Differences in the Association of Calmodulin with Cyclic Nucleotide Phosphodiesterase in Relaxed and Contracted Arterial Strips†

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ABSTRACT: Changes in the concentration of cytosolic Ca^{2+} are assumed to alter the activity of Ca^{2+}–calmodulin-sensitive cyclic nucleotide phosphodiesterase in intact cells. However, this assumption is based on indirect evidence and by analogy from studies of enzyme activities in broken cell systems. We have developed a procedure for estimating the fraction of Ca^{2+}–calmodulin-sensitive phosphodiesterase that is in an activated, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) sensitive state in intact porcine coronary artery strips. The experimental approach involves homogenization of the strips and assay of cyclic guanosine monophosphate (cyclic GMP) phosphodiesterase activity under conditions that retard changes in the amount of the complex Ca^{2+}–calmodulin–phosphodiesterase. Our findings indicate that cyclic GMP phosphodiesterase in intact coronary artery strips does associate with Ca^{2+}–calmodulin and that interventions that change the concentration of Ca^{2+} in the cytosol of the intact strip change the extent of this functional association. Exposure to histamine (10 or 100 μM) or 50 mM KCl caused contraction and an increase in EGTA-sensitive cyclic GMP phosphodiesterase activity. Isoproterenol-induced relaxation of tissues that had been caused to contract with 10 μM histamine was accompanied by a reduction in EGTA-sensitive cyclic GMP phosphodiesterase activity to the same level as that present before contraction was initiated.

Calmodulin, a Ca^{2+}-binding protein, has a broad distribution and can alter the activities of several enzymes in cell-free systems in a Ca^{2+}-dependent fashion, and the regulation by Ca^{2+} of a variety of cell processes seems to be mediated by calmodulin (Cheung, 1980; Kakiuchi et al., 1982; Klee & Vanaman, 1982). A model of some such systems, including a form of cyclic nucleotide phosphodiesterase and smooth muscle myosin light chain kinase, holds that Ca^{2+} binds reversibly to calmodulin, and the Ca^{2+}–calmodulin complex in turn binds to target enzymes with much greater affinity than does calmodulin without Ca^{2+}; the Ca^{2+}–calmodulin–enzyme complex has greater activity than the uncomplexed enzyme (Wang et al., 1975). This model is well supported by many studies in cell-free systems. There is less evidence, however, to indicate that calmodulin actually regulates in intact cells the enzymes that it can activate in cell-free systems.

Agents (e.g., phenothiazines and naphthalenesulfonamides) that can bind to the Ca^{2+}–calmodulin complex and prevent its functional association with target enzymes have been used as tools to obtain evidence in support of the regulation of myosin light chain kinase by Ca^{2+}–calmodulin in intact smooth muscle [e.g., see Hidaka et al. (1979), Asano et al. (1982); and Silver & Stull (1983)]. In cultured astrocytoma cells, cholinergic agonists seem to lower adenosine cyclic 3′,5′-phosphate (cyclic AMP)[1] levels through a Ca^{2+}-mediated activation of phosphodiesterase (Meeker & Harden, 1982). Selective inhibitors of a Ca^{2+}–calmodulin-sensitive isozyme of cyclic nucleotide phosphodiesterase attenuate Ca^{2+}-dependent effects of muscarinic cholinergic agonists to lower cyclic AMP levels in thyroid (Miot et al., 1984).

When a tissue is homogenized without a Ca^{2+} chelator, calmodulin is exposed to higher Ca^{2+} concentrations than exist in cell cytoplasm, and enzymes that can be activated by Ca^{2+}–calmodulin are exposed to the complex. Even if an enzyme in an intact cell is in its "basal" state because there is insufficient cytosolic Ca^{2+} available to promote its interaction with calmodulin, this enzyme may become fully associated with Ca^{2+}–calmodulin and thereby activated immediately upon disruption of the cell. Conversely, if the tissue is homogenized in the presence of EGTA, the Ca^{2+}–calmodulin–enzyme complex rapidly dissociates, as EGTA has a much greater affinity for Ca^{2+} than does calmodulin. To estimate the fraction of an enzyme in a Ca^{2+}–calmodulin-activated state in an intact cell, it thus is necessary to homogenize a tissue in a way that will prevent the association of any enzyme and Ca^{2+}–calmodulin that were not associated in the intact cell and also prevent the functional dissociation of any of the ternary complex that did exist in the intact cell. It also is necessary to assay the activity of the enzyme so as to get estimates of basal and calmodulin-stimulated activities.

Most cells or tissues contain a mixture of isozymes of cyclic nucleotide phosphodiesterases, only some of which are Ca^{2+}–calmodulin sensitive (Wells & Hardman, 1977). In some tissues, a major fraction of the cyclic GMP phosphodiesterase activity is Ca^{2+}–calmodulin sensitive. In coronary arteries from pigs, virtually all of the cyclic GMP phosphodiesterase activity seems to be Ca^{2+}–calmodulin sensitive (Wells et al., 1975). We have used these vessels in an attempt to answer two questions: (1) does cyclic GMP phosphodiesterase associate with Ca^{2+}–calmodulin in intact cells, and (2) if so, does a change in cytoplasmic Ca^{2+} concentration change the extent of this association? Our approach has been to homogenize rapidly frozen tissues and measure cyclic GMP phosphodiesterase activity in the presence of trifluoperazine,

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[1] Abbreviations: cyclic AMP (cAMP), adenosine cyclic 3′,5′-phosphate; cyclic GMP (cGMP), guanosine cyclic 3′,5′-phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DEAE-cellulose, diethylaminoethylcellulose; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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a phenothiazine known to bind calmodulin (Levin & Weiss, 1977) at 4 °C. The ternary Ca^{2+}–calmodulin–phosphodiesterase complex dissociates slowly at 30 °C (Wang et al., 1980) and would be expected to dissociate even more slowly at 4 °C, and trifluoperazine prevents association of the enzyme with Ca^{2+}–calmodulin (LaPorte et al., 1980). Our data indicate that trifluoperazine binds to Ca^{2+}–calmodulin that is not associated with the enzyme, as suggested by LaPorte et al. (1980), but does not bind to the Ca^{2+}–calmodulin–enzyme complex.

Our results indicate that the Ca^{2+}–calmodulin complex does associate with cyclic GMP phosphodiesterase in intact cells and, furthermore, that the extent of this association is altered by changes in cytoplasmic Ca^{2+} concentration.

Experimental Procedures

Materials. Cyclic GMP, EGTA, and histamine dihydrochloride were purchased from Sigma Chemical Co. Tritiated cyclic GMP was purchased from New England Nuclear Corp. and was purified by chromatography on Dowex-50 cation-exchange resin columns (Hardman & Sutherland, 1969) before use. Trifluoperazine dihydrochloride was kindly supplied by Smith, Kline and French Laboratories. (*R,S*)-Isoproterenol was purchased as Isuprel from Breon Laboratories, Inc., and other chemicals were of analytical grade from commercial sources. Calmodulin from porcine testes was purified to apparent homogeneity by the procedure of Jamieson & Vanaman (1979), with slight modification. Calmodulin-deficient cyclic GMP phosphodiesterase was prepared from the 48000*g* supernatant fraction from porcine coronary arteries by DEAE-cellulose chromatography (Wells et al., 1975).

Tissue Preparation. Hearts from pigs of varying ages and both sexes were obtained from a local slaughterhouse and transported on ice to the laboratory. Right coronary arteries were dissected as described previously (Kramer & Wells, 1979) within 2 h of slaughter of the animals. Before use, tissues were stored for 18–42 h at 4 °C in Krebs–Ringer bicarbonate buffer with 10 mM glucose and 1 mM pyruvate, bubbled with 95% O_2–5% CO_2 at pH 7.4.

Measurement of Tension Development. Helical strips (3 × 25 mm) of the coronary arteries, dissected in a way that disrupts functional endothelium (Furchgott, 1982), were mounted under 1-g resting tension in organ baths containing 27 mL of incubation buffer (defined above) at 37 °C. Isometric force development was monitored by a Statham strain gauge transducer connected to a Gould-Brush 2400 recorder. Tissues were incubated for 2 h, during which time the tension was readjusted several times to 1 g until no more spontaneous relaxation occurred. Tissues were then further conditioned with exposure to 50 and 20 mM KCl as described by Kramer & Wells (1979).

Tissue Homogenation and Assay of cGMP Phosphodiesterase Activities. At the appropriate times, the baths were lowered from the tissue, and within 2 s (with no observable change in force), the tissues were clamped between aluminum blocks that had been cooled in liquid nitrogen. The frozen tissues were stored at −70 °C prior to the procedure that follows. Frozen samples (60–80 mg) were placed in plastic capsules with plastic pestles (L. D. Caulk Co.) that had been cooled in liquid nitrogen. The cold capsules and their contents were agitated in a Wig-L-Bug dental amalgamator (Crescent Dental Manufacturing Co.) at maximum speed for three 15-s periods. The capsules were cooled in liquid nitrogen for 15–20 s between agitations and after the tissue had been converted to powder. The powdered tissues were kept at the temperature of liquid nitrogen until homogenization. Subsequent procedures were conducted in a cold room that was maintained at 5–8 °C. The frozen, powdered tissues were added to 25 volumes (milliliters per gram of frozen tissue) of medium containing 10 mM Tris·HCl (pH 7.5) and 3 mM $Mg(OAc)_2$ (with or without 150 μM trifluoperazine as indicated in the text and figure legends). The medium had been cooled in a NaCl–ice–water bath to about −3 °C. The suspension was immediately homogenized, for 5 s at speed setting 8, with a Polytron homogenizer (PCU-2; Kinematica GmbH Switzerland). The Polytron probe was cooled in ice–water before use, and the powder suspension was cooled in a salt–ice–water bath during the homogenization. Aliquots (175 μL) of homogenates containing trifluoperazine were assayed immediately for cyclic GMP phosphodiesterase activity. Trifluoperazine was added 2 min after homogenization to the homogenates that were made without trifluoperazine, and the preparations were then assayed. This 2-min incubation in the absence of trifluoperazine allowed maximal expression of the cyclic GMP phosphodiesterase activity of the homogenate (see Results). An aliquot of each homogenate (each now containing 150 μM trifluoperazine) was incubated at 30 °C for 5 min in the presence of 400 μM EGTA and then returned to an ice bath for 3 min prior to assay for basal cyclic GMP phosphodiesterase activity. This 30 °C preincubation was necessary for maximal EGTA inhibition of phosphodiesterase activity.

Cyclic GMP phosphodiesterase activity was assayed as described previously (Keravis et al., 1980). The phosphodiesterase-catalyzed reaction (250 μL) was conducted in an ice–water bath for 3 min. The temperature of the reaction mixture was about 4 °C. Assays were conducted at dilutions of the enzyme that gave 5–24% hydrolysis of 1 μM cyclic GMP. For assays carried out with whole homogenates, enzyme activities are expressed relative to the protein content of the 48000*g* supernatant fractions, which contained nearly all of the cyclic GMP phosphodiesterase activity in these preparations (Wells et al., 1975). Protein content was assayed by the method of Lowry et al. (1951). The bovine serum albumin standards contained 150 μM trifluoperazine, when appropriate.

Statistical Methods. Data were evaluated by using the Student's *t* test for comparison of unpaired data with equal sized groups.

Results

The rates of both inactivation by EGTA and reactivation by Ca^{2+} of cyclic GMP phosphodiesterase activity in whole homogenates of porcine coronary arteries could be reduced substantially by lowering the temperature of the assay system to 4 °C. At 30 °C, the rate of cyclic GMP hydrolysis was reduced to that of EGTA-pretreated homogenates within 1–2 min after addition of EGTA (Figure 1A). Development of inhibition by EGTA was considerably slower at 4 °C, presumably reflecting slower dissociation of the Ca^{2+}–calmodulin–enzyme complex. Indeed, when EGTA was added at 4 °C, the rate of substrate hydrolysis was indistinguishable from that in the absence of EGTA for about 30 s and was only slightly slower between 60 and 120 s (Figure 1B). All data reported herein as "basal" refer to enzyme preparations that were incubated with EGTA for 5 min at 30 °C prior to assay at 4 °C.

The restoration by Ca^{2+} of maximal activity in EGTA-pretreated whole homogenates also was not immediate at 4 °C. When Ca^{2+} in excess of EGTA was added simultaneously with substrate to EGTA-pretreated whole homogenates, 10–30 s elapsed before the rate of cyclic GMP hydrolysis became equal to that of enzyme that was not EGTA pretreated (Figure

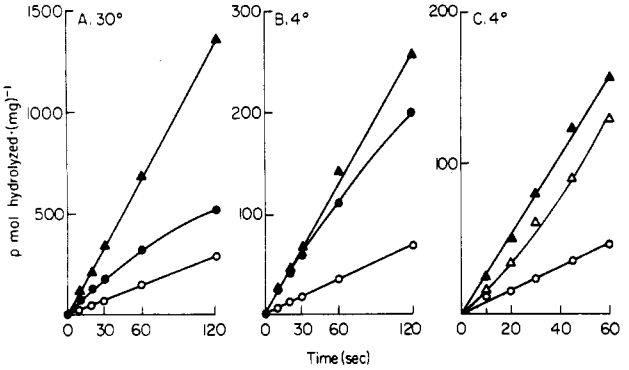

FIGURE 1: Inhibition (A, B) or activation (C) of cGMP phosphodiesterase activity in whole homogenates of porcine coronary artery strips. Tissues were homogenized in 125 (A) or 25 (B, C) volumes (milliliters per gram of tissue) of cold buffer consisting of Tris·HCl (20 mM), $Mg(OAc)_2$ (1 mM), and dithiothreitol (1 mM), and the homogenates were incubated with (○, △) or without (●, ▲) 400 μM EGTA at 30 °C for 5 min. Aliquots (175 μL) of the homogenates were then assayed at 30 °C (A) or cooled and assayed at 4 °C (B, C) for the times indicated. (A, B) The phosphodiesterase assay was initiated by the addition of 75 μL of substrate with (●) or without (○, ▲) EGTA (400 μM in the assay). (C) The assay was initiated by the addition of 75 μL of substrate with (△) or without (▲, ○) $CaCl_2$ (600 μM in the assay). Values are means of duplicate determinations.

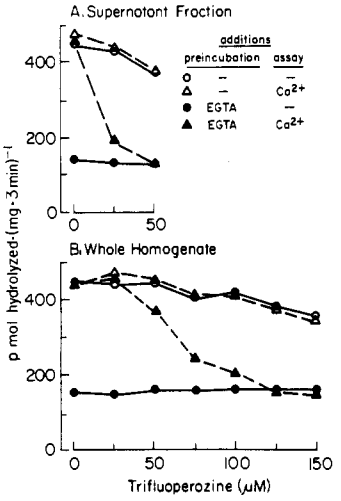

FIGURE 2: Inhibition of Ca^{2+}-induced activation of cGMP phosphodiesterase activities of the supernatant fraction (A) or whole homogenate (B) from porcine coronary arteries. Aliquots (150 μL) of the supernatant fraction (A) and homogenate (B) were incubated at 30 °C for 5 min in the presence (●, ▲) or absence (○, △) of 400 μM EGTA and then cooled at 4 °C. Fifty microliters of a mixture of trifluoperazine (5 times the indicated concentrations) and 3 mM $CaCl_2$ (△, ▲) or H_2O (○, ●) was added, and the mixture was allowed to equilibrate at 4 °C for 8 min before the 3-min (4 °C) phosphodiesterase assays were initiated by the addition of 50 μL of 5 μM [^{3}H]cGMP. Values are the means of duplicate determinations.

1C). This lag time was not detectable at 30 °C (data not shown).

When trifluoperazine was added simultaneously with Ca^{2+} to EGTA-pretreated homogenates or supernatant fractions at 4 °C, the ability of Ca^{2+} to restore activity was inhibited (Figure 2). Trifluoperazine completely inhibited restoration of activity at concentrations of about 50 and 125 μM in the supernatant fraction and whole homogenate, respectively. Basal activity (that detected after preincubation with EGTA) was not significantly altered by trifluoperazine. Maximal activity (that detected in the absence of EGTA or in the presence of EGTA plus excess Ca^{2+}) was inhibited by trifluoperazine, but only by about 20% and 25% by 50 (Figure 2A) and 150 μM (Figure 2B) trifluoperazine in the supernatant fraction and whole homogenate, respectively. We interpret these data as being consistent with the concept that trifluoperazine inhibits phosphodiesterase activity by binding to free calmodulin but not to calmodulin bound to the enzyme. It follows that the slight inhibition (20–25%) of maximally activated phosphodiesterase activity was caused by trifluoperazine binding to calmodulin that slowly (at 4 °C) dissociated from the enzyme during the experiment, and the more extensive inhibition when trifluoperazine was added simultaneously with Ca^{2+} was caused by the drug binding to calmodulin *before* calmodulin bound to the enzyme.

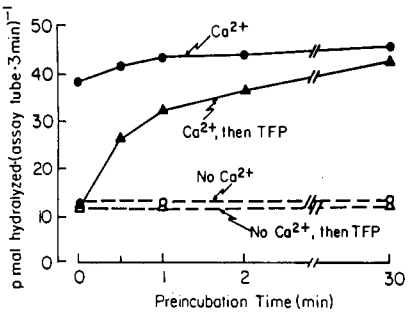

FIGURE 3: Time course of activation by Ca^{2+} of partially purified cGMP phosphodiesterase activity in the presence of calmodulin. Partially purified, calmodulin-deficient phosphodiesterase and 50 μL of the boiled supernatant fraction of a homogenate of porcine coronary arteries were incubated at 30 °C for 5 min with EGTA (214 μM) in a volume of 175 μL and then cooled at 4 °C. Calcium chloride (25 μL of 2.25 mM) (●, ▲) or 25 μL of H_2O was added at zero time, and the tubes were preincubated for the times shown in the figure. Fifty microliters of 5 μM [^{3}H]cGMP (●, ○) or 50 μL of a mixture of trifluoperazine (TFP) (750 μM) and [^{3}H]cGMP (△, ▲) was added simultaneously with Ca^{2+} or H_2O (zero time) or after the indicated preincubation times. The ensuing 3-min assays were conducted at 4 °C. Values are means of duplicate determinations. This phosphodiesterase preparation contained bovine serum albumin for enzyme stability; values, therefore, are presented as picomoles of cyclic GMP hydrolyzed per assay tube.

Higher concentrations of trifluoperazine were required to inhibit stimulation by Ca^{2+} of the phosphodiesterase of the whole homogenate than of the supernatant fraction. The concentration–response curve for trifluoperazine was shifted to the right as the amount of protein or particulate matter present in the assay was increased. Qualitatively similar shifts in the concentration–response curves for trifluoperazine were observed when bovine serum albumin was added to assays of a partially purified preparation of calmodulin-dependent phosphodiesterase in the presence of purified calmodulin (data not shown).

The data presented in Figures 1 and 2 indicate that, at 4 °C, the dissociation of calmodulin from phosphodiesterase is relatively slow, in accordance with the data of Wang et al. (1980). The data in Figure 2 confirm that, in the presence of sufficient trifluoperazine, activation of the enzyme by Ca^{2+}–calmodulin can be prevented. Thus, the addition of trifluoperazine at various times after the addition of Ca^{2+} and calmodulin to a calmodulin-deficient phosphodiesterase preparation should reveal a time course of activation similar to that shown in Figure 1C. Figure 3 shows the results of an experiment in which partially purified, calmodulin-deficient phosphodiesterase (Wells et al., 1975) and a crude preparation of calmodulin were incubated with EGTA at 30 °C for 5 min and then cooled to 4 °C. Ca^{2+} was added at zero time, and trifluoperazine and cyclic GMP were added at various times after the Ca^{2+}. The activity at the zero time point was determined by adding Ca^{2+}, trifluoperazine, and cyclic GMP simultaneously. The rates of cyclic GMP hydrolysis determined in the presence of Ca^{2+} but in the absence of trifluo-

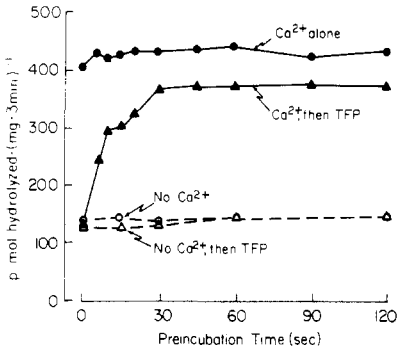

FIGURE 4: Time course of activation of cGMP phosphodiesterase after addition of Ca^{2+} to an EGTA-treated whole homogenate of porcine coronary arteries. Aliquots (150 μL) of the whole homogenate of porcine coronary arteries were mixed with 25 μL of 4 mM EGTA, incubated for 5 min at 30 °C, and then cooled at 4 °C. Twenty-five microliters of 6 mM $CaCl_2$ (●, ▲) or H_2O (○, △) was added at zero time, and the tubes were incubated for the times indicated in the figure. Three-minute assays (4 °C) were initiated by addition of 50 μL of a solution containing 5 μM [^{3}H]cGMP and 625 μM trifluoperazine (TFP) (△, ▲) or H_2O (○, ●) simultaneously with Ca^{2+} or water (zero time) or after the indicated preincubation times. Values are means of duplicate determinations.

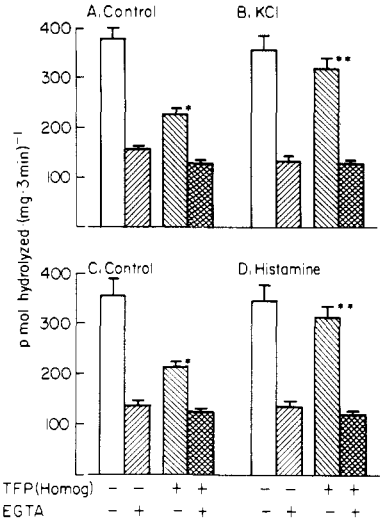

FIGURE 5: Effects of KCl and histamine on phosphodiesterase activities of porcine coronary artery strips. Coronary artery strips were mounted in 27 mL of incubation buffer and conditioned as described under Experimental Procedures. Tissues were quickly frozen 5 min after being changed to fresh buffer (A) or after being changed to an incubation buffer in which 50 mM KCl had been substituted for an equivalent concentration of NaCl (B) or 5 min after addition of 270 μL of 10 mM histamine (D) or H_2O (C). Frozen strips were powdered and homogenized (as described under Experimental Procedures) in the presence or absence, as indicated under the figure, of 150 μM trifluoperazine [TFP(Homog)]. Aliquots of homogenates prepared in the presence of trifluoperazine were assayed immediately. Two minutes after homogenization, trifluoperazine was added to homogenates that were prepared in the absence of this agent, and aliquots were assayed (clear bars). Twenty-five microliters of 4 mM EGTA was added to 175-μL aliquots of each homogenate, and the mixtures were incubated for 5 min at 30 °C before they were cooled and assayed (identified under the figure). Data are means ± standard errors of the mean for eight strips. One asterisk, $p < 0.005$ as compared to control that was homogenized in the absence of trifluoperazine; two asterisks, $p < 0.005$ as compared to control tissues that were homogenized in the presence of trifluoperazine.

perazine indicate that the enzyme activity was not maximal during the entire 3-min assay at 4 °C, as would be expected from the data in Figure 1. The data obtained by adding trifluoperazine at various times after Ca^{2+} indicate that, under the conditions of this experiment, the enzyme was half-maximally activated by about 30 s. The addition of purified calmodulin (143 ng) instead of the boiled supernatant fraction gave results indistinguishable from those presented in Figure 3 (data not shown). The time required to activate the enzyme fully was shortened in the presence of higher concentrations of calmodulin (data not shown), as has been suggested by Wang et al. (1980). Figure 4 shows the results of a similar experiment done with a whole homogenate that had been preincubated with EGTA and presumably, therefore, contained dissociated calmodulin and phosphodiesterase. The time course of restoration of cyclic GMP phosphodiesterase activity by Ca^{2+} at 4 °C was again determined by adding trifluoperazine simultaneously with, and at various times after, the addition of Ca^{2+} to the EGTA-pretreated whole homogenate. Under the conditions of this experiment, the time required to activate the enzyme fully was about 30 s, and the enzyme was half-maximally activated by 6–10 s. The concentrations of calmodulin-sensitive phosphodiesterase and of calmodulin in this experiment were essentially identical with those in the experiment shown in Figure 1B,C, and the times required for maximal activation appear to be virtually the same, even though determined by different methods.

If the calmodulin-sensitive form of phosphodiesterase in intact cells is dissociated from calmodulin when the cytoplasmic levels of Ca^{2+} are low and is associated with calmodulin when Ca^{2+} concentration is high, rapid homogenization of the tissue in the presence and absence of trifluoperazine at cold temperatures followed quickly by assay at a low temperature should yield an estimate of the extent of activation of the enzyme in the tissue just prior to homogenization. To test this possibility, strips of arteries were equilibrated in a tissue bath under 1-g basal tension and then quickly frozen. These tissues should have contained a cytosolic free Ca^{2+} concentration (ca. 0.1 μM) below that required to activate the calmodulin-sensitive phosphodiesterase. The tissues were pulverized while frozen, and the powder was homogenized in the presence or absence of 150 μM trifluoperazine and then immediately assayed with a 3-min incubation at 4 °C. As indicated in Figure 5A,C, the preparations that contained no trifluoperazine during homogenization contained total measurable cyclic GMP phosphodiesterase activity that was significantly higher than did the preparations that had been homogenized with trifluoperazine. The EGTA-sensitive activity of the homogenates prepared in the presence of trifluoperazine was only 41% of that in the homogenates that had been prepared in the absence of trifluoperazine. When the frozen powder was allowed to thaw by adding it to buffer at room temperature and the suspension then cooled in a NaCl–ice–water bath before homogenization, the activities of the homogenates prepared with and without trifluoperazine were indistinguishable (data not shown). Thus, these data indicate that this procedure can be used to detect phosphodiesterase activity that is less than fully activated in the intact cell. Whether the amount of EGTA-sensitive activity measured (40–45% of maximal) was a reflection of the extent of activation of the enzyme in the intact, relaxed tissue preparation or a reflection of the extent of activation that occurred during homogenization, but before trifluoperazine could bind to calmodulin, is unclear.

Figure 5 shows the phosphodiesterase activity in homogenates of tissues that had been freeze-clamped after addition of 0.1 mM histamine (D) or an equal volume of water (C) to the muscle bath. Tissues treated with histamine had generated maximal force by 4 min. The EGTA-sensitive activity of the histamine-contracted tissues was virtually the same when trifluoperazine was present during the homogenization as when the drug was absent (Figure 5D). Comparable results were

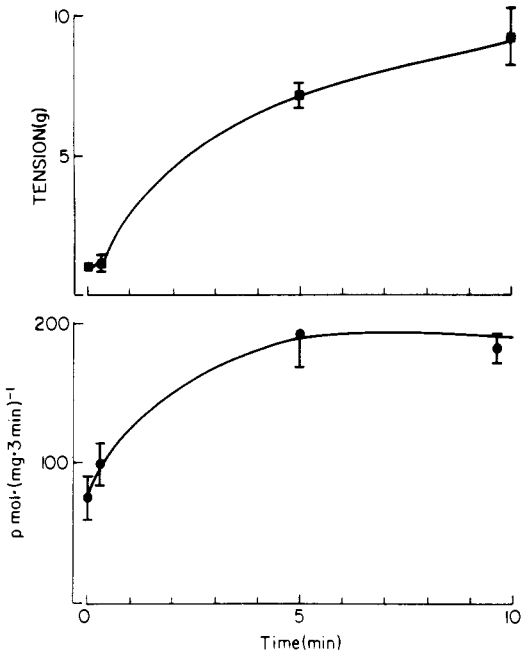

FIGURE 6: Tension development and cGMP phosphodiesterase activity in response to KCl. Temporal changes in isometric force (top panel) and EGTA-sensitive cGMP phosphodiesterase activity (bottom panel). Enzyme activity is expressed as the activity that is sensitive to EGTA inhibition (activity of the enzyme prepared in the presence of trifluoperazine minus the activity of the same preparation in the presence of 400 μM EGTA). Tissues were mounted in a tissue bath under 1-g tension, conditioned, and caused to contract to 50 mM KCl as described in the legend to Figure 5. At the times indicated, the tissues were quickly frozen, homogenized, and assayed, as described in the legend to Figure 5. Values in the upper and lower panels are from the same strips and, in both cases, are means ± standard errors of the mean for six strips. The mean of all phosphodiesterase activities (24 strips) assayed in the presence of EGTA was 103 ± 6 pmol·(mg·3 min)$^{-1}$.

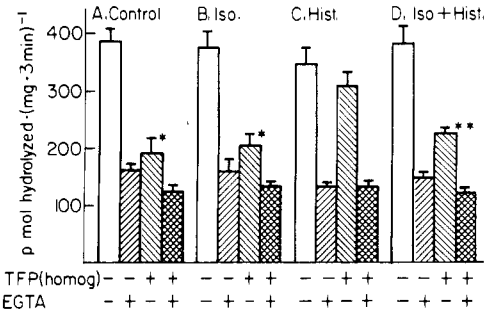

FIGURE 7: Effect of isoproterenol on phosphodiesterase activity of coronary artery strips. The methods used were as described in the legends to Figures 5 and 6. All strips were conditioned and equilibrated under 1-g resting tension as described under Experimental Procedures and quickly frozen after the following treatments: (B) Uncontracted strips were exposed to 10 μM isoproterenol for 3 min. This treatment reduced the resting tension from 1 g to approximately 400 mg. (C) Strips were exposed to 10 μM histamine for 4 min; this concentration gives rise to approximately 50% of the maximal histamine-inducible tension. (D) Strips were exposed to 10 μM histamine for 4 min as in (C), but after 1 min, the tissues were also exposed to 10 μM isoproterenol. When isoproterenol was added, the tissues had attained about 35% of the tension that they eventually attained in the presence of 10 μM histamine alone. Isoproterenol attenuated the increase in tension (to 50% of that attained in the presence of 10 μM histamine alone) and at the time of freezing had caused the tension to decline to 30% of the tension attained in its absence. (A) Control strips were frozen at the same time as those described in (B) through (D). Values are the mean of duplicate determinations with four strips ± standard errors of the mean. One asterisk, $p < 0.005$ as compared to strips that were treated in an identical fashion but were homogenized in the absence of trifluoperazine; two asterisks, $p < 0.01$ as compared to strips that were exposed to histamine alone and were homogenized in the presence of trifluoperazine.

obtained when contraction of the tissue was induced by 50 mM KCl (Figure 5B). These data indicate that, under conditions of increased cytosolic Ca^{2+} concentration, the calmodulin-sensitive phosphodiesterase became maximally active (or nearly so), as would be expected if the activity of this enzyme were regulated by changes in the intracellular Ca^{2+} concentration.

Figure 6 shows the time courses of force generation by the tissues (upper panel) and of the increase in EGTA-sensitive phosphodiesterase activity (lower panel) in response to 50 mM KCl. Phosphodiesterase activity appeared to reach a maximal value before maximal force was attained.

Isoproterenol caused relaxation of control tissues and inhibited the generation of force caused by 10 μM histamine. Addition of 10 μM isoproterenol to tissues under 1 g of basal tension caused the tissues to relax to about 400 mg of tension. This relaxation was not reflected in a reduction of the EGTA-sensitive phosphodiesterase activity below that measured in control tissues (Figure 7; compare parts A and B). The addition of isoproterenol to strips that were in the process of contracting to 10 μM histamine attenuated the contraction and then caused the strips to relax. This effect of isoproterenol on the contractile response to histamine was accompanied by a reduction in the EGTA-sensitive phosphodiesterase activity (Figure 7; compare parts C and D).

Discussion

We interpret our findings to indicate that (1) Ca^{2+}–calmodulin and cyclic GMP phosphodiesterase do indeed associate in intact cells of porcine coronary arteries and (2) the extent of this association is greater in contracted arteries, when cytoplasmic Ca^{2+} concentration should be high, than in relaxed arteries, when cytoplasmic Ca^{2+} concentration should be relatively low. The assumptions that underlie our conclusions are the following: (1) The fraction of the total measured cyclic GMP phosphodiesterase that is inhibited by EGTA is equal to the Ca^{2+}–calmodulin-activated fraction of activity. (2) Trifluoperazine predominantly associates with "free" Ca^{2+}–calmodulin and not with Ca^{2+}–calmodulin that is complexed with phosphodiesterase. (3) The dissociation of the Ca^{2+}–calmodulin–phosphodiesterase complex at 4 °C is slow, and (4) there is no formation of the Ca^{2+}–calmodulin–phosphodiesterase complex when trifluoperazine is present (or, if the complex does form, the phosphodiesterase is not activated as a result).

We infer that the higher EGTA-sensitive activity seen in homogenates of tissues that were made to contract with KCl or histamine resulted from increased cytoplasmic Ca^{2+}-promoting formation of the ternary complex. Conversely, we also infer that the reduction by isoproterenol of histamine-increased EGTA-inhibitable activity resulted from a reduction in cytoplasmic Ca^{2+} concentration [we cannot, of course, exclude a role for reduced myosin light chain kinase activity via cyclic AMP induced phosphorylation (Adelstein & Hathaway, 1979) in isoproterenol-induced relaxation].

Our most significant assumption, that the EGTA sensitivity defines the fraction of total activity that is calmodulin activated, has been supported by the recent finding (J. Miller and J. N. Wells, unpublished results) that values comparable to those we report here are obtained when maximum activity in trifluoperazine-containing homogenates is estimated by adding an excess of purified calmodulin.

The approximately 40% of maximum EGTA-sensitive activity that was observed consistently in relaxed arteries (with or without isoproterenol) could indicate that there is some

association of calmodulin with phosphodiesterase under basal, relaxed conditions. However, our data show that, even at 4 °C, the complex of Ca^{2+}–calmodulin and phosphodiesterase forms extremely rapidly (maximum association can take less than 10 s). Thus, the "basal" EGTA-sensitive activity could reflect formation of the complex during homogenization, before the trifluoperazine binds to calmodulin. Our results do not favor either possibility.

We have used the term *association* here to mean an interaction of Ca^{2+}–calmodulin with phosphodiesterase that results in activation of the enzyme and the term *dissociation* to mean reversal of activation. We do not mean to imply that an association of the components cannot occur without activation (Newton et al., 1983). We believe our results demonstrate a functional interaction between cyclic GMP phosphodiesterase and Ca^{2+}–calmodulin that is regulated by changes in cytoplasmic Ca^{2+} concentration.

Applications of the approach we have used to other cells or tissues will require that the enzyme activity measured be predominantly a form that is Ca^{2+}–calmodulin sensitive, that concentrations of homogenate protein and trifluoperazine (or a similarly acting drug) be adjusted carefully, and that dissociation of the activated complex be slow enough to permit estimation of its activity. An attempt to apply this approach to an analogous study with myosin light chain kinase in the same kind of blood vessels has not succeeded, because dissociation of the Ca^{2+}–calmodulin–kinase complex is too fast (J. Miller and J. N. Wells, unpublished results).

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Registry No. cGMP phosphodiesterase, 9068-52-4; Ca, 7440-70-2.

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