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Am J Physiol Heart Circ Physiol 295:953-961, 2008. First published Jul 11, 2008;
doi:10.1152/ajpheart.00520.2008

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Conditioning the heart induces formation of signalosomes that interact with mitochondria to open mitoK_{ATP} channels

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Submitted 15 May 2008; accepted in final form 6 July 2008

Quinlan CL, Costa AD, Costa CL, Pierre SV, Dos Santos P, Garlid KD. Conditioning the heart induces formation of signalosomes that interact with mitochondria to open mitoK_{ATP} channels. *Am J Physiol Heart Circ Physiol* 295: H953–H961, 2008. First published July 11, 2008; doi:10.1152/ajpheart.00520.2008.—Perfusion of the heart with bradykinin triggers cellular signaling events that ultimately cause opening of mitochondrial ATP-sensitive K⁺ (mitoK_{ATP}) channels, increased H₂O₂ production, inhibition of the mitochondrial permeability transition (MPT), and cardioprotection. We hypothesized that the interaction of bradykinin with its receptor induces the assembly of a caveolar signaling platform (signalosome) that contains the enzymes of the signaling pathway and that migrates to mitochondria to induce mitoK_{ATP} channel opening. We developed a novel method for isolating and purifying signalosomes from Langendorff-perfused rat hearts treated with bradykinin. Fractions containing the signalosomes were found to open mitoK_{ATP} channels in mitochondria isolated from untreated hearts via the activation of mitochondrial PKC-ε. mitoK_{ATP} channel opening required signalosome-dependent phosphorylation of an outer membrane protein. Immunodetection analysis revealed the presence of the bradykinin B₂ receptor only in the fraction isolated from bradykinin-treated hearts. Immunodetection and immunogold labeling of caveolin-3, as well as sensitivity to cholesterol depletion and resistance to Triton X-100, attested to the caveolar nature of the signalosomes. Ischemic preconditioning, ischemic postconditioning, and perfusion with ouabain also led to active signalosome fractions that opened mitoK_{ATP} channels in mitochondria from untreated hearts. These results provide initial support for a novel mechanism for signal transmission from a plasma membrane receptor to mitoK_{ATP} channels.

bradykinin; mitochondria; cardioprotection; protein kinase C; ouabain

BRADYKININ protects the heart against ischemia-reperfusion injury by a signaling process whose sequence has been well described by Downey and coworkers (33). The interaction of bradykinin with its receptor causes the activation of phosphatidylinositol 3-kinase, which phosphorylates membrane phospholipids to yield phosphatidylinositol trisphosphate, which then activates phospholipid-dependent kinases, which in turn cause the phosphorylation and activation of Akt. Akt phosphorylates endothelial nitric oxide (NO) synthase (eNOS), causing it to generate NO. NO activates soluble guanylyl cyclase, causing it to synthesize cGMP, which then activates PKG. PKG acts on the mitochondrial outer membrane (MOM), causing the opening of inner membrane mitochondrial ATP-sensitive K⁺ (mitoK_{ATP}) channels (8).

These temporal events take place within complex spatial domains. In particular, the signal must be transmitted from the plasma membrane to mitochondria through the cytosol. How does this occur? Cytosolic proteins are extensively hydrated, and the organization of this water causes a phase separation from bulk cell water. Minimization of the phase boundary, in turn, causes proteins to coalesce within their common hydration phase (17). If the proteins of the cardioprotective signaling pathway were randomly distributed in the cytosol, they too would coalesce within the hydration phase. The concerted sequential reactions of cardioprotection would be unlikely to take place by random diffusional collisions in this milieu. Accordingly, we suggest that the signaling cascade is compartmentalized in a manner to promote metabolic channeling and that the entire reaction sequence moves through the cytosol as a unit. Thus, our working hypothesis was that cytosolic transmission of the signal to mitochondria takes place via vesicular, multimolecular signaling complexes called signalosomes (5, 16, 48). This hypothesis agrees with and extends the proposal by Ping and coworkers (41, 56) that intracellular signaling involves the assembly and regulation of multiprotein complexes.

We showed first that bradykinin treatment of perfused rat hearts caused a persistent open state of mitoK_{ATP} channels that was reversed by exposing the inner membrane to protein phosphatase 2A (PP2A). If this open state was caused by an in situ interaction of signalosomes with mitochondria, as hypothesized, the signalosomes should be recoverable for study. Indeed, we were able to recover them from the mitochondrial suspension obtained from treated hearts. Fractions containing the putative signalosomes from bradykinin-perfused hearts were added to mitochondria from untreated hearts and found to be functionally active. That is, they opened mitoK_{ATP} channels to the same extent as diazoxide and also caused inhibition of the mitochondrial permeability transition (MPT). These effects were blocked by the specific PKG inhibitor KT-5823, confirming that PKG is the terminal kinase of the bradykinin signalosome. Purified signalosomes were dissolved by the cholesterol-binding agent methyl-β-cyclodextrin, and they were resistant to Triton X-100, indicating their caveolar nature. This was confirmed by immunodetection analysis showing the presence of caveolin-3, a marker for muscle caveolae (52). The bradykinin B₂ receptor was also found in signalosomes isolated from bradykinin-perfused hearts. The vesicular nature of signalosomes

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somes was verified by electron microscopy, which revealed entities between 110 and 160 nm in diameter. Immunogold labeling showed a clear enrichment of the vesicles with caveolin-3. Signalosome formation appears to be a general phenomenon. We also recovered active signalosomes from hearts treated with ischemic preconditioning, ischemic postconditioning, and ouabain.

MATERIALS AND METHODS

Isolated heart perfusions. Hearts from male Sprague-Dawley rats (200–220 g) were perfused for 50–55 min as previously described (38). Control hearts were perfused for 50 min with Krebs-Henseleit buffer containing (in mM) 118 NaCl, 5.9 KCl, 1.75 CaCl₂, 1.2 mM MgSO₄, 0.5 EDTA, 25 NaHCO₃, and 16.7 glucose at pH 7.4. The perfusate was gassed with 95% O₂-5% CO₂. Treated hearts were perfused for 25 min with Krebs-Henseleit buffer and then with buffer containing 100 nM bradykinin or 50 μM ouabain (38) for 15 min, followed by 10 min of washout. The ischemic preconditioning protocol was established by two cycles of 5-min global ischemia followed by 5 min of reperfusion. Mitochondria were isolated promptly after the second reperfusion. Ischemic postconditioning was performed as described by Tsang et al. (55), with six cycles of 10-s ischemia plus 10 s of reperfusion. Infarct size was also measured after each of these treatments and found to be reduced to an extent similar to that reported elsewhere (26, 37, 55). The experimental protocols used in this study were performed in compliance with the American Physiological Society's "Guiding Principles in the Care and Use of Animals" and were approved by the Institutional Animal Care and Use Committee at Portland State University.

Mitochondrial isolation. Mitochondria from treated and untreated donor rat hearts were isolated immediately after 50 min of Langendorff perfusion and used to isolate signalosomes. Mitochondria from untreated assay rat hearts were isolated without previous perfusion, purified in a Percoll gradient, and used to assay for signalosome activity. A standard differential centrifugation isolation protocol was used (8). Mitoplasts were prepared from isolated mitochondria by digitonin treatment as described by Schnaitman et al. (47). Briefly, an ice-cold 2% digitonin solution was added stepwise to mitochondria stirring on ice for 8 min. The digitonin solution was then washed out by dilution with mitochondrial isolation buffer [250 mM sucrose, 10 mM HEPES (pH 7.2), and 1 mM EGTA], and the sample was pelleted by centrifugation at 10,000 *g*. Verification that the MOM was disrupted was determined by a respiratory assay in the presence and absence of cytochrome *c*. Interfibrillar mitochondria (IFM) were prepared using a protocol slightly modified from Palmer et al. (34). Briefly, the first low-speed pellet from the standard isolation was resuspended in 150 mM KCl and 50 mM HEPES medium and rehomogenized with a tight-fitting Teflon pestle. After this step, the standard isolation protocol was followed (8). The mitochondrial protein concentration was estimated using the Biuret reaction (19).

Signalosome purification. To determine if caveolar bodies associated with mitochondria after bradykinin treatment, donor mitochondria were further purified in a self-generated 24% Percoll gradient, resulting in a purified mitochondrial fraction and a low-density fraction called the light layer (LL). The LL was postulated to contain signalosomes (see Fig. 1) but also contained plasma membrane fragments and broken mitochondria. To purify signalosomes, we used the nondetergent caveolae isolation protocol of Smart et al. (50). Briefly, the LL fraction was adjusted to 2 ml, mixed with 50% Optiprep in *buffer A* [250 mM sucrose, 20 mM Tris·Cl (pH 7.8), and 1 mM EDTA], and placed in the bottom of a 12-ml centrifuge tube [the sonication step of the previously published protocol (50) was eliminated]. A 20–10% Optiprep gradient was layered on top, and tubes were centrifuged at 52,000 *g* for 90 min. After centrifugation, the top 5 ml were collected and mixed with 4 ml of 50% Optiprep

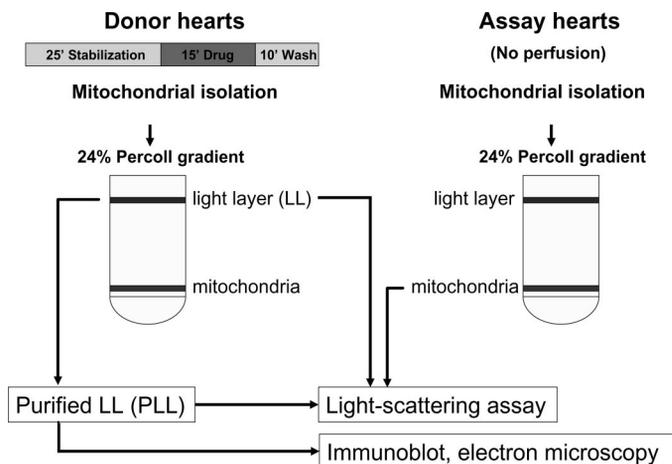


Fig. 1. Schematic representation of the experimental protocol. Each experiment required mitochondria from a perfused donor heart and mitochondria from untreated assay hearts. Mitochondrial fractions from both hearts were purified on a 24% Percoll density gradient. Light layers (LL) from donor hearts were assayed for their ability to open mitochondrial ATP-sensitive K⁺ (mitoK_{ATP}) channels in assay mitochondria. In separate experiments, LL were further purified [purified LLs (PLLs)] and used for light scattering assays, immunoblot analysis, and electron microscopy.

solution. This mixture was then overlaid with 5% Optiprep solution and centrifuged again at 52,000 *g* for 90 min. The signalosome fraction was identified as an opaque band at the 5% interface. Validation of the purity of this fraction was demonstrated by Western blots that showed very little mitochondrial voltage-dependent anion channel (VDAC) contamination and an enrichment of caveolins (see Fig. 8).

mitoK_{ATP} assays. mitoK_{ATP} activity causes mitochondrial swelling due to respiration-driven uptake of K⁺ salts and water, and these volume changes were followed by light scattering, as previously described (6, 8, 11). Mitochondria from untreated assay hearts were added at 0.1 mg/ml to medium containing K⁺ salts of 120 mM Cl⁻, 10 mM HEPES (pH 7.2), 10 mM succinate, and 5 mM phosphate supplemented with 0.5 mM MgCl₂, 5 μM rotenone, and 0.67 μM oligomycin. LLs or purified LLs were added as indicated in the figures. Data are summarized in bar graphs as "mitoK_{ATP} activity (in %)," given by the following equation:

$$100 \times (V_x - V_{ATP}) / (V_0 - V_{ATP})$$

where V_x is the observed steady-state volume at 120 s under the given experimental conditions and V_{ATP} and V_0 are observed values in the presence and absence of ATP, respectively. Statistical significance of the difference of the means was assessed using an unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant. It should be noted that mitoK_{ATP} channel-dependent K⁺ flux was validated by five independent measurements: light scattering, direct measurements of K⁺ flux, H⁺ flux, respiration, and H₂O₂ production. Each of these was found to yield quantitatively identical measures of K⁺ flux using valinomycin-induced K⁺ flux as a calibrating control (1, 11).

LLs were expected to contain microsomes and peroxisomes (30), which may be a source of H₂O₂. Because H₂O₂ opens mitoK_{ATP} channels in a PKC-ε-dependent manner (23), we examined whether catalase (10 U/ml) and mercaptopyrionylglycine (MPG; 0.3 mM) inhibited LL-dependent mitoK_{ATP} channel opening. This treatment had no effect on the ability of LLs to open mitoK_{ATP} channels ($n = 5$).

LL-dependent mitoK_{ATP} channel opening depends on the amount of LLs added. The concentration dependence was determined, and the amount used in the assay was sufficient to give a near- V_{max} response. Importantly, the yield of LLs from one donor preparation was just sufficient to treat the mitochondria from one assay preparation.

Diazoxide was added at 30 μ M, sufficient to yield a V_{\max} response (18).

MPT assays. Opening of the MPT was synchronized by sequential additions to the assay medium described above of CaCl₂ (100 μ M free Ca²⁺), ruthenium red (0.5 μ M, to block further Ca²⁺ uptake), and CCCP (250 nM, to synchronize MPT opening) (9). Rates of volume change were obtained by taking the linear term of a second-order polynomial fit of the light scattering trace, calculated over the initial 2 min after MPT induction by CCCP. MPT inhibition was calculated by determining Ca²⁺-induced swelling rates in the presence and absence of 1 μ M cyclosporin A as 100% and 0%, respectively.

Immunoblot analysis. Immunoblots were performed as described previously (23). Protein (5 μ g) was separated by SDS-PAGE using 10% acrylamide precast gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Millipore) by semidry transfer at 12 V for 50 min. Membranes were blocked with 5% nonfat dry milk in 20 mM Tris-buffered saline (TBS) and 0.5% Tween 20 (TBS-T) and incubated with primary antibody overnight in TBS-T containing 5% BSA. The membrane was then incubated for 1 h with the appropriate secondary antibody conjugated to horseradish peroxidase in TBS-T. Membranes were exposed to autoradiograph films, which were scanned. The primary antibody dilutions were as follows: bradykinin B₂ receptor, 1:5,000 (BK2; Santa Cruz Biotechnology); caveolin-3, 1:10,000 (Affinity Bioreagents); VDAC, 1:5,000 (Affinity Bioreagents); PKG, 1:20,000 (Stressgen); and eNOS, 1:500 (BD Transduction Labs).

ImageQuant version 5.0 (Molecular Dynamics) was used to quantify Western blots. Quantification of gel band intensities was initiated by drawing rectangles around the appropriate gel bands from the Western blots. Using the method of local averages for background correction, the software reports band intensities for each rectangle. The SE of the mean for gel band intensities was determined from four to six replicate Western blots and represents the cumulative error for the total Western blot analysis assay.

Electron microscopy. For immunogold labeling, aliquots were washed in 20 mM KCl, mounted onto carbon-coated copper grids, and fixed for 10 min in 3% paraformaldehyde. Grids were then incubated with TBS containing 0.2% BSA (TBS-BSA) for 30 min and subsequently incubated with the primary antibody (20 μ g/ml mouse monoclonal caveolin-3, BD Transduction Labs) in TBS-BSA for 30 min. Grids were washed three times for 5 min each in TBS-BSA and then incubated with the secondary antibody (1:100 dilution of 10-nm gold-conjugated goat anti-mouse IgG, Sigma) in TBS-BSA for 30 min. Grids were washed once in TBS-BSA and five more times in distilled H₂O. Control experiments were performed by omission of the primary antibody (14, 45, 50, 57). Grids were stained with 2% uranyl acetate and examined with a JEOL TEM 100CX microscope.

Chemicals. KT5823 and PP2A were purchased from Calbiochem. The PKC isoform-specific peptides ϵ V₁₋₂ and ψ RACK were synthesized by EZ Biolabs (Westfield, IN) according to previously published amino acid sequences (7). Optiprep was purchased from Axis-Schield (Oslo, Norway). Bafilomycin A₁, methyl- β -cyclodextrin, and all other chemicals were purchased from Sigma.

RESULTS

Bradykinin perfusion opens mitoK_{ATP} channels by phosphorylation. Bradykinin's cardioprotection is blocked by 5-hydroxydecanoate (5-HD) and therefore presumed to require mitoK_{ATP} channel opening (33, 38); however, this has not previously been demonstrated. Bradykinin treatment acts via PKG, which interacts with mitochondria to open mitoK_{ATP} channels via a PKC- ϵ -mediated phosphorylation (8). Thus, bradykinin perfusion should cause a persistent open state of mitoK_{ATP} channels that is reversed by dephosphorylation. We isolated mitochondria from bradykinin-perfused and sham-

perfused hearts and prepared mitoplasts to permit access of the phosphatase PP2A to the inner membrane, which cannot cross the MOM. The results are shown in Fig. 2. In mitoplasts from untreated hearts (sham perfusion), mitoK_{ATP} channels were inhibited by ATP. As expected, ATP inhibition was reversed by diazoxide, and PP2A had no effect (not shown). In mitoplasts from bradykinin-treated hearts (bradykinin perfusion), mitoK_{ATP} channels were resistant to inhibition by ATP unless PP2A was present (Fig. 2). Bradykinin-dependent mitoK_{ATP} channel opening was maximal, as shown by the fact that diazoxide had no further effect and by the finding that the bradykinin-dependent open state was equal to that observed in the presence of PP2A plus ATP plus diazoxide. The data shown in Fig. 2 demonstrate, for the first time, that the mitoK_{ATP} channel is opened *ex vivo* when the heart is perfused with a cardioprotective agent.

Purified LLs from bradykinin-treated hearts open mitoK_{ATP} channels via PKG and mitochondrial PKC- ϵ . To test whether the mitochondria-associated LL contained signaling enzymes capable of opening mitoK_{ATP} channels, we added LL aliquots to purified mitochondria from untreated assay hearts. The representative traces shown in Fig. 3 show that both LLs and purified LLs from bradykinin-treated hearts reversed the ATP inhibition and opened mitoK_{ATP} channels. LLs from untreated hearts (sham LLs) and residual LL from bradykinin-treated hearts had no effect. (Residual LLs contain the proteins that remained after purified LLs were isolated from LLs.)

Light scattering data from an extensive series of experiments are shown in Fig. 4. LLs from control hearts (sham LLs) and isolated plasma membrane caveolae had no effect on mitoK_{ATP} channels or on the effect of diazoxide ($n = 10$). LLs from bradykinin-treated hearts opened mitoK_{ATP} channels to the same extent as diazoxide ($n = 10$). The addition of diazoxide plus LLs from bradykinin-treated hearts had no further effect,

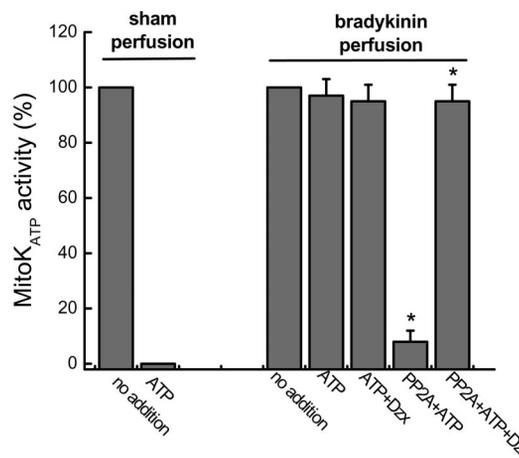


Fig. 2. Bradykinin perfusion causes a persistent open state of mitoK_{ATP} channels. Shown are changes in mitoK_{ATP} channel activity (in %) measured in mitoplasts prepared from mitochondria isolated from perfused rat hearts. Mitoplasts from sham-perfused (no treatment) and bradykinin-perfused hearts were compared for the response of mitoK_{ATP} channels to ATP (0.2 mM), ATP + protein phosphatase 2A (PP2A; 11 ng/ml), ATP + diazoxide (DZX; 30 μ M), and ATP + PP2A + DZX. mitoK_{ATP} channels from bradykinin-perfused hearts were open and unresponsive to ATP unless PP2A was added. This indicates that the persistence of the open state was due to phosphorylation. Data are averages \pm SD of 3 experiments from 3 separate perfusions. * $P < 0.05$ vs. bradykinin-treated mitochondria without PP2A treatment.

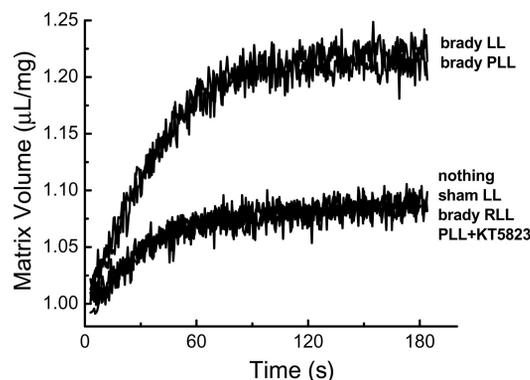


Fig. 3. LLs and PLLs from bradykinin (brady)-treated hearts open mitoK_{ATP} channels in isolated mitochondria. Mitochondrial matrix volume is plotted versus time. Aliquots of LLs (10–15 µg/ml), PLLs (0.5 µg/ml), and residual LLs (RLLs; 10–15 µg/ml) were added to the medium 1 s after mitochondria. ATP (0.2 mM) was present in each run. KT-5823, a PKG inhibitor, was added at 0.5 µM. Traces are representative of at least 5 independent experiments. Six separate traces were plotted, and some of them are superimposed.

so the bradykinin treatment of LLs opened mitoK_{ATP} channels maximally. mitoK_{ATP} channel opening by bradykinin-treated LLs was blocked by 5-HD acting directly on the channel. It was also blocked by εV₁₋₂, showing that LL-dependent mitoK_{ATP} channel opening was mediated via PKC-ε. A complete set of experiments (*n* = 8) was also carried out with bradykinin-treated purified LLs (not shown), which exhibited results identical to those from bradykinin-treated LLs. For the practical reason that purified LLs are more time consuming to prepare, we carried out the majority of functional experiments using LLs.

Expressed per milligram of mitochondrial protein, LLs from bradykinin-perfused and sham-perfused hearts were 130 and

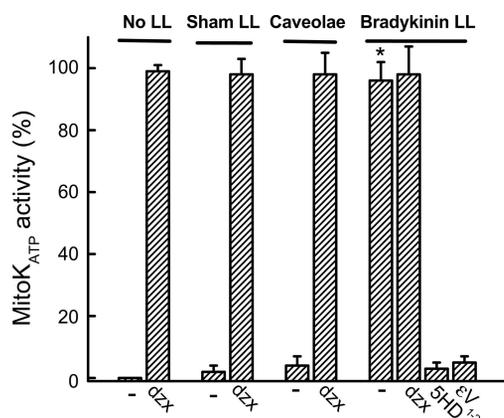


Fig. 4. Properties of LL-induced mitoK_{ATP} channel opening. Shown are changes in mitoK_{ATP} channel activity (in %). Standard mitoK_{ATP} channel activity was observed in the absence of LLs (no LLs). LLs from sham-perfused hearts (sham LLs) had no effect on mitoK_{ATP} channel activity (*n* = 3). Caveolae isolated from untreated hearts (50) did not open mitoK_{ATP} channels at amounts up to 700 µg/mg mitochondrial protein. (Shown are data using 300 µg/mg caveolae; *n* = 3.) Bradykinin treatment of LLs induced mitoK_{ATP} channel opening that was inhibited by 5-hydroxydecanoate (5-HD; 1.0 mM) and εV₁₋₂ (0.5 µM). Note that a higher concentration of 5-HD was required to block mitoK_{ATP} channels after they had been opened by phosphorylation (8). Bradykinin-treated LLs opened mitoK_{ATP} channels to the same extent as DZX, and DZX had no additional effect in the presence of bradykinin-treated LLs. Bradykinin data are averages ±SD of at least 4 independent experiments. **P* < 0.05 vs. sham LLs or caveolae.

100 µg/mg, respectively. Purified LLs from bradykinin-treated and sham hearts were 8 and 3.7 µg/mg, respectively. Thus, the LL purification removed 94% of the LL protein, and the 16-fold increase in specific activity of the purified LL shows a strong purification of functionality. The 1:125 protein ratio (purified LL to mitochondrial protein) permits a rough estimate to be made of the size of the putative signalosomes. Assuming that the protein ratio is roughly equal to the volume ratio, we calculated the diameter of the signalosomes to be ~140 nm, in excellent agreement with the size observed by electron microscopy described below.

LLs recovered from hearts after ouabain perfusion, ischemic preconditioning, and ischemic postconditioning open mitoK_{ATP} channels. We carried out experiments with other models of cardioprotection to estimate the generality of this mechanism. We performed ischemic preconditioning, ischemic postconditioning, or perfused the hearts with the known protective ligand ouabain (38), isolated the LL, and then determined the effects on mitochondria from untreated hearts. As shown in Fig. 5 (first bar of each group), each of these treatments led to signalosomes capable of opening mitoK_{ATP} channels. LL-dependent mitoK_{ATP} channel opening by each treatment was blocked by 5-HD and εV₁₋₂, acting at the level of inner membrane mitoK_{ATP} channels and PKC-ε, respectively (23) (not shown).

As shown in Fig. 5, the effects of bradykinin perfusion, ischemic preconditioning, and ischemic postconditioning were blocked by KT-5823, indicating that these G protein receptor-coupled protective mechanisms act on mitochondria via PKG contained in the signalosome. The mitoK_{ATP} channel opening effects of ouabain-treated LLs were not blocked by KT-5823, indicating that PKG is not the terminal kinase of these signalosomes. This is in agreement with the finding in perfused hearts that KT-5823 blocked cardioprotection by bradykinin but did not block cardioprotection by ouabain (38). In further experiments not shown, we found that other models of cardioprotection, including acetylcholine and elevated perfusate Ca²⁺, also led to LLs that opened mitoK_{ATP} channels (*n* = 1–3 for each treatment).

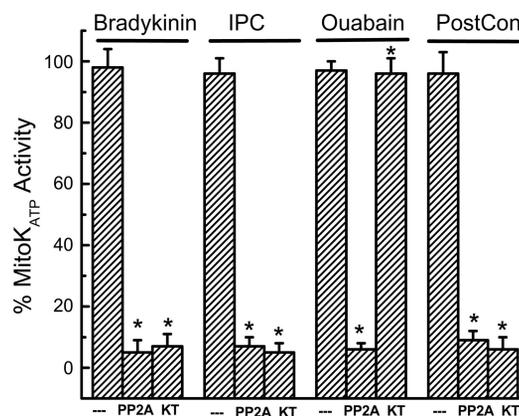


Fig. 5. LL-induced mitoK_{ATP} channel opening after ischemic preconditioning (IPC) and ouabain treatment. Shown are changes in mitoK_{ATP} channel activity (in %) when LLs treated with bradykinin, IPC, ouabain, or ischemic postconditioning (PostCon) were added to assay mitochondria. Also shown are the effects of PP2A (11 ng/ml) and KT-5823 (KT; 0.5 µM) added to the assay. Note that PP2A cannot cross the mitochondrial outer membrane (MOM) of intact mitochondria, so the effect is on the MOM. Data are averages ±SD of 3–4 independent experiments. **P* < 0.05 vs. LLs alone.

Role of the MOM in LL signaling. As shown in Fig. 5, LL-induced mitoK_{ATP} channel opening was blocked in each case by PP2A added to the assay medium. In these experiments, in contrast to those of Fig. 2, the MOM was intact. Because PP2A cannot cross the MOM, the results indicate that signalosome activity required the phosphorylation of a MOM protein. We used mitoplasts to study the effects of MOM removal on mitoK_{ATP} channel opening by various treatments. The findings (not shown) may be summarized as follows: MOM removal had no effect on diazoxide-induced mitoK_{ATP} channel opening, but it abolished the abilities of PKG plus cGMP and bradykinin-, ouabain-, and ischemic precondition-treated LLs to open mitoK_{ATP} channels. Thus, the MOM is essential for the action of the LL, and the effect of PP2A (Fig. 5) indicates that the LL phosphorylates a MOM protein whose identity is not yet known.

LLs from IFM of bradykinin-treated hearts open mitoK_{ATP} channels. Cardiomyocytes contain two populations of mitochondria: subsarcolemmal (SSM) and IFM, which exhibit subtle bioenergetic differences (34). Standard isolation yields a primarily SSM population. We investigated whether active LLs could also be isolated from IFM, which largely supply ATP to contractile ATPases. We found that LLs from IFM opened mitoK_{ATP} channels to the same extent as diazoxide and otherwise behaved identically to LLs from SSM (data not shown; *n* = 5). Separation of SSM and IFM is imperfect; however, the fact that aliquots of LLs from each fraction opened mitoK_{ATP} channels maximally indicates that we were not studying a mixture of active and inactive fractions.

Bradykinin treatment of LLs inhibits the MPT. The elevated calcium and ROS that occur during ischemia-reperfusion cause MPT opening, and this is inhibited by cardioprotective agents (22). PKG-dependent MPT inhibition is a result of mitoK_{ATP} channel opening, an increase in mitochondrial ROS production, and activation of inner membrane PKC-ε that inhibits MPT (9) (see Fig. 10). Thus, ROS are necessary for both the initiation and prevention of MPT opening (9). As shown in Fig. 6, bradykinin treatment of LLs inhibited MPT opening and did so to the same extent as diazoxide. MPT inhibition by the bradykinin-treated LLs follows the same pattern as was observed in

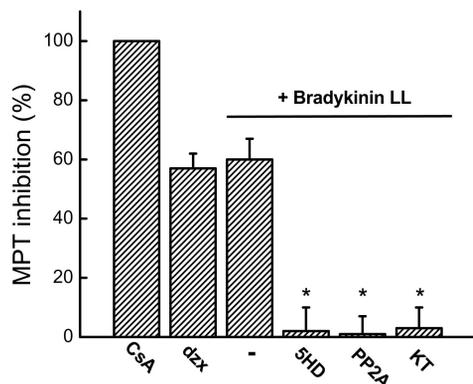


Fig. 6. LL-induced inhibition of the mitochondrial permeability transition (MPT). LL inhibition of the MPT in isolated heart mitochondria and the effects of various antagonists are shown. Data are plotted as MPT inhibition (in %). The following additions were made: LLs (10–15 μg/ml), Dzox (30 μM), 5-HD (0.3 mM), KT (0.5 μM), and PP2A (11 ng/ml). Data are shown as averages ±SD of at least 4 independent experiments. **P* < 0.05 vs. bradykinin-treated LLs.

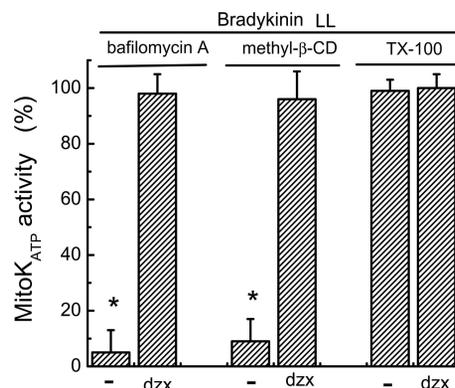


Fig. 7. Bafilomycin A or methyl-β-cyclodextrin (methyl-β-CD) abolish LL-induced mitoK_{ATP} channel opening; Triton X-100 does not. Shown are changes in mitoK_{ATP} channel activity (in %). These experiments examined the effects of bafilomycin A (50 nM), methyl-β-CD (5%), and Triton X-100 (1%) on mitoK_{ATP} channel opening induced by bradykinin-treated LLs. Bafilomycin A was added to the heart perfusate before the isolation of LLs. Methyl-β-CD, a cholesterol-binding agent, was incubated with LLs at 30°C for 5 min before the assay. Triton X-100 was incubated with purified LLs for 10 min at 4°C. Before the assay, vesicles were pelleted in a microcentrifuge (16,000 g for 15 min), washed twice in dilute salt solution, and resuspended in buffer without Triton X-100. Dzox (30 μM) was added at the same time as the aliquot of LLs (10–15 μg/ml). Data are averages ±SD of at least 3 independent experiments. **P* < 0.05 vs. corresponding LLs.

Fig. 4 for mitoK_{ATP} channel opening: it was abolished by 5-HD, KT-5823, and PP2A.

Activity of bradykinin-treated LLs is sensitive to methyl-β-cyclodextrin and bafilomycin A₁ but not to Triton X-100. Hearts were treated with bafilomycin A₁, an inhibitor of receptor recycling (24), for 5 min before and during treatment with bradykinin. LLs and purified LLs obtained from these hearts failed to open mitoK_{ATP} channels (Fig. 7). Treatment of LLs with the cholesterol-binding agent methyl-β-cyclodextrin abolished LL-induced mitoK_{ATP} channel opening (Fig. 7) and dissolved purified LLs, as revealed by the absence of a pellet after centrifugation. Neither of these treatments affected diazoxide-dependent mitoK_{ATP} channel opening, showing that the observed effects were due to inactivity of the LL and not to mitochondrial damage caused by adding treated LLs. To further characterize the nature of the purified LL, we investigated its response to the nonionic detergent Triton X-100. Activity of purified LLs from bradykinin-treated hearts was not affected by treatment with 1% Triton X-100 (Fig. 7).

Presence of the bradykinin receptor and caveolin-3 in purified LLs from treated hearts. Mitochondria and purified LLs from donor hearts perfused with medium containing no bradykinin (sham perfusion) or bradykinin were obtained as described in Fig. 1 and MATERIALS AND METHODS. As shown in Fig. 8A, the bradykinin B₂ receptor was detected uniquely in bradykinin-treated purified LLs, and caveolin-3 was strongly enriched. eNOS, a known component of bradykinin signal transduction (33), was also found to be enriched in the bradykinin fraction. PKG was found in all samples. Because the bradykinin-treated purified LL contains twice as much protein as the sham purified LL and the same amount of protein was used for each sample, we estimated that purified LLs from bradykinin-treated hearts contained about twice as much total PKG as purified LLs from sham-treated hearts (Fig. 8B). VDAC was used as a mitochondrial marker, and its immuno-

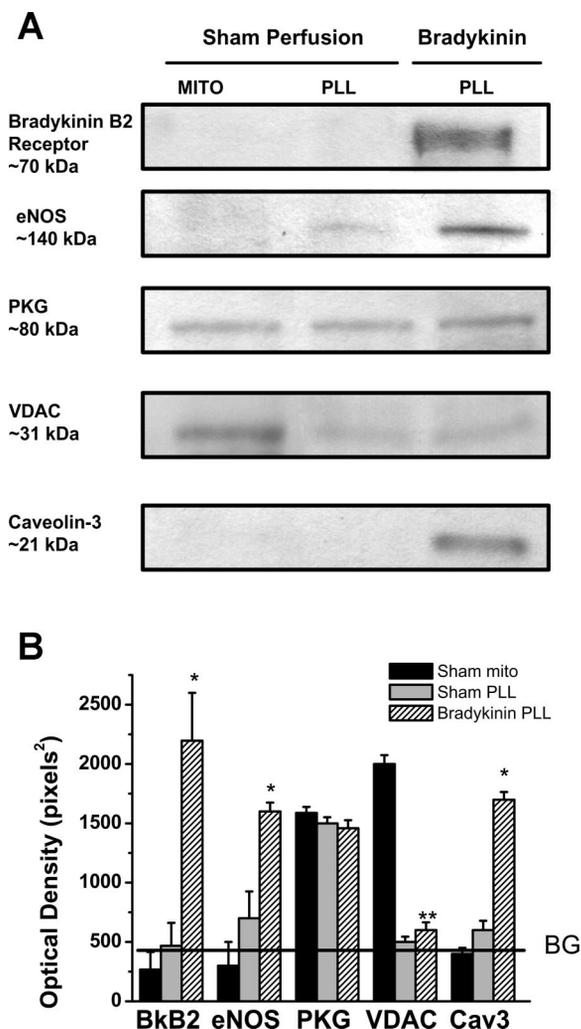


Fig. 8. Immunodetection analysis of bradykinin-treated signalosomes. *A*: representative immunoblots of 6 independent experiments. Mitochondria protein (MITO; 5 μ g) from perfused untreated hearts or 5 μ g of PLLs from untreated (sham perfusion) or bradykinin-treated hearts, obtained as described in MATERIALS AND METHODS, were loaded. For each antigen, the signal shown was obtained on samples loaded on the same gel and processed under the same conditions. eNOS, endothelial nitric oxide synthase; VDAC, voltage-dependent anion channel. *B*: pixel density analysis performed on each band of $n = 6$ Western blots. BG, pixel density of a protein-free region of the blot. * $P < 0.05$ vs. sham perfusion; ** $P < 0.05$ vs. mitochondria.

blots demonstrated minimal mitochondrial contamination in both purified LLs. Figure 8*B* shows the results of a densitometric analysis of the bands from six independent Western blots.

Immunogold labeling of caveolin-3 in purified LLs. We performed immunogold labeling experiments using an antibody to caveolin-3. As shown by the representative example in Fig. 9*A*, each vesicle was decorated with several gold particles, indicating multiple copies of caveolin-3 in the vesicles. Figure 9*B*, obtained in the absence of primary antibody, shows no nonspecific binding of colloidal gold. Transmission electron microscopy of Triton X-100-purified LLs was also performed to measure vesicle dimensions. The mean diameter of the roughly spherical vesicles was 137 ± 25 nm ($n = 48$). Residual LLs contained no vesicles. Purified LLs from sham-perfused hearts contained 15–20%

as many vesicles as purified LL from bradykinin-treated hearts, but these were ineffective in opening mitoK_{ATP} channels (Fig. 3).

DISCUSSION

The cardioprotective signal arising from bradykinin treatment is transmitted from the bradykinin receptor to the MOM, where it initiates the intramitochondrial signaling pathway described in recent publications (1, 8–10, 23) and in Fig. 10. The objective of the present study was to understand how the signal passes through the cytosol to mitochondria. We propose that the pathway is mediated by signalosomes, which are vesicular, multimolecular signaling complexes that are assembled in caveolae and deliver signals to cytosolic targets, including mitochondria. We first established that bradykinin treatment caused phosphorylation-dependent mitoK_{ATP} channel opening in donor mitochondria (Fig. 2). Using the protocol

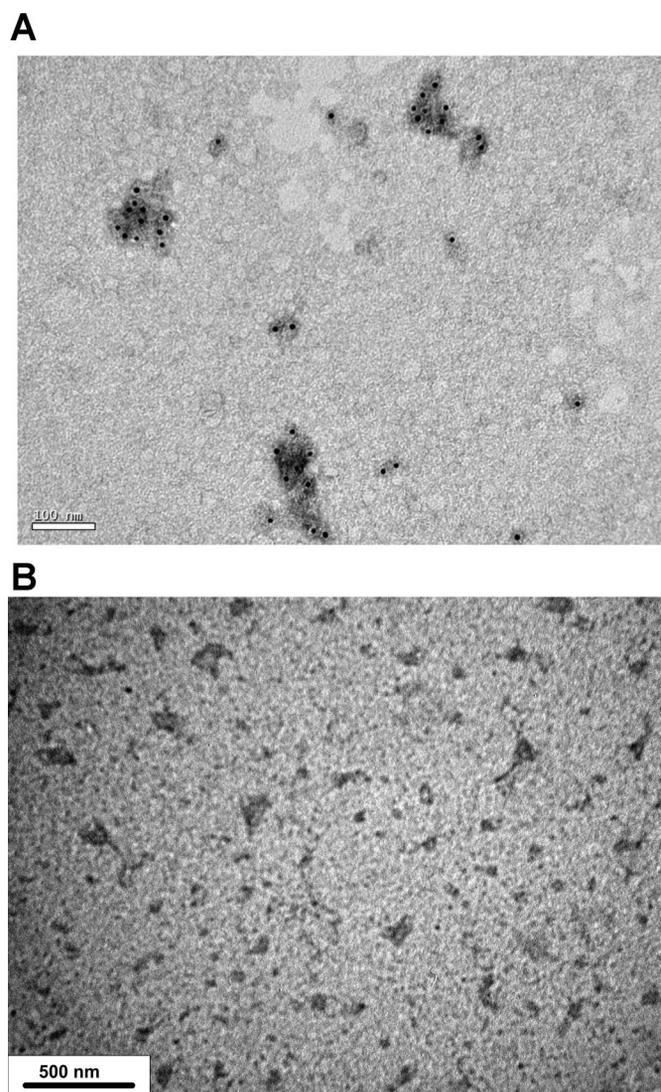


Fig. 9. Immunogold staining of bradykinin-treated signalosomes. Transmission electron microscopic analysis of PLLs from bradykinin-perfused hearts is shown. Samples were applied to carbon-coated grids, fixed, and processed for the presence of immunogold labeling when treated either with the primary antibody to caveolin-3 (*A*) or without the primary antibody as a negative control (*B*). Magnification: $\times 100,000$ in *A* and $\times 67,000$ in *B*.

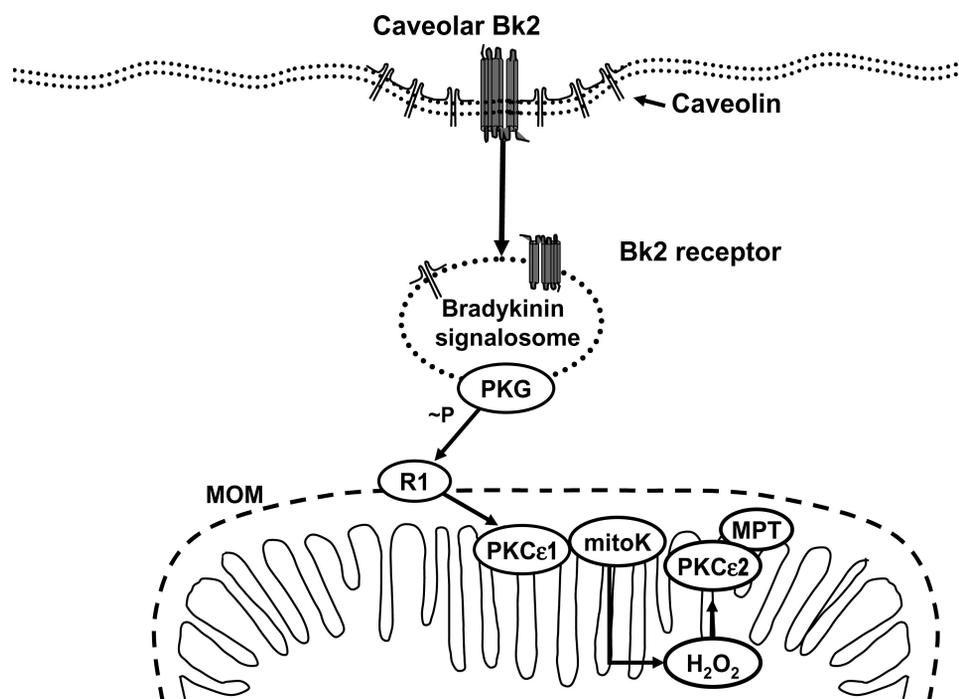


Fig. 10. The signalosome hypothesis. It is proposed that the interaction of bradykinin with its receptor [bradykinin B_2 receptor (Bk2)] induces the formation of a vesicular caveolar signaling platform (signalosome) that phosphorylates a receptor (R1) on the MOM. The identity of R1 is unknown. The terminal kinase of the bradykinin-treated signalosome is PKG, which phosphorylates R1 ($\sim\text{P}$) at a serine/threonine residue. After phosphorylation of the MOM receptor, the signal is transmitted across the intermembrane space to activate PKC- ϵ_1 on the mitochondrial inner membrane. PKC- ϵ_1 , which is constitutively expressed in mitochondria (9) and localized in close association with the $\text{mitoK}_{\text{ATP}}$ channel (23), causes $\text{mitoK}_{\text{ATP}}$ channel opening (8) and a consequent increase in H_2O_2 production (1). H_2O_2 then activates a second PKC- ϵ , PKC- ϵ_2 , which leads to a reduction in necrosis through inhibition of MPT (9).

described in Fig. 1, we then isolated functionally active signalosomes from the same donor mitochondria. These were found to open $\text{mitoK}_{\text{ATP}}$ channels in mitochondria from untreated hearts (Fig. 3). This finding is consistent with, but does not prove, the suggestion that these vesicles were responsible for the $\text{mitoK}_{\text{ATP}}$ channel opening that was observed in the experiments shown in Fig. 2.

The remaining experiments of this study focus on the functional effects of the isolated signalosomes, on their physical properties, and on the generality of the signalosome mechanism. Signalosome-containing LLs and purified LLs were able to open $\text{mitoK}_{\text{ATP}}$ channels in mitochondria from untreated hearts to maximum capacity (Figs. 3 and 4). As expected from previous results (8, 9), $\text{mitoK}_{\text{ATP}}$ channel opening was blocked by 5-HD and ϵV_{1-2} acting at the level of the inner membrane (Figs. 4 and 10). Importantly, three different control preparations had no effect on $\text{mitoK}_{\text{ATP}}$ channels: a caveolar preparation isolated from cardiac sarcolemma (Fig. 4), purified LLs from untreated hearts (Figs. 3 and 4), and residual LLs obtained after removal of purified LLs from the LL preparation (Fig. 3).

$\text{mitoK}_{\text{ATP}}$ channel opening by bradykinin-treated LLs was blocked by the PKG inhibitor KT-5823 (Fig. 5), confirming previous studies showing that PKG is the terminal cytosolic kinase of the bradykinin signaling pathway (8, 9). LL-dependent $\text{mitoK}_{\text{ATP}}$ channel opening was also blocked by the serine-threonine phosphatase PP2A (Fig. 5), indicating that phosphorylation of a MOM protein is an essential step in transmitting the signal to mitochondria. This was confirmed by the further finding that removal of the MOM abolished all effects of LLs on $\text{mitoK}_{\text{ATP}}$ channel activity (not shown). Thus, the effects of bradykinin treatment of LL on $\text{mitoK}_{\text{ATP}}$ channels were identical to the previously reported effects of addition of exogenous PKG plus cGMP (8).

We have not yet identified the outer membrane target of PKG (R1 in Fig. 10), and we can only speculate on the nature

of the link between cytosolic PKG and mitochondrial PKC- ϵ . Several kinases have been observed to be associated with mitochondria in the cardioprotected phenotype. Increased phosphorylation of mitochondrial MAPK has been observed, and PKC- ϵ has been shown to phosphorylate VDAC, but the functional consequences of these observations are not well understood (2, 3). Akt has been shown to phosphorylate hexokinase-II and modulate the MPT (32). Current experiments in our laboratory indicate a role for a MOM MAPK in cardioprotective conditioning mediated by the ouabain signalosome, but this MAPK is not involved in PKG signaling. We have found that PKG-dependent signaling from R1 to PKC- ϵ (PKC- ϵ_1 in Fig. 10) is not prevented by MPG, and therefore this step does not involve ROS. The mechanism of PKC- ϵ_1 activation is unknown; however, PKC- ϵ_1 is activated by $\Psi\epsilon$ -receptor for activated C kinase (RACK) (8), and we speculate that the connection between R1 and PKC- ϵ_1 operates by a pseudo-RACK mechanism (46).

Signal transmission by the signalosome appears to be a general phenomenon. Ischemic preconditioning, ischemic post-conditioning, and ouabain perfusion each led to LLs that opened $\text{mitoK}_{\text{ATP}}$ channels (Fig. 5). A limited survey showed perfusion with acetylcholine or elevated Ca^{2+} also led to LLs that opened $\text{mitoK}_{\text{ATP}}$ channels.

The results shown in Fig. 6 showed that bradykinin treatment of LLs causes inhibition of MPT, which is widely thought to be responsible for cell death from ischemia-reperfusion injury (13). We infer from this result that $\text{mitoK}_{\text{ATP}}$ channel opening by the bradykinin-treated signalosome mediates the MPT inhibition afforded by bradykinin (35).

Both clathrin-mediated and caveolar-mediated endocytosis and recycling depend on endosomal acidification by vacuolar H^+ -ATPase (24, 31, 51), which occurs when vesicles pinch off from the plasma membrane (31). Consistent with this, bafilomycin A_1 , a specific inhibitor of vacuolar H^+ -ATPase, prevented signalosome function (Fig. 7). Interestingly, Western

blots of the bradykinin-treated purified LLs were identical to those obtained in the absence of bafilomycin A₁ (data not shown), in agreement with the finding that bafilomycin A₁ blocks receptor return to the plasma membrane without affecting receptor internalization (4, 24). This suggests that signalosome-mediated delivery of the cardioprotective signal to mitochondria is a dynamic process that requires both internalization and return to the plasma membrane. Further investigation is warranted to test this hypothesis.

Resistance to Triton X-100 and sensitivity to methyl- β -cyclodextrin (Fig. 7), together with the Western blot data showing enrichment with caveolin-3 (Fig. 8), are consistent with a caveolar origin of the signaling platform (36, 49). The immunoblot analyses shown in Fig. 8 showed that purified LLs from bradykinin-perfused hearts contain the bradykinin B₂ receptor, caveolin-3, PKG, and eNOS. Finally, electron microscopy of immunogold-labeled purified LLs revealed that the vesicles contain multiple copies of caveolin-3 (Fig. 9).

In general support of the signalosome hypothesis, there is considerable evidence from a variety of cell types that activated G protein-coupled receptors first migrate to caveolae, where caveolins compartmentalize receptors and signaling molecules (12, 15, 27, 28, 39, 43), assemble them into a signaling platform (20, 56), and deliver the platform to the cytosol by internalization (21, 28, 29, 42, 43, 54). The hypothesis is also consistent with the findings of Tong et al. (53), who showed that receptor internalization and recycling are essential for cardioprotection by ischemic preconditioning. Signalosome-mediated signaling has previously been postulated to occur in the heart in conditions other than cardioprotection (5, 16, 40, 48). The data presented here appear to be the first demonstration of a specific functional property of purified cardiac signalosomes.

These findings represent an initial step in our effort to apprehend the organization of the complex signaling pathways of cardioprotection. The signalosome hypothesis offers a new perspective for understanding and studying cardioprotective signaling in the heart; however, the concept remains a working hypothesis that will require additional critical experiments to test its predictions and validity.

ACKNOWLEDGMENTS

A preliminary report of this work has appeared in abstract form (44). Present address of A. D. T. Costa: Carlos Chagas/Fundação Oswaldo Cruz, Instituto de Biologia Molecular do Paraná, Rua Prof. Algacyr Munhoz Mader, 3775, Curitiba, PR 81350-010, Brazil.

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-67842 and HL-36573.

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