Rate-limiting Steps in the Tension Development of Freeze-glycerinated Vascular Smooth Muscle

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ABSTRACT A method for “skinning” arterial smooth muscle is presented which yields isometric tension development typically 60-80% of maximum physiological tension in the presence of micromolar Ca" and millimolar Mg-ATP, while retaining essentially the native protein content. Using the methods of “Ca jump,” the time-course of Ca"-activated tension development in the skinned artery can be made identical to, but not faster than, the rate of tension development in the intact artery. In the skinned artery, activating free [Ca"] does not substantially alter the rate at which tension development approaches the final steady tension attained at that free [Ca"] (<25% decline in speed for a 10-fold decrease in [Ca"]). These observations are taken to mean that the rate-limiting step in isometric tension development in arterial smooth muscle does not depend directly on Ca".

INTRODUCTION

Much work has recently appeared using preparations of “skinned” smooth muscle to study the processes of activation and contraction (Sparrow et al., 1981; Kerrick et al., 1980; Peterson, 1980; Mrwa et al., 1979; Gordon, 1978; Endo et al., 1977). Skinning processes are primarily methods by which membranes are rendered highly permeable so that the intracellular milieu can be controlled by altering the extracellular environment, while nonetheless leaving the contractile apparatus functional. Data obtained from such systems that retain contractility therefore lie midway between studies on isolated contractile proteins and studies on physiologically intact smooth muscle.

To obtain fair comparison between studies on skinned and intact smooth muscle, it should be established that the contractile properties of the skinned preparations are at least comparable to those of the intact preparations. In many studies in the literature, this has not been done. In this report, I describe a skinning method that renders the smooth muscle cells of hog carotid artery highly permeable to low molecular weight substances, but retains essentially the contractile properties and native protein content of the intact artery.

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Maximum isometric tension development of the skinned artery segments in micromolar Ca\(^{++}\) and millimolar Mg-ATP ranges from 60 to 80\% of the observed maximum physiological tension development.

Studies on isolated smooth muscle proteins to identify the rate-limiting steps in the activation process have been equivocal. Mrwa and Hartshorne (1980) have reported that whereas isolated myosin light chains are phosphorylated much more rapidly than the actomyosin cross-bridge cycling rate, light chains attached to whole myosin (a situation more similar to the in vivo case) are phosphorylated at about the same rate or slower than the cross-bridge cycle rate. This allows the possibility that phosphorylation is the rate-limiting step. A mathematical model of this possibility has recently been presented and verified for the case of skinned guinea pig taenia coli smooth muscle (Peterson, 1982b). Driska et al. (1981), on the other hand, have shown in the intact hog carotid artery that myosin light chain phosphorylation proceeds more rapidly than tension development, which suggests that other steps are rate limiting.

In these studies with skinned vascular smooth muscle, by the use of appropriate solutions designed to minimize rate limitations due to the inward diffusion of activating Ca\(^{++}\), the time-course of isometric tension development can be made identical to that of the physiologically intact artery, but not faster. This suggests that the in vivo rate-limiting step in tension development is not directly dependent on Ca\(^{++}\); that is, some step after Ca\(^{++}\) activation is rate limiting for tension development in vascular smooth muscle.

**MATERIALS AND METHODS**

In earlier work, the glycerination method used successfully with skeletal muscles (prolonged soaking in cold glycerol solutions) gave contractile responses in smooth muscles only 5–10\% of maximum physiological tension development (Filo et al., 1965; Mrwa et al., 1974). A modification of this method ("freeze-glycerination") gives contractile responses 40–80\% of full physiological tension development in a variety of smooth muscles upon application of micromolar Ca\(^{++}\) in the presence of millimolar Mg-ATP (Peterson, 1980).

**Tissue Preparation**

Hog carotid arteries were collected at an abattoir within 15 min of animal death, cleaned of blood and loose adventitia, and stored in a cold or room temperature physiological saline solution (PSS). Small segments of artery were prepared by first dissecting out the smooth muscle-containing media layer following the method described by Glück and Paul (1977), laying the media strip out flat on Parafilm, and slicing off thin (0.1–0.3 mm) transverse sections in the direction of smooth muscle orientation with a razor blade. In tests of physiological contractility, this method proved more reliable than "pulling out" media strips, which appears to overstretch the tissue and leads to reduced tension development (cf. Driska et al., 1981). This method produces artery segments typically 8 mm long with a rectangular cross-section \(~0.1–0.3\; \text{mm}\) (as desired) by 0.6–0.8 mm with a wet weight of 1–2 mg. For comparative studies, \(~20\; \text{pieces per artery were prepared and allowed to equilibrate in oxygenated PSS at 37\degree C for 1 h. The pieces were then gently drained of PSS, immersed in 5 ml of cool (5\degree C) glycerinating solution, and allowed 10 min to}\)
equilibrate. The beaker was then placed in a freezer at -25°C. Artery segments were
left in the freezer until needed, then rinsed with relaxing solution and mounted in an
isometric tension apparatus. Within ~15 min in the freezer, physiological contractility
(i.e., responsiveness to normal agonists such as norepinephrine, histamine, or high
K+) was completely abolished and isometric tension development depended wholly
on externally provided Ca++ and Mg-ATP. Tissues stored frozen as long as 1 yr
showed no deterioration in properties such as absolute force generation or the force
[Ca++] relation.

Permeability (as measured by the equilibration rate with [3H]ATP) is high: DATP
≈2 × 10⁻⁶ cm²/s. Due to the nature of the method, this high permeability is probably
due to extensive membrane cracking rather than wholesale membrane dissolution.
That the freezing (or thawing) step produces the permeability increase is evidenced
by the fact that artery segments tested after the 5-10 min equilibration soak in
glycerinating solution showed normal physiological contractility and no contractile
response to millimolar Mg-ATP and micromolar Ca++. Preservation of some mem-
brane structure for force transmission, better retention of native protein content, and
the fact that the "skinning" occurs homogeneously throughout the preparation rather
than progressively (as in detergent treatments, which can require 16 h at room
temperature to be effective; Gordon, 1978) perhaps account for the superior tension
development with this skinning method.

Solutions

Because the stability of isometric tension in the skinned artery segments was found to
be very sensitive to high ionic strength, solutions were prepared to maintain ionic
strength as low as feasible and always <0.1 M. The relaxing solution contained 20
mM imidazole, 5 mM EGTA, 5 mM Na₂ATP, 5 mM MgCl₂ and, as ATP-regenerating
system, 5 mM K-phosphoenolpyruvate with 20 U/ml pyruvate kinase (Sigma type
III; Sigma Chemical Co., St. Louis, Mo.). Solutions were titrated with KOH to pH
6.85 to 7.0 as desired at 37°C and KCl was added to a final ionic strength of 0.085 M.
Under these conditions, Mg²⁺ binding to ATP and phosphoenolpyruvate provides a
reasonably well-buffered free [Mg++] of ~0.4 mM. Activating solutions contained
added CaCl₂ with reduced KCl and increased KOH, so that ionic components (except
for Ca-EGTA and free Ca++) were virtually identical to relaxing solution. Free Ca++
concentration (pCa = -log[Ca++] ) and other ionic components were computed from
the data of White and Thorson (1972). In the "Ca-jump" relaxing solution, EGTA
was reduced to 0.1-0.2 mM and the ionic strength was compensated by the addition
of HDTA (1,6-diamino-hexan-n,n,n',n'-tetraacetic acid; Fluka AG, Switzerland) to a
constant tetraacid concentration of 5 mM (Moisescu, 1976).

The freeze-glycerination solution contained 50% relaxing solution; 50% glycerol
(vol/vol) with 2 mM dithiothreitol added.

Tension Measurements

The small skinned smooth muscle segments were attached horizontally between a
fixed, bent glass rod and a thin carbon filament-epoxy extension rod from an AE 801
force transducer (Aksjeselskapet Mikroelektronikk, Horten, Norway), using a small
amount of fast water-setting cyanoacrylate glue. System compliance amounted to
<0.2% shortening at maximum isometric force (typically 10-20 mN) and the trans-
ducer speed exceeded 8 kHz. Tissue segments were mounted in relaxing solution,
stretched to a peak force ~10 mN, and allowed to stress relax. This cycle of stretch
and relaxation was repeated twice (~20 min) and the tissue length was then adjusted
to a passive force of ~1 mN. Tissues were incubated in 0.9-ml glass cups of the appropriate solutions, which were rotated at ~1 Hz to provide stirring and thermostated at 37°C (±1°). The cross-section area was computed from the segment wet weight/length.

**Diffusion Measurements**

Small segments of arteries were preweighed and mounted for force measurements as described. Tissues were then incubated for 8 min in relaxing solution (pCa > 8) without ATP and then for 10 min in ATP-free contracting solution (pCa 6.4); ionic strength was maintained with added KCl and free [Mg²⁺] with MgCl₂. In the absence of ATP, Ca²⁺ produced no contraction. At time \( t = 0 \), 2 mM Mg-ATP with [2,8-³H] ATP at specific activity 0.05 Ci/mmol or trace amounts of [³H(G)]inulin were added (New England Nuclear, Boston, Mass.), mixed, and an aliquot of the bathing solution was taken. At various times after the onset of tension development, the bathing solutions were again sampled and tissues were removed from the labeled ATP solutions, gently blotted to remove adherent surface solution, weighed, and dissolved in 0.5 ml Soluene-350 (Packard Instrument Co., Inc., Downers Grove, Ill.) at 50°C. Two aliquots of the tissue extract and the incubating solutions were then counted in 5 ml Dimilume (Packard Instrument Co., Inc.). The ratio of average counts per unit volume in the artery piece (assuming density = 1.0) to average counts per unit volume in the incubating solution was used as an index of equilibration rate.

**Tissue ATPase Measurements**

Because of the ATP-regenerating system used in the incubating solutions, the total ATPase of the skinned artery segment appears as accumulation of pyruvate (ADP + phosphoenolpyruvate \( \rightarrow \) ATP + pyruvate with pyruvate kinase). In control experiments, the artery segments metabolized no pyruvate added directly to the incubating solutions in the absence of added NADH. In each ATPase measurement, two 0.9-ml incubation cups were filled with appropriate solutions; one cup contained the tissue and the other served as control for spontaneous decomposition of ATP and phosphoenolpyruvate. After a chosen time interval (usually 10 min), both solution cups were brought to 0.25 mM NADH, 37.5 mM Tris, 7.5 mM MgCl₂ at pH 8.6 by the addition of 15 μl concentrate. The difference in absorbance change between the two cups at 340 nm upon the addition of 10 μl lactate dehydrogenase (Sigma type II) was equated to the ATP hydrolyzed using a calibration factor 6 (±0.2) A₃₄₀ units/mM ADP.

**Protein Content**

The total protein content of various solutions and tissue extracts were determined by the biuret method with bovine serum albumin as standard. Protein was extracted by homogenization and continual stirring for 12-16 h at 4°C in 80 mM KCl, 20 mM imidazole, 5 mM EGTA, 10 mM ATP, and 1 mM dithiothreitol (pH 7.2), followed by centrifugation at 20,000 g for 15 min.

**RESULTS**

**Protein Content**

A comparison was made between intact and "freeze-glycerinated" arteries with regard to contractile protein content both by protein assay and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Artery segments were prepared as described in Methods, some freeze-glycerinated and others
left intact in PSS. Several small pieces of each (typically 4–6 mg) were finely minced and incubated with shaking for 0.5 h at 50°C in 1% SDS, 1% mercaptoethanol, 20 mM imidazole, and 5 mM EGTA (pH 6.85); they were then briefly centrifuged, and the supernatant was applied to 6% polyacrylamide gels (0.1 M phosphate, 0.1% SDS, pH 7). The resulting gels showed a multitude of peaks (~40). In repeated trials, visual comparison of densitometer tracings gave no substantial differences between the glycerinated and living samples.

Similarly, larger samples of artery (typically 2–3 g) were freeze-glycerinated or left intact, and treated with conditions used to extract crude actomyosin (cf. Russell, 1973). The extractable protein content of the intact arteries averaged 46.8 (± 4.3 SD, n = 4) mg protein/g wet tissue, which is comparable to the value determined by Cohen and Murphy (1978) for the content of actin + myosin + tropomyosin in hog carotid artery (43 ± 5 mg protein/g wet tissue). Batches of freeze-glycerinated arteries were extracted under identical conditions, giving an average extractable protein content of 49.2 (± 8.9 SD, n = 3) mg protein/g wet tissue. Gel densitometry of the crude extract run on SDS polyacrylamide gels as above (Fig. 1) shows that the contractile proteins constitute the bulk of the protein extractable from freeze-glycerinated arteries. The two very prominent bands at ~32,000 and 23,000 mol wt are not identified, but can be greatly reduced by precipitation of the crude actomyosin by dialysis against ATP-free buffer.

Differences in important proteins of a regulatory nature present in low concentrations would not be detected by the above methods. To test freeze-glycerinated arteries for protein elution under the experimental conditions, a large batch of skinned artery segments (~4 g) were incubated with gentle shaking for 1 h at 37°C in 2 ml of normal relaxing solution, which was then assayed for protein content. The value determined was <0.5 mg/ml, which indicates that ~0.5% of extractable protein is eluted from the skinned artery segments during a typical experimental time period.

**Dependence of Tension Development on [Ca++]**

Fig. 2 shows the average measured dependence of stable isometric tension development in nine skinned artery segments from five arteries as a function of free [Ca++]}, where the data have been grouped according to pCa and averaged as illustrated by the standard error bars shown. Maximum Ca++-activated force (pCa 4.5) at 5 mM Mg-ATP was typically 8–9 mN, or ~60% of the maximum force measured in identically prepared artery segments that were not freeze-glycerinated and stimulated with high K+ and 10−5 M histamine (cf. Peterson, 1982). In individual experiments, however, tensions as high as 90% of maximum physiological tension were occasionally observed. The sigmoidal shape of the force [Ca++] relation is as expected for a muscle preparation and is well fit by the functional form used for cooperative binding models, 1/(1 + (K/[Ca++]^N). The observed value of N, which determines the steepness of the relation, was estimated from a linearized least-squares fit and is not substantially different from that reported for other skinned smooth muscle.
muscle preparations. Gordon (1978) found a value of $\sim 3$ for detergent-skinned rabbit taenia coli, whereas the data of Filo et al. (1965) indicate a value $\sim 2.5$ for hog carotid artery. The value of $K$, which in some sense represents an apparent binding constant for activating Ca\textsuperscript{++} (cf. Edsall and Wyman, 1958), is shifted substantially to the left relative to other smooth muscles. Data from visceral smooth muscles (Gordon, 1978; Endo et al., 1977) indicate that the vascular preparation is some four- to fivefold more Ca\textsuperscript{++} sensitive. In a direct comparison, Endo et al. (1977) observed a similar difference in pK for the force [Ca\textsuperscript{++}] relations measured in rabbit taenia coli and pulmonary artery.

![Figure 1](image)

**Figure 1.** Densitometer tracing of the protein extracted from freeze-glycerinated arteries and subjected to SDS polyacrylamide gel electrophoresis and stained with Coomassie Blue. The molecular weight scale shown (X 10\textsuperscript{2} mol wt) was obtained from calibrations using cross-linked hemoglobin and cross-linked albumin (Sigma Chemical Co.).

The saponin-treatment used there, however, led to a rapid decline in maximum Ca\textsuperscript{++}-activated tension development upon repeated contractions. Maximum tension development in the freeze-glycerinated artery preparation, on the other hand, was usually reproducible to better than 10% with repeated Ca\textsuperscript{++} activation. Long-term incubation at 37°C, however, led to a failure to relax completely upon subsequent Ca\textsuperscript{++} removal. The force [Ca\textsuperscript{++}] relation here is somewhat steeper than and shifted to the left of the relation reported for hog carotid artery treated 12–16 h in cool 50% glycerol, which developed a maximal tension of $\sim 0.4$ N/cm\textsuperscript{2} (Mrwa et al., 1974).
Dependence of Tension Development on \([\text{Mg-ATP}]\)

Fig. 3 shows the average dependence of tension development on externally provided Mg-ATP at pCa \(\sim 6.0\) measured in eight artery segments from four arteries. It is interesting to note that isometric tension is maximized by 2 mM Mg-ATP, which is about the concentration of high-energy phosphates in intact artery (cf. Butler and Davies, 1980). Below 1 mM external Mg-ATP, tension falls off sharply with decreasing ATP availability. Unlike the force \([\text{Ca}^{2+}]\) dependence, which represents some equilibrium-binding relation, the force \([\text{ATP}]\) relation results from a diffusion-limited reaction rate. Below 2 mM Mg-ATP, the diffusion rate of ATP into the tissue becomes limiting to the actomyosin ATPase. This is directly illustrated in Fig. 4, which shows that under these conditions, total ATPase falls off directly with tension when ATP is the limiting substrate (A). The response of measured ATPase to external [Mg-ATP] is similar to that of the isometric tension (B). With [ATP] greater than 2 mM, however, isometric tension remains maximized or declines slightly while ATPase continues to increase somewhat, perhaps indicating the contribution to total ATPase of non-actomyosin ATPases. Alternatively, increasing ATPase at constant maximum tension could reflect an increased rate of cross-
FIGURE 3. Active isometric tension at various external Mg-ATP concentrations and pCa 6.0 is expressed relative to the tension observed in each artery segment at 2 mM [Mg-ATP]o. The data from eight artery segments have been averaged at each [ATP]o (usually four to six measurements); standard deviation is shown. For Mg-ATP concentrations of <5 mM, some MgCl₂ was added. With these additions, between 0 and 5 mM [Mg-ATP], free [Mg²⁺] is calculated to increase from 0.28 to 0.43 mM.

FIGURE 4. The measured tissue ATPase at pCa ~6.0 in three skinned artery segments is normalized to the maximum ATPase observed in each artery segment (~1.2 µmol ATP/min·g wet artery) and plotted against the external Mg-ATP concentration (B) and the relative isometric force developed at various external Mg-ATP concentrations (A).
bridge cycling, although the average number of attached cross-bridges (and therefore tension) is not greatly altered. A similar dissociation of ATPase and tension has been reported for chemically skinned cardiac muscle fibers (Herzig et al., 1981).

**Diffusion Measurements**

The diffusion rate of $[^3H]$ATP into the preparation was measured directly and compared with the time-course of tension development under the same conditions. As detailed in Methods, ATP-free segments were first equilibrated with activating concentrations of Ca$^{++}$ so that equilibrium Ca$^{++}$ binding could be reached without tension development. This ATP-free Ca$^{++}$ incubation was found to reduce subsequent tension development upon addition of ATP, relative to activation in the opposite order. As shown in Fig. 5, upon addition of labeled 2 mM Mg-ATP, tension development and ATP permeation throughout the tissue rise with similar time-courses (as indicated by the half-times to saturation). Average tissue [ATP] is expressed as counts per gram wet artery segment, while bath [ATP] is counts per cubic centimeter. In separate experiments, the water content of hog carotid arteries was estimated from the ratio of dry weight to wet weight. The water content averaged 0.76 ($\pm$ 0.03 SD, $n = 21$) of the total weight, in agreement with the observation that ATP equilibrates in 5 min with $\sim$75% of the total artery volume as computed from segment weight (that is, dry weight excludes $\sim$25% of the tissue volume). As an approximation, using the diffusion equation for a plane of infinite extent (cf. Crank, 1956), the data obtained are fit by the solid line with a diffusion constant of $1.4 \times 10^{-6}$ cm$^2$/s, although the fit is not very sensitive to the exact number (as illustrated by the marks at 1 and 3.5 min, which show the range of the fit for $D_{ATP}$ between 1 and $2 \times 10^{-6}$ cm$^2$/s).

Even though tissue segments were lightly blotted with filter paper to remove adherent labeled solution before counting, the data appear to start at a relatively high value, which suggests that about one-third of the available water space equilibrates almost instantaneously on the time scale of these experiments. The segments for these experiments were purposely cut thick so that permeation would be slow, thus facilitating the measurements. Recalling, however, the rectangular cross-sectional profile of the segments (0.4 X 0.8 mm in these experiments), a simple calculation shows that surface ATP penetration to a depth of only 40 $\mu$m could account for this extent of rapid equilibration. This is also perhaps indicative that by cutting off artery segments, surface disruption becomes important.

In several experiments, the equilibration with labeled inulin was determined for the freeze-glycerinated artery. In agreement with the protein elution experiments, which suggest that high molecular weight material is retained by the skinned preparation, labeled inulin in the artery segment after 15 min rose to a value of 27% ($\pm$ 3 SD, $n = 3$) of the bathing solution concentration; a value that, when taken relative to the total water space (75%), is slightly less than that estimated for the extracellular space in hog carotid artery (Murphy...
et al., 1974). Whereas materials of molecular weight on the order of 500 (Mg-ATP and Ca-EGTA, for example) penetrate into the smooth muscle cells quite readily, inulin with a molecular weight of ~5,000 appears to be excluded. This extracellular space could also play some part in the very rapid partial equilibration with labeled ATP.

**Figure 5.** Top: the tension development upon addition of 2 mM Mg-ATP to 18 skinned artery segments prepared from a single artery is shown. The pCa was 6.0. Absolute tension development, which was impaired by this procedure, is shown at various times and expressed in gram weight (1 gwt = 0.01 N). Bottom: the counts per unit volume from [3H]ATP in the tissue relative to counts per unit volume in the incubating solution is shown as a function of time for the same tissue samples. The solid line shown is the theoretical fit starting from the high initial value, which is discussed in the text.
Dependence of Tension Development Rates on Ca\(^{++}\) and ATP

It was noted in the above experiments that after pre-equilibration with activating Ca\(^{++}\) in the absence of ATP, tension develops rapidly upon addition of Mg-ATP (time to half-maximal tension development, \(t_{0.5}\), was 69 s at 2 mM external Mg-ATP). Similar experiments in which tissues remained equilibrated with 5 mM Mg-ATP and in which EGTA was abruptly replaced by equimolar Ca-EGTA (pCa 6.0-6.3 to effect activation) led to very much slower rates of isometric tension development (\(t_{0.5}\) typically 250 s) after a substantial delay in the onset of activation. Ashley and Moisescu (1973) have developed a technique for reducing the time to produce a step-change in internal free [Ca\(^{++}\)] in skinned skeletal muscle fibers. By first incubating relaxed tissues in very low EGTA solutions to reduce Ca\(^{++}\)-buffering capacity and then "jumping" to very highly buffered Ca-EGTA-activating solutions (50 mM), changes of internal [Ca\(^{++}\)] could be produced within 200 ms in fibers ~0.05 mm in diameter. Although the smooth muscle segments used here are three to five times thicker and the ionic strength considerations limited the high Ca\(^{++}\)-buffering capacity of the activating solutions to <20 mM, the much slower time-course of smooth muscle contraction nonetheless permits this method to be used to make the Ca\(^{++}\) diffusion time short compared with the half-time of isometric tension development. Assuming that diffusion time goes as the square of the thickness, we estimate that the internal [Ca\(^{++}\)] step occurs in <5 s, while \(t_{0.5}\) for isometric tension development in the intact artery activated with high K\(^{+}\) and histamine averages 34 s (± 8 SD, \(n = 9\)).

Calling "jump intensity" the ratio of [Ca-EGTA] in the activating solution to [EGTA] in the relaxing solution, it was found for the freeze-glycerinated artery segments that increasing jump intensity led to progressively faster rates of isometric tension development (Fig. 6), as did increasing the external [ATP] in experiments with skinned artery segments pre-equilibrated with activating [Ca\(^{++}\)], i.e., "ATP jumps." As shown in Table I, increasing the jump intensity progressively increased the rate of isometric tension development until the time-course of tension development saturated at essentially that found for the intact artery supramaximally activated. To allow for possible changes in the shape of the tension-time relation, two characteristic time constants for tension development have been tabulated in Table I (times to 50 and 90% of final tension). Further increases in Ca\(^{++}\) jump intensity did not cause the rate of tension development to become faster than that observed in the intact artery preparation. A similar progression of tension development rate was observed for ATP jumps.

Having found Ca\(^{++}\)-activating conditions that maximized the rate of isometric tension development, the effect of varying activating free [Ca\(^{++}\)] was examined. The results are illustrated in Fig. 7 for a series of "Ca jumps" to various free Ca\(^{++}\) concentrations. Over the full range of activating [Ca\(^{++}\)], the isometric contractions proceed with a time-course that is essentially unaffected by [Ca\(^{++}\)]. This is shown in the lower panel, in which the time-courses of the contractions at pCa 6.1 and 7.1 have been normalized to the final tension attained at each pCa. The time-courses virtually superimpose (the other
isometric contractions, which lie between the two shown, have been deleted for clarity), differing by only ~25% in the initial steady rate of isometric tension development for a 10-fold change in activating $[\text{Ca}^{++}]$.

**Table I**

| Time constant | Skinned artery Ca jump intensity | Intact artery K$^+$ + histamine |
|---------------|---------------------------------|---------------------------------|
| $s$           | $1:1$                           | $10:1$                          | $100:1$                         | $200:1$                         |
| $t_{50}$      | $250\pm10$                      | $102\pm16$                     | $34\pm4$                       | $36\pm8$                       | $34\pm8$                       |
| $t_{90}$      | $675\pm147$                     | $347\pm37$                     | $84\pm12$                      | $91\pm8$                       | $92\pm28$                      |

**ATP jump $[\text{Mg-ATP}]_0$**

| $t_{50}$      | $2\text{ mM}$                  | $5\text{ mM}$                  | $10\text{ mM}$                 |
|---------------|--------------------------------|--------------------------------|--------------------------------|
| $t_{50}$      | $69\pm8$                       | $56\pm3$                       | $34\pm3$                       | $34\pm8$                       |
| $t_{90}$      | $188\pm21$                     | $104\pm12$                     | $93\pm17$                      | $92\pm28$                      |

$t_{50}$ and $t_{90}$ are the times in seconds for isometric tension to reach 50 and 90% of final tension, respectively. Errors shown are standard deviations measured from three to four experiments for each value, rounded off to the whole second. Data for the intact artery are from nine experiments.
DISCUSSION

From the measurements of total tissue ATPase (~1.2 μmol/min·gram artery when maximally activated) and the ATP diffusion constant (~1.4 × 10⁻⁶ cm²/s), the ATP concentration profile throughout the thickness of the skinned
artery segment can be estimated from the steady-state reaction-diffusion equation for an infinite plane sheet (cf. Crank, 1956). At 2 mM Mg-ATP in the bathing solution, the concentration of ATP in the tissue is apparently adequate to support maximum tension generation, since increasing [ATP]o does not increase tension (Fig. 3). From the values presented above, the central ATP concentration for artery segments 0.3 mm thick would be on the order of 0.5 mM. Below this minimum internal [ATP], tension apparently cannot be maximally maintained. For comparison, Murphy (1969) has found for actomyosin isolated from hog carotid artery that actomyosin ATPase declines by only ~15% when [ATP] is reduced from 1 to 0.5 mM, but by ~70% when [ATP] is decreased from 0.5 to 0.1 mM. Taking 0.5 mM ATP as the minimum concentration needed to support full tension and using the estimated diffusion constant, then for artery segments given ample time to equilibrate with activating [Ca++] in ATP-free solution, the abrupt addition of 10 mM [ATP], would cause internal [ATP] to exceed 0.5 mM everywhere in the tissue in ~30 s (±10). Whereas [ATP] adequate to support maximum tension generation is therefore available in ~30 s, isometric tension under the same conditions requires ~90 s to approach maximum tension. ATP availability does not, apparently, limit the rate of isometric tension development.

It was shown above that the rate of isometric tension development in skinned hog carotid artery can be made identical to the rate of isometric tension development in the intact artery if the methods of “Ca jump” are used. Under these conditions, making intracellular Ca++ rapidly available for activation (within ~5 s) does not cause the skinned preparation to develop tension more rapidly than does the intact artery activated hormonally and by membrane depolarization (the half-time is ~35 s in both cases). Furthermore, the rate of tension development relative to the final tension attained is not affected by the activating free [Ca++] (Fig. 7). These observations indicate that the rate-limiting process in isometric tension development is not directly dependent on Ca++. That is, processes related to the sudden appearance of activator-Ca++ (such as binding) are rapid compared with subsequent steps such as myosin light chain phosphorylation and actomyosin cross-bridge formation. Since the observed rates of isometric tension development in the skinned preparation are identical to those found in the intact preparation, it seems fair to extrapolate this conclusion to intact arterial smooth muscle. After activation of physiologically intact vascular smooth muscle, the rate at which intracellular free [Ca++] increases is not rate-limiting for tension development.

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