediated stimulation of IK and SK channels to promote vasodilation. These results support the concept that Ca2+- influx through single TRPV4 channels is leveraged by the amplifier effect of cooperative channel gating and the high Ca2+ sensitivity of IK and SK channels to cause vasodilation.

Endothelial cells (ECs) line all blood vessels and regulate the smooth muscle contractile state (tone). The concentration of intracellular free calcium ([Ca2+]i) in ECs is increased by influx and by release from intracellular stores through inositol trisphosphate receptors (IP3Rs) in the membrane of the endoplasmic reticulum. Although Ca2+-influx pathways are incompletely characterized, members of the transient receptor potential (TRP) family of nonselective cation channels have been implicated in this function. In particular, results from gene-knockout studies suggest that the vanilloid (TRPV) family member TRPV4 is involved in endothelium-dependent vascular dilation in response to flow and acetylcholine (ACh) (1–5).

Increases in endothelial [Ca2+]i activate EC pathways that terminate in the release of soluble factors or initiation of processes that hyperpolarize the membrane of adjacent vascular smooth muscle cells, and thus promote dilation. These Ca2+-dependent vasodilatory influences fall into three broad categories: (i) nitric oxide (NO), a gas produced in the endothelium that causes smooth muscle relaxation (6); (ii) prostaglandins, produced through cyclooxygenase (COX) (7); and (iii) endothelial-derived hyperpolarizing factor (EDHF), characterized by its strict dependence on the activity of EC intermediate-conductance KCa3.1 channels (8). Although a number of factors have been suggested as EDHF, accumulating evidence points to the importance of electrotonic spread of EC IK and/or SK channel–mediated hyperpolarizing current to smooth muscle cells through gap junctions (8, 9).

Studies of Ca2+-signaling in ECs using conventional Ca2+-binding fluorescent dyes (e.g., Fluoro-4) are limited by interference from the vigorous Ca2+-signaling activity of adjacent smooth muscle cells, which also readily take up such dyes. A recently developed alternative is a transgenic mouse that expresses a genetically encoded Ca2+-sensor (GCaMP2) exclusively in the endothelium of the vascular wall (10, 11). GCaMP2 is a fusion protein of the Ca2+-binding protein calmodulin and a circularly permuted enhanced green fluorescent protein (EGFP) that fluoresces when Ca2+ binds to calmodulin. The GCaMP2 protein is homogeneously expressed throughout the EC (11) and allows long, stable recordings of intracellular Ca2+ in ECs in the intact blood vessel wall, without contamination of signals from smooth muscle. Using this model, we previously identified local, IP3R-mediated Ca2+ events in ECs, termed Ca2+ pulsars (10), that had previously gone undetected with conventional imaging protocols.

To identify Ca2+-influx pathways in the ECs of resistance arteries (i.e., arteries important in regulating peripheral resistance and blood pressure), we imaged Ca2+–fluorescence in isolated, small (100 μm diameter) mesenteric arteries from GCaMP2 mice using confocal microscopy (12). Isolated arteries were surgically opened and pinned down with the EC surface facing up (en face preparation) to improve optical resolution (10). In a single field of view, local Ca2+ signals in ~14 individual ECs could be recorded simultaneously with high spatial (0.3 μm) and temporal (15 ms) resolution. Events were analyzed offline by measuring the fluorescence intensity over time within defined 1.7-μm2 regions of interest on images corresponding to active sites.

With IP3R-mediated signaling eliminated by pretreatment with the sarcoplasmic reticulum/endoplasmic reticulum Ca2+-ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA), or the

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Supplementary Materials

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Materials and Methods

Figs. S1 to S7

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phospholipase C (PLC) blocker, U73122 (10), local Ca\(^{2+}\) signals distinct from pulsars could be detected (Fig. 1, A (left) and B (upper left); fig. S2A (left); and movie S1), albeit at a very low frequency (2.8 ± 0.8 (SEM) event sites per field per 2 min; n = 5), possibly reflecting extracellular Ca\(^{2+}\) influx. Exposure of the endothelial surface to the selective TRPV4 channel agonist GSK1016790A (GSK; 10 nM) (13, 14) induced a marked increase [30.4 ± 2.5 (SEM)–fold, n = 5] in the activity of these Ca\(^{2+}\) signals [Fig. 1, A (right) and B (upper right); fig. S2, A and B; and movie S1], without increasing global [Ca\(^{2+}\)] (Fig. 1B and movies S1 to S4).

Unlike Ca\(^{2+}\) pulsars, which are brief (<300 ms), spikelike signals, GSK-induced Ca\(^{2+}\) events exhibited a plateau phase with discrete amplitudes (Fig. 1C). Their spatial spread within an EC was 11.2 ± 0.4 (SEM) μm\(^2\) (50 sites, n = 5 arterioles), or about 0.6% of the total cell surface area, and they occurred repetitively at the same sites (Fig. 1, A and B). Also in contrast to Ca\(^{2+}\) pulsars, which predominantly occur at “holes” in the internal elastic lamina (IEL) corresponding to the locations of endothelial projections to smooth muscle (10), TRPV4-mediated Ca\(^{2+}\) signals were approximately evenly distributed between such holes (39%) and the ends of cells (31%; fig. S2B).

4α-Phorbol 12,13-didecanoate (4α-PDD), a pharmacological activator of TRPV4 channels, and 11,12-epoxyeicosatrienoic acid (11,12-EET), an endogenous activator of TRPV4 channels, induced IP\(_R\)-independent Ca\(^{2+}\) signals comparable to GSK-induced events (Fig. 1D and fig. S3C). The selective TRPV4 channel antagonist HC-067047 (HC; 1 μM) (13, 15), and the non-selective TRPV4 pore blocker ruthenium red (RuR; 5 μM), inhibited GSK (10 nM)–induced Ca\(^{2+}\) signals by about 93%, reducing both the number of sites and the activity per site (Fig. 1E and figs. S2D and S3B). Moreover, GSK had no effect on arterioles from TRPV4\(^{-}\) mice [P = 0.88, n = 5; paired two-sample t test (12)]. Notably, rapid removal of external Ca\(^{2+}\) eliminated GSK-induced Ca\(^{2+}\) events (n = 4; fig. S2C). Thus, these IP\(_R\)-independent optical signals reflect Ca\(^{2+}\) influx through plasmamembran TRPV4 channels.

TRPV4-mediated Ca\(^{2+}\) signals exhibited characteristics of single channels, including square, discrete amplitudes (Fig. 1, A to C; fig. S2, A, C, and D; and fig. S3, A to C). Such fluorescence signals can be inferred to represent Ca\(^{2+}\) influx through single channels if they meet the following criteria (16): (1) Recording volume is very small (<1 μL); (2) events are quantal (i.e., exhibit fixed amplitude steps); (3) amplitude steps depend on the Ca\(^{2+}\) electrochemical gradient; (4) amplitude steps do not depend on the concentration or nature of the agonist or antagonist; (5) durations of events are exponentially distributed; and (6) channels have high Ca\(^{2+}\) permeability and single-channel conductance. The first criterion may exist naturally for native ECs, which are about 0.5 μm thick (yielding an approximate recording volume of 0.8 fL), and satisfaction of the last criterion has been demonstrated for TRPV4 channels (17). A fit of multiple Gaussians to an all-points histogram of fluorescence signals (Fig. 2A) (12) revealed quantal amplitudes (criterion 2), with evenly spaced ΔF/Δt increments (ΔF/Δt: change in fluorescence relative to baseline fluorescence) of 0.19 with 2 mM extracellular Ca\(^{2+}\). Increasing extracellular Ca\(^{2+}\) from 2 to 10 mM increased the amplitude of these events (Fig. 2A), and membrane depolarization with 100 mM K\(^{+}\) decreased their amplitude (Fig. 2B), indicating a dependence on the Ca\(^{2+}\) electrochemical gradient (criterion 3). Moreover, these events were independent of the concentration and nature of the agonist or antagonist used (criterion 4): Increasing GSK concentration from 3 to 10 nM increased activity (fluorescence integral) 3.7 ± 0.8 (SEM)–fold (n = 3 arterioles; fig. S3A, left), but did not increase amplitude (fig. S3A, right); the specific TRPV4 inhibitor HC reduced activity, but not amplitude (fig. S3B); and other TRPV4 channel activators (4α-PDD and 11,12-EET) did not differentially affect amplitudes (fig. S3C). Finally, the durations of GSK (3 nM)–induced events were exponentially distributed (criterion 5), with a time constant of 37.0 ± 0.7 ms (Fig. 2C) (12). Thus, each quantal level appears to represent Ca\(^{2+}\) influx through a single TRPV4 channel in the plasma membrane of a vascular EC. By analogy to the L-type Ca\(^{2+}\) channel–mediated events first described in cardiac myocytes (18), we use the term TRPV4 sparklet to refer to the optically detected influx of extracellular Ca\(^{2+}\) through a single TRPV4 channel.

Each sparklet site exhibited up to four quantal levels interspersed with long quiescent periods. If these discrete amplitude steps represent independently functioning channels, their probabilities should follow a binomial distribution. The observed distribution deviated significantly from a binomial distribution (P < 0.01; χ\(^2\) test), with an excess of double, triple, and quadruple events.

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**Fig. 1.** GSK-induced Ca\(^{2+}\) signals represent Ca\(^{2+}\) influx through plasmamembran TRPV4 channels. (A) Ca\(^{2+}\) imaging of an en face preparation of third-order mesenteric arteries from a GCaMP2-expressing mouse, showing changes in the activity of local, IP\(_R\)-independent Ca\(^{2+}\) signals recorded over time (traces, below) from regions of interest (1.7 μm\(^2\)), denoted by boxes in images (above). An EC in the field is outlined by red dashes (above, left). Scale bar: 2 s. Calibration bar at right indicates intensity of signals (F/F\(_0\)). Top panel: Representative traces illustrating differences in the kinetic properties of local, IP\(_R\)-independent Ca\(^{2+}\) signals recorded over time (traces, below) from regions of interest (1.7 μm\(^2\)), denoted by boxes in images (above). An EC in the field is outlined by red dashes (above, left). Scale bar: 2 s. Calibration bar at right indicates intensity of signals (F/F\(_0\)). (B) Top panels: Pseudocolor overlay images showing all IP\(_R\)-independent Ca\(^{2+}\) signaling events detected in the absence or presence of GSK (10 nM) in a single field over a 94-s interval. Scale bar: 10 μm. Bottom panel and trace: Pseudo–line-scan image and associated trace recorded from a single site over the same interval. Scale bar: 2 s. Calibration bar at right indicates intensity of signals (F/F\(_0\)). (C) Representative traces illustrating differences in the kinetic properties of IP\(_R\)-mediated Ca\(^{2+}\) pulsars (left) and TRPV4-mediated Ca\(^{2+}\) events (right) from the same field of view in the presence of 10 nM GSK (without CPA). (D) Increased numbers of sites per 2 min per field and activity per field (right; n = 4 to 6 arteries) in the presence of GSK (10 nM), 4α-PDD (5 μM), or 11,12-EET (1 μM). (E) Inhibition of GSK-induced increases in activity per site and activity per field by HC (1 μM; n = 5) and RuR (5 μM; n = 3). Error bars (C and D), SEMs.
(Fig. 2D) (12), indicating that TRPV4 channels open cooperatively, likely reflecting potentiation of nearby TRPV4 channels by intracellular Ca2+ (19). The degree of spread and termination of sparklet activity may reflect inhibition by higher concentrations of intracellular Ca2+ (17). A fifth level, which should have been resolvable under the imaging conditions used, was not reliably detected, suggesting that TRPV4 channels form a four-channel metastructure.

Ca2+-sensitive IK and SK channels are potentially important targets of Ca2+ influx through TRPV4 channels (20). Measurement of membrane currents in freshly isolated ECs using the perforated-patch configuration of the patch-clamp technique (12) showed that, at physiological salt concentrations and voltages (−50 mV), GSK (10 nM) induced an outward current that was substantially reduced by the IK blocker charybdotoxin (ChTx) and further reduced by subsequent addition of the SK blocker apamin (Fig. 3A). Using a voltage-ramp protocol (−100 to +40 mV, 200 ms), we found that activation of TRPV4 channels with GSK (10 nM) increased IK and SK channel current densities to 15.6 ± 2.5 and 3.6 ± 0.9 pA/pF (±SEM; n = 5 cells), respectively, at 0 mV, values similar to maximal IK and SK current densities obtained by dialyzing cells with 3 μM Ca2+ (Fig. 3B).

Our imaging data indicated that GSK (≤10 nM) activates a small number of channels per cell [average activity, 2.8 ± 0.4 (SEM) sparklets per cell with 10 nM GSK; n = 15 cells from five fields]. To estimate the number of TRPV4 channels per cell, we measured whole-cell K+ currents through TRPV4 channels at +100 mV under conditions that minimize Ca2+ entry through TRPV4 (12). Under these conditions, TRPV4 current was 63 ± 27 pA with 10 nM GSK and 1228 ± 242 pA with 100 nM GSK (+SEM; n = 5 cells each; fig. S4), currents that correspond to the activation of 8 and 152 channels per cell, respectively.

Although TRPV4 activation by 3 to 10 nM GSK opened only a few TRPV4 channels in an EC (fig. S5 and movie S5), it hyperpolarized the smooth muscle membrane potential by about 10 mV (fig. S6) and led to a maximal, endothelium-dependent dilation of pressurized arteries (Fig. 3C) (12). GSK-induced dilations were blocked by RuR and HC and were absent in arteries from TRPV4−/− mice (Fig. 3C), conditions that did not affect dilations in response to the direct IK and SK channel agonist NS309 (Fig. 3C); they were also eliminated by removing the endothelium and unaffected by inhibition of smooth muscle Ca2+-sensitive K+ (BK) channels with paxilline (Fig. 3C). Notably, TRPV4-mediated dilations induced by 10 nM GSK were unaffected by inhibition of NO and COX with nitro-L-arginine (L-NNA) and indomethacin, respectively (Fig. 3C). Block of IK channels with ChTx eliminated the dilation in response to 3 nM GSK and greatly reduced the dilation in response to 10 nM GSK (Fig. 3D), indicating that the greater activation of TRPV4 channels at these higher GSK concentrations resulted in increased signaling through SK channels. Thus, local Ca2+ influx through TRPV4 channels acts through IK and SK channels and not through eNOS or COX, demonstrating a specific link between TRPV4 activation and the EDHF pathway of endothelial-mediated vascular dilation.

Muscarinic receptor activation by ACh or carbachol (CCh) induces vasodilation through both NO and EDHF mechanisms (1–6, 8, 9). The NO-dependent component of ACh-induced dilation is modestly reduced in larger mesenteric arteries from TRPV4−/− mice, whereas the EDHF-mediated dilation is more considerably reduced (3–5). Thus, NO-mediated dilation in response to muscarinic receptor activation may be driven primarily by IP3-R-mediated increases in global Ca2+ and Ca2+ pulsars, whereas the EDHF component may reflect the action of local increases in Ca2+ influx through a small number of TRPV4 channels. Global Ca2+ and Ca2+ pulsars were increased by muscarinic receptor stimulation and were eliminated by blocking PLC or SERCA (Fig. 4A) (10). ACh (5 μM) alone increased basal sparklet activity in the absence of IP3-R-mediated Ca2+ release and TRPV4 agonist [2.3 ± 0.3 (SEM)-fold, n = 4 arteries], indicating that the muscarinic signaling pathway can increase Ca2+ influx through TRPV4 channels. In the presence of a submaximal concentration of GSK
Fig. 3. Activation of IK and SK channels and induction of EDHF-dependent vasodilation by Ca²⁺ influx through TRPV4 channels. (A) Inhibition of GSK (10 nM)–induced outward current by 300 nM ChTx (n = 5 cells) or 300 nM apamin (n = 5 cells). (B) Densities of IK and SK channel currents in cells treated with GSK (10 nM) or in cells dialyzed with 3 μM Ca²⁺ (n = 8 cells). Conv. W. C., conventional whole-cell configuration; Perf. Patch, perforated-patch configuration. (C) Top trace: GSK (3 nM)–induced vasodilation of pressurized (80 mmHg) mesenteric arteries; middle trace: effects of 100 μM L-NNa + 10 μM indomethacin (Indo; n = 5), or 1 μM paxilline (n = 3) on GSK-induced dilations; bottom trace: effects of the TRPV4 antagonist HC (1 μM; n = 4) and RuR (5 μM; n = 4), and TRPV4 knockout (n = 3) on dilations induced by the IK and SK agonist NS309 (1 μM); bar graph (right): quantification of effects of CPA + L-NNa (n = 5), L-NNa + Indo (n = 5), paxilline (n = 3), endothelium removal (n = 5), RuR (n = 5), HC (n = 5), and TRPV4 knockout (n = 4) on GSK (10 nM)–induced dilations. (D) Vasodilation in response to GSK (3, 10, 30 nM) in the presence or absence of 200 nM ChTx (n = 7), or ChTx + 300 nM apamin (Apa). Effects on dilation [bar graphs in (C) and (D)] determined relative to initial tone [26 ± 1% (SEM), n = 10], defined as the percentage decrease in arterial diameter to pressure (80 mmHg) relative to the diameter in external Ca²⁺-free solution. Error bars (B to D), SEMs.

Fig. 4. Effects of muscarinic receptor activation on global Ca²⁺ and TRPV4 sparklets. (A) Global Ca²⁺, shown as fractional fluorescence from outlined whole cells treated with CCh (10 μM) with and without CPA (30 μM). (B) GSK (3 nM)–induced sparklets in the presence or absence of 5 μM ACh (left) and 1 μM HC (right; n = 5). (C) Effects of 200 nM ChTx + 300 nM apamin (Apa; n = 5), 100 μM L-NNa (n = 6), L-NNa + HC (n = 5), ChTx + Apa + L-NNa (n = 4) on dilations in response to the muscarinic agonist CCh (1 μM). Error bars (B and C), SEMs. Except where indicated by a bracket (Student’s t test), P-values are for comparison to control (one-way analysis of variance).

(3 nM), ACh (5 μM) increased TRPV4 sparklet activity by 2.9 ± 0.4 (SEM)–fold (n = 5), an effect that was inhibited by the TRPV4 antagonist HC (Fig. 4B). Endothelial-dependent dilation by CCh of arteries constricted by intravascular pressure (80 mmHg) was dependent on NO (34%) and EDHF (66%) (Fig. 4C). Our demonstration that muscarinic receptor stimulation induces TRPV4 sparklets suggests that this pathway should cause vasodilation. Indeed, TRPV4 inhibition by HC reduced muscarinic receptor–induced EDHF dilations by 76% (Fig. 4C), indicating that the EDHF component of these dilations largely reflects TRPV4 sparklet–mediated activation of IK and/or SK channels.

Our results demonstrate that a small number of active TRPV4 channels (about three to eight per cell) mediate local Ca²⁺ signals that activate IK and SK channels (primarily IK) to cause maximal dilation of resistance arteries. We propose that cooperative activation of TRPV4 channels in a cluster leverages the large Ca²⁺ influx through a single TRPV4 channel to produce a more substantial Ca²⁺ signal (fig. S1), which is further boosted by the high Ca²⁺ sensitivity of IK and SK channels, conferred by calmodulin (20). The current caused by activation of IK and SK channels is likely spread to the surrounding smooth muscle through myoendothelial gap junctions, resulting in hyperpolarization of smooth muscle cells and vasodilation (8, 9). IK channels are localized to endothelial projections where a portion (39%) of TRPV4 sparklets occur and myoendothelial gap junctions are concentrated (10, 21, 22), an arrangement that may facilitate activation of IK channels by a local Ca²⁺ signal.
Whereas low-level activation of TRPV4 channels with synthetic agonists (e.g., 3 to 10 nM GSK) or via muscarinic receptor stimulation caused significant vasodilation (P < 0.0001; paired two-sample t test), higher-level activation (100 nM GSK) led to rapid global Ca2+ overload in ECs and oscillations of blood-vessel diameter (fig. S7 and movie S6). Notable in this context, systemic activation of TRPV4 channels by GSK causes a reduction in blood pressure and generalized circulatory failure (14). Collectively, these observations indicate that small numbers of EC TRPV4 channels regulate vascular physiology and suggest that pathologies characterized by blood-pressure reduction and vascular permeability increases (e.g., septic shock) may involve excessive activation of EC TRPV4 channels.

References and Notes

Multidimensional Optimality of Microbial Metabolism
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Although the network topology of metabolism is well known, understanding the principles that govern the distribution of fluxes through metabolism lags behind. Experimentally, these fluxes can be measured by 13C-flux analysis, and there has been a long-standing interest in understanding the principles that govern this functional network operation from an evolutionary perspective. On the basis of 13C-determined fluxes from nine bacteria and multi-objective optimization theory, we show that metabolism operates close to the Pareto-optimal surface of a three-dimensional space defined by competing objectives. Consistent with flux data from evolved Escherichia coli, we propose that flux states evolve under the trade-off between two principles: optimality under given condition and minimal adjustment between conditions. These principles form the forces by which evolution shapes metabolic fluxes in microorganisms’ environmental context.

As a network of about a thousand enzymatic reactions, metabolism fuels growth by converting nutrients into building blocks and energy, but our understanding of the principles that govern the functional distribution of fluxes through this network is limited. Experimentally, intracellular fluxes can be determined by 13C-based flux analysis (1, 2). Based on empirically derived optimality principles (3, 4), stoichiometric models of metabolism (3, 4) can predict condition-dependent flux phenotypes (5-9) as the outcome of single environment evolution (10, 11). However, a concept that integrates such incidental empirical observations into a consistent framework is lacking (12). Although cost-benefit theory indicates that evolution in a constant environment minimizes the expression of enzymes (10, 13), in reality microbes must cope with continuous environmental changes. Thus, we investigated whether the incidental objectives of metabolic operation (5-10) can be integrated into a general optimality framework that explains an organism’s evolution toward particular distributions of fluxes under fluctuating conditions. The basis of our analysis is a stoichiometric reaction model of Escherichia coli central metabolism (table S1) that constrains metabolic fluxes at steady state in a convex space of feasible solutions (3, 4). Assuming that optimality goals are tailored to conditions and that different, eventually competing, objectives cannot be optimized simultaneously, cells face a trade-off that is described by the Pareto surface (14) on which each point is Pareto optimal; that is, the value of one objective can be increased only at the cost of another. To identify the axes of such a multidimensional optimality space, we computationally predicted flux distributions with 54 single objective functions and quantified the deviation to 44 reported in vivo flux distributions (fig. S1a and table S2) obtained from 13C-labeling experiments (15-19) (table S3). Five of the objective functions were found to be consistent with the in vivo fluxes under some conditions: maximum adenosine triphosphate (ATP), biomass, acetate, and carbon dioxide yields and minimum sum of absolute fluxes (fig. S1a). For all possible pairs and triplets of these, we computed the Pareto surface (fig. S1b). Although no dual combination could describe all measured fluxes adequately, the combination of the two efficiency objectives, maximum ATP yield and maximum biomass yield, with the optimal resource allocation objective minimum sum of absolute fluxes achieved the highest optimality, evidenced by all 44 in vivo flux distributions being very close to the Pareto surface (fig. 1, A and E, and figs. S1b and S2).

On the Pareto surface, the in vivo flux distributions occupied distinct regions that cluster into biologically meaningful groups (fig. 1A). Whereas aerobic cultures of various E. coli strains grown with nonlimiting glucose (blue) clustered in the upper right corner, cultures in which glucose was continuously supplied at a limiting amount (green) stretched according to their growth rate diagonally between the maximum ATP and biomass yield axes. The proximity of

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