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# Lack of Correlation between Activation of Cyclic AMP-Dependent Protein Kinase and Inhibition of Contraction of Rat Vas Deferens by Cyclic AMP Analogs

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**SUMMARY**

The effects of $N^6$,$O^2$-dibutyryl-adenosine-3′,5′-cyclic monophosphate (db-cAMP) and 8-bromo-adenosine-3′,5′-cyclic monophosphate (8-Br-cAMP) on tension and cAMP-dependent protein kinase (PKA) activities in rat vas deferens were investigated. A soluble enzyme fraction obtained from the vas deferens was found to contain both type I and type II isozymes of PKA, whereas a particulate fraction contained only the type II isozyme. Exposure of the vas deferens to db-cAMP (1–100 μM) for 30 min caused a concentration-dependent inhibition of phenylephrine-induced contractions, with an $EC_{50}$ of less than 10 μM. 8-Br-cAMP had no significant effect on contractions over a similar concentration range. Both of the analogs were able to activate PKA significantly at a concentration of 10 μM, and the magnitude of the PKA activation was greater with 8-Br-cAMP than with db-cAMP. Charcoal was added to the homogenization buffer in these experiments to prevent the artifactual activation of PKA by cAMP analogs trapped in the extracellular space. The ability of db-cAMP, but not 8-Br-cAMP, to inhibit the contraction of vas deferens could not be explained on the basis of differential activation of soluble or particulate PKA or of specific isozymes of the enzyme. It is, therefore, concluded that activation of PKA is not responsible for the relaxant effects of cAMP analogs in some smooth muscle.

cAMP has been generally recognized as a second messenger that mediates the effects of many hormones and neurotransmitters. Until very recently, all of the known actions of cAMP in mammalian cells were thought to be produced by activation of PKA (1–3). In smooth muscle cells, there is a substantial body of evidence indicating that cAMP is the mediator of the smooth muscle-relaxing effects of a variety of drugs and hormones (reviewed in Refs. 4–7). However, the precise mechanism by which cAMP elevation might cause relaxation in smooth muscles has not been elucidated. A number of possible mechanisms have been suggested, including lowering of cytoplasmic calcium by intracellular sequestration (8), stimulation of $Na^+$,$K^+$-ATPase activity, resulting in acceleration of $Na^+$-$Ca^{2+}$ exchange and increased calcium efflux from the cytoplasm (9), and interference with contraction at the level of the contractile proteins (10). All of these mechanisms are assumed to operate via the activation of PKA, which presumably catalyzes the phosphorylation of some key enzymes or cellular proteins involved in the control of smooth muscle contraction. However, very few studies have attempted to examine the role of PKA in smooth muscle relaxation directly. Some of the most convincing evidence for such a role was provided by Glenn *et al.* (11), who showed that the catalytic subunit of PKA was able to inhibit $Ca^{2+}$-induced contraction of skinned smooth muscle. In one of the few studies in which the kinase activity was measured in intact muscles, Silver *et al.* (12) reported a good correlation between activation of PKA by isoproterenol and relaxation of vascular smooth muscle.

One of the criteria usually used in assigning a role for cAMP as a mediator of a particular response is the ability of cAMP and its analogs, such as db-cAMP and 8-Br-cAMP, to mimic the response. These analogs are believed to penetrate cell membranes more effectively than cAMP itself and are more resistant to phosphodiesterases (4, 13, 14). In one type of smooth muscle, the rat vas deferens, db-cAMP was shown to inhibit norepinephrine-induced contractions, whereas 8-Br-cAMP was without any effect (15). If the activation of PKA is indeed the mechanism for cAMP-dependent smooth muscle relaxation, as is generally accepted, then db-cAMP, but not 8-Br-cAMP, should be capable of activating PKA in the rat vas deferens. The objective of the present study was to test this hypothesis. Our results do not support this hypothesis and

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**ABBREVIATIONS:** PKA, cAMP-dependent protein kinase; db-cAMP, dibutyryl-cAMP; 8-Br-cAMP, 8-bromo-cAMP; DTT, (*dl*)-1,4-dithiothreitol; IBMX, 3-isobutyl-1-methylxanthine; PE, *l*-phenylephrine; mb-cAMP, $N^6$-monobutyryl-cAMP; cGMP-dependent protein kinase.

instead suggest that PKA is not the mediator of smooth muscle relaxation by cAMP analogs in this tissue. A preliminary report of this study was presented at the annual meeting of the Canadian Federation of Biological Societies in Calgary, Alberta, in June 1989 (16).

## Materials and Methods

**Preparation of rat vas deferens.** Rat vas deferens were prepared as described by Schultz *et al.* (15). Segments (3–4 cm) of vas deferens were suspended in 10-ml tissue baths containing Krebs-Henseleit solution, which was maintained at 37° and bubbled with 5% $CO_2$ in $O_2$. The tissues were equilibrated for 90 min at a resting tension of 2 g. The changes in tension were recorded isometrically using a force-displacement transducer (Grass model FT.03) coupled to a polygraph (Grass model 7C). Vas deferens preparations were contracted by 1–3 μM PE, which was washed out when the peak tension was reached. This was repeated twice to establish the control contractile response. The tissues were then treated with various concentrations of db-cAMP or 8-Br-cAMP for 30 min, after which the tissues were again contracted with PE three times. Analogs were readded to the tissue bath after each wash. Some tissues were freeze-clamped at this point for PKA assay.

**Assay of PKA.** PKA activity was determined using a modification of the method described by Corbin and Reimann (17), as modified by Giembycz and Diamond (18, 19). Briefly, the frozen tissue was homogenized with a Polytron (type T 10 29 350D) at setting 8 for 15 sec, in 20 volumes of ice-cold 10 mM phosphate buffer (pH 6.8) containing 10 mM EDTA, 10 mM DTT, 500 μM IBMX, and 150 mM NaCl. Activated neutralized charcoal (10 mg/ml) was also present in the homogenization buffer, as suggested by Corbin *et al.* (20), to prevent the artifactual activation of PKA by cAMP trapped in the extracellular space. The homogenate was centrifuged in a Sorvall RC2-B centrifuge, using a SM-24 rotor, at 31,000 × *g* for 15 min at 4°. The supernatant was used to assay soluble PKA activity. The pellet was resuspended in the same homogenization buffer containing 0.2% Triton X-100. The pellet was stirred gently for 60 min at 4° to extract particulate PKA, and the resulting solution was centrifuged again at 31,000 × *g* for 15 min. The supernatant from this step was used to estimate particulate PKA activity.

The assay was initiated by addition of 25 μl of soluble or particulate PKA solution to 65 μl of reaction medium consisting of 20 mM phosphate buffer (pH 6.8), 10 mM magnesium acetate, 500 μM IBMX, 71 μM kemptide, and 100 μM ATP, containing 100 cpm/pmol [γ-$^{32}$P]ATP, in the presence or absence of 10 μM cAMP. The reaction was allowed to proceed for 8 min at 30° and was stopped by the spotting of 70 μl of the reaction medium onto phosphocellulose paper squares (2 × 2 cm; Whatman P81), which were immersed in phosphoric acid (1%) and then extensively washed four times for 5 min each time. The paper squares were dehydrated by immersion in 95% ethanol for 5 min and in diethyl ether for another 5 min and then were allowed to dry. The bound radioactivity was determined by placement of the papers in scintillation vials containing 5 ml of ACS (Amersham Corp) and counting in a liquid scintillation counter (Packard Tricarb 460 CD). The PKA activity was expressed as pmol of phosphate transferred into kemptide/min/mg of protein. The extent of PKA activation was assessed by determination of PKA activity in the absence and presence of added cAMP (10 μM) and was expressed as an activity ratio.

The ability of charcoal to prevent the artifactual activation of PKA by cAMP analogs that were trapped in the extracellular space was examined by addition of the analogs directly to tubes containing frozen control muscles and then homogenization in the presence or absence of charcoal. Kinase activity was then determined as described above. The time course of the activation of PKA by 8-Br-cAMP was also investigated by freeze-clamping of vas deferens at various time points after the tissues were incubated with the analog. The tissues were then homogenized in the absence or presence of charcoal for the PKA assay.

**Determination of PKA isozyme profile in rat vas deferens.** The PKA isozymes were separated by DEAE-cellulose chromatography, as previously described (19). About 200 mg of rat vas deferens were homogenized in 10 volumes of 5 mM phosphate buffer (pH 6.8) containing 1 mM EDTA, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml soybean trypsin inhibitor, and 20 μg/ml benzamidine. The homogenate was centrifuged at 31,000 × *g* to form soluble and particulate fractions, as described above. The fractions were loaded onto a 1.5- × 8-cm DEAE column (DE-52; Whatman) that was pre-equilibrated with the homogenization buffer. After the column was washed with 100 ml of the same buffer, it was eluted with a linear NaCl gradient running from 0 to 400 mM, in 45 fractions, with a total volume of 90 ml. Each of the 45 fractions was then assayed for PKA activity to obtain the isozyme distribution profiles for the rat vas deferens. The protein concentrations were determined according to the Lowry procedure, using bovine serum albumin as a standard.

**Drugs and reagents.** PE, EDTA, DTT, activated neutralized charcoal, IBMX, phenylmethylsulfonyl fluoride, Triton X-100, and synthetic peptide protein kinase inhibitor (rabbit sequence) were obtained from Sigma. [γ-$^{32}$P]ATP was obtained from Amersham. All other reagents were obtained from BDH.

**Statistical analysis.** One-way analysis of variance, followed by Newman-Keul's multiple comparison test, was used to compare control and various drug-treated groups. Significant difference was accepted when $p$ was $<0.05$. All values are presented as mean ± standard error.

## Results

**Partial characterization of PKA isozyme profile in rat vas deferens.** Two peaks of PKA were resolved by DEAE chromatography of the soluble fraction of tissue homogenates, corresponding to type I and type II isozymes of PKA, with approximately 74% of total kinase activity being represented by the type II isozyme (Fig. 1 A). However, only one peak was resolved in the particulate fraction at high ionic strength (Fig. 1B), indicating that particulate PKA in vas deferens is almost exclusively the type II isozyme, as expected for membrane-associated kinase (2). The free catalytic subunits of PKA were eluted with washing buffer, the initial ionic strength of which was 2 mmhos/cm. Soluble PKA represented 70% of total PKA activity in these preparations.

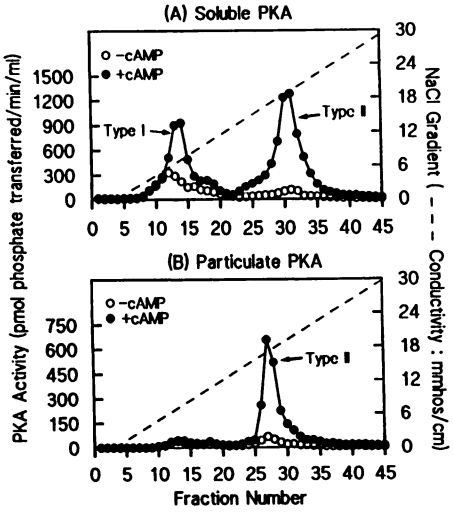


**Fig. 1.** Isozyme profile of PKA in rat vas deferens. Tissues were homogenized and the soluble and particulate fractions were prepared as described in Materials and Methods. The fractions were loaded onto DEAE ion exchange columns and eluted with a linear NaCl gradient running from 0 to 400 mM. Each fraction was then assayed for PKA activity in the absence and presence of 10 μM cAMP.

In addition, a synthetic protein kinase inhibitor specific for PKA (21) was shown to inhibit 97% of the kinase activity, as determined in our assays (Fig. 2).

**Prevention of spurious activation of PKA by cAMP analogs in vas deferens by the use of charcoal in the homogenization buffer.** When the vas deferens segments were treated with cAMP analogs for 30 min at 37°, certain amounts of the analogs were trapped in the extracellular space. These analogs were then released during homogenization of the tissues, leading to the *in vitro* activation of PKA. Charcoal was, therefore, added to the homogenization buffer to prevent this spurious activation of PKA, by removal of the analogs released during homogenization. The effectiveness of charcoal in this regard was examined by addition of the analogs (10 μM solutions in a volume equivalent to 70% of the tissue weight) directly to previously untreated frozen tissues, which were then homogenized in the absence or presence of charcoal (Table 1). PKA was found to be significantly activated by the exogenously added cAMP analogs when the homogenization was performed in the absence of charcoal, but with the inclusion of 10 mg/ml charcoal in the homogenization buffer this activation of PKA was completely prevented. The total activity of PKA (with cAMP) was not significantly changed by the addition of charcoal, although the basal activity ratio was lower in its presence, suggesting the possibility that some endogenous cAMP might have been sequestered in the control tissues and released during the process of homogenization. It is also possible that charcoal may have reduced the activation ratio of PKA, as discussed below. In either case, the addition of charcoal was able to prevent the spurious activation of PKA by released cAMP or added cAMP analogs.

Concerned that addition of cAMP analogs to frozen tissues may not have exactly simulated the actual experimental conditions, we designed an experiment to more closely mimic the conditions under which tissues were treated with cAMP analogs. Vas deferens segments were incubated with 10 μM 8-Br-cAMP at 37° for various periods and freeze-clamped for PKA assay. The homogenization was then performed in the presence or absence of charcoal. As shown in Fig. 3, when charcoal was absent from the homogenization buffer, PKA was shown to be significantly activated after a 1-min exposure to 8-Br-cAMP. In contrast, when charcoal was present, there was no significant activation of PKA until the tissues had been exposed to 8-Br-cAMP for 30 min, suggesting that activation of PKA by 8-Br-cAMP was an artifact at 1 min of exposure, presumably due to the release of 8-Br-cAMP trapped in the extracellular space. Because charcoal could prevent this artifactual activation of PKA, it was included in the homogenization buffers for tissues that were incubated with cAMP analogs.

**Effects of cAMP analogs on the tension and PKA activities in rat vas deferens.** Incubation of rat vas deferens with 10 μM db-cAMP for 30 min significantly inhibited PE-induced contractions of rat vas deferens (>87%), whereas incubation with the same concentration of 8-Br-cAMP did not have any effect on the contractions (Fig. 4A). However, both analogs activated soluble PKA significantly, with 8-Br-cAMP having a greater effect (Fig. 4B). Furthermore, 8-Br-cAMP also significantly activated particulate PKA. The activation of particulate PKA by db-cAMP was not statistically significant.

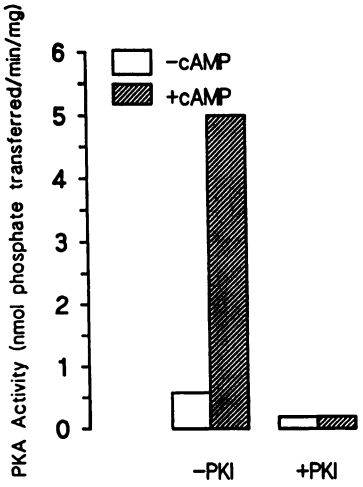


**Fig. 2.** Effects of protein kinase inhibitor (*PKI*) on PKA activity in rat vas deferens. PKA activity was determined in the absence and presence of the synthetic peptide protein kinase inhibitor (2.5 μg/ml). *Bars*, values from the average of two separate experiments.

TABLE 1

**Prevention by charcoal of PKA activation by cAMP analogs in rat vas deferens *in vitro***

These experiments were performed on frozen tissues that were not previously treated with cAMP analogs. Tissues were exposed to the analogs (10 μM in a volume equivalent to 70% of the wet weight of the tissues) and were then homogenized immediately, in the absence or presence of charcoal (10 mg/ml). The values represent the mean ± standard error for the number of preparations shown (*n*).

| Addition | PKA activity | | −cAMP/+cAMP ratio | *n* |
|---|---|---|---|---|
| | −cAMP | +cAMP | | |
| | *pmol $^{32}$P transferred/min/mg of protein* | | | |
| −Charcoal | | | | |
| None | 275 ± 36 | 1092 ± 63 | 0.26 ± 0.04 | 6 |
| db-cAMP | 432 ± 42 | 1292 ± 50 | 0.33 ± 0.03 | 3 |
| 8-Br-cAMP | 1155 ± 335 | 1472 ± 269 | 0.76 ± 0.10 | 3 |
| +Charcoal | | | | |
| None | 109 ± 21 | 1312 ± 129 | 0.08 ± 0.01 | 7 |
| db-cAMP | 117 ± 18 | 1303 ± 148 | 0.09 ± 0.2 | 4 |
| 8-Br-cAMP | 158 ± 36 | 1414 ± 272 | 0.11 ± 0.02 | 4 |

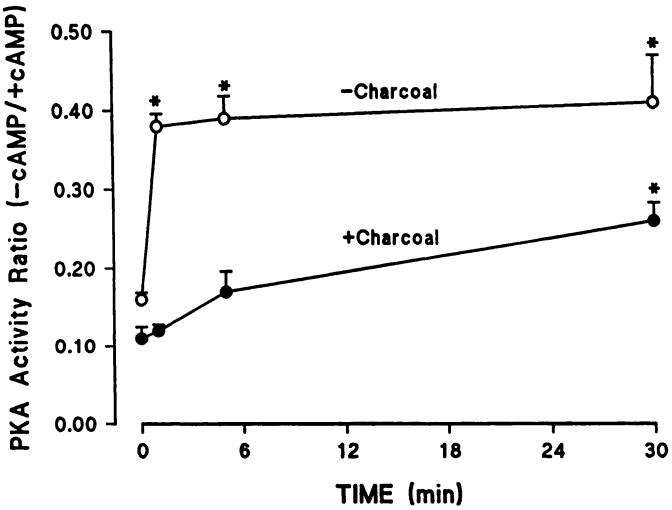


**Fig. 3.** Time course of PKA activation by 8-Br-cAMP in rat vas deferens. The tissues were incubated with 10 μM 8-Br-cAMP at 37° for various time points, between 1 and 30 min, and freeze-clamped. Tissues were homogenized in the absence (○) or the presence (●) of charcoal (10 mg/ml) and assayed for PKA activity. *Points*, mean ± standard error of six or seven experiments. *, Significantly different from the corresponding zero time controls ($p < 0.05$).

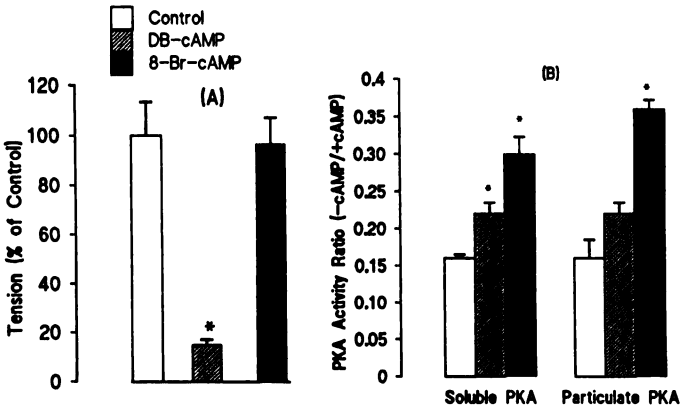


**Fig. 4.** A, Effects of cAMP analogs on PE-induced contraction in rat vas deferens. Vas deferens were prepared as described in Materials and Methods. The tissues were treated with PE (1 $\mu$M) three times, and the responses were taken as control values. Some of the tissues were then incubated with cAMP analogs (10 $\mu$M each) for 30 min, at which time they were reexposed to PE three times and the responses were recorded. B, Effects of cAMP analogs on PKA activity in rat vas deferens. The tissues were treated as in A and freeze-clamped after the second set of responses to PE had been obtained (i.e., in the presence and absence of cAMP analogs). PKA activities were then determined, and the activity ratios are indicated by the *bars*. Values in both figures represent mean ± standard error of five to seven experiments. *, Significantly different from control values ($p < 0.05$).

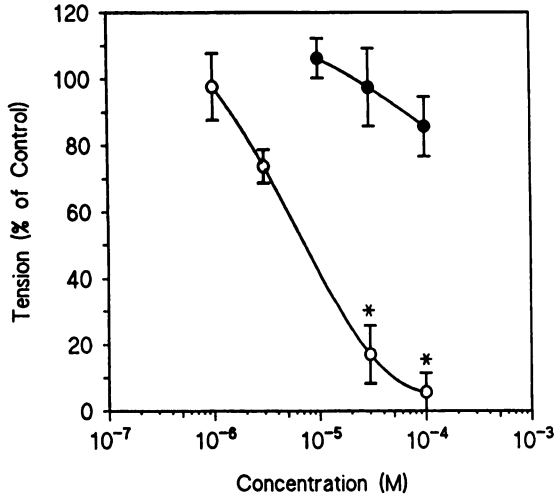


**Fig. 5.** Dose-response curves for inhibition of PE-induced contraction of rat vas deferens by db-cAMP (○) and 8-Br-cAMP (●). The tissues were treated as described in Fig. 4A, using a PE concentration of 3 $\mu$M. *Points*, mean ± standard error of three experiments. *, Significantly different from control values ($p < 0.05$).

Even at higher concentrations, 8-Br-cAMP still failed to produce any appreciable effects on the PE-induced contractions of vas deferens, whereas higher concentrations of db-cAMP essentially eliminated the contraction (Fig. 5). The activation of PKA was not determined for concentrations of the analogs above 10 $\mu$M, because preliminary experiments indicated that the highest concentration of charcoal that did not alter the total activity of PKA was unable to prevent the artifactual activation of PKA by the extracellularly bound cAMP analogs at these higher concentrations.

Because a previous study (14) reported that db-cAMP was deacylated by tissues into mb-cAMP, which is the active form in the activation of PKA, the effects of mb-cAMP on the tension and PKA activity were also observed. PKA was significantly activated after exposure to 10 $\mu$M mb-cAMP for 30 min. The activity ratio was increased from a control value of 0.06 ± 0.01 ($n = 5$) to 0.12 ± 0.01 ($n = 7$) ($p < 0.05$). The contractile responses in these preparations were diminished by approximately 25%. db-cAMP (10 $\mu$M) increased the PKA activity ratio by about the same amount in two preparations from this same group of vas deferens (from 0.06 to 0.12 and 0.16) and inhibited contractions by more than 80%.

In a separate set of experiments, using DEAE column-purified PKA, 8-Br-cAMP and mb-cAMP were found to be capable of fully activating both type I and type II PKA *in vitro*, whereas db-cAMP *per se* did not activate type II PKA and was a very weak activator of type I PKA (data not shown).

## Discussion

Two major isozymes of PKA (type I and type II) are present in various tissues and can be separated by DEAE anion exchange chromatography (2, 22). Because the optimal assay conditions for the type I and type II isozymes are different (23), it was important to determine the isozyme profile of PKA in the rat vas deferens, in order to determine the conditions under which the enzyme activity should be measured. Because we found that rat vas deferens contains both types of isozymes, it was decided that 150 mM NaCl should be used in the homogenization buffer, to prevent the reassociation of the type II isozyme as well as to limit the dissociation of the type I isozyme (1, 23).

The PKA activity ratio in crude tissue extracts has been considered to be a valid indication of the activation state of the enzyme in the tissue (2). A very important requirement for such activity ratio determinations is prevention, or minimization, of changes in the protein kinase activation state following homogenization. One of the difficulties in the determination of PKA activation by cAMP analogs in intact tissues is that the cAMP analogs may be nonspecifically bound within the tissues or may be trapped in the extracellular space of the tissues. The amount of analog released during the process of homogenization may be sufficient to cause artifactual activation of PKA, which may not have occurred in the intact tissues. In order to prevent this spurious activation of PKA, charcoal was added to the homogenization buffer in our experiments, to take advantage of its known ability to bind free nucleotides such as cAMP, ADP, and ATP (20). We estimated that the amount of analog nonspecifically bound to the surface of the tissues or trapped in the extracellular space following freeze-clamping of preparations exposed to cAMP analogs for 30 min would not exceed 70% of the wet weight of the tissues in terms of volume. Therefore, a volume of a 10 $\mu$M solution of cAMP analog equivalent to 70% of the tissue weight was added to frozen control tissues, to demonstrate the ability of charcoal to prevent the activation of PKA *in vitro*. As shown in Table 1, 10 mg/ml charcoal was able to prevent activation of PKA by the addition of exogenous cAMP analogs to the homogenization buffer. It may still be argued that addition of cAMP analogs to the homogenization buffer may not exactly simulate the conditions of the experiment in which tissues were exposed to cAMP analogs for 30 min at 37°. The latter procedure would have allowed enough time for cAMP analogs to distribute into the extracellular space, whereas simple addition of analogs to the frozen tissues immediately before homogenization would not allow time for the analogs to distribute into the extracellular

space before homogenization. A time course for PKA activation in tissues exposed to 8-Br-cAMP at 37° was, therefore, determined (Fig. 3). The distribution of cAMP analogs into the extracellular space is presumably a rapid process at 37°. However, even after a 5-min exposure to 8-Br-cAMP, a time at which the analog was presumably well distributed into the extracellular space, charcoal was still able to prevent the activation of PKA by the analog trapped in the extracellular space. The presence of charcoal in the homogenization buffer did not prevent the activation seen after a 30-min exposure to 8-Br-cAMP, a time period sufficient to allow a significant amount of the analog to enter the cells. Thus, the use of charcoal enables us to differentiate the activation of PKA by cAMP analogs that are in the extracellular space and bound nonspecifically within the tissues from that caused by cAMP analogs that have permeated into the cells.

It is worth noting that the concentration of charcoal used in our experiments appears to lower the basal activity ratio of PKA. It is, therefore, possible that charcoal may have reversed the activation of PKA by cAMP or cAMP analogs in intact tissue, i.e., charcoal may be able to remove cAMP that is bound to the regulatory subunit of PKA, leading to the reassociation of the catalytic and regulatory subunits and, thus, underestimation of the PKA activation. Preliminary experiments using purified PKA suggest that this may be the case. Addition of charcoal to purified PKA that had been submaximally activated by prior exposure to 30 nM 8-Br-cAMP reduced the apparent activity ratio by approximately 2-fold (data not shown). Nevertheless, we found that PKA activity ratios were significantly increased, even with charcoal in the homogenization buffer, when tissues were treated with the cAMP analogs for 30 min. This indicates that PKA was significantly activated by the analogs under our experimental conditions.

Considering the number of protein kinases that are present in smooth muscles, the PKA activity measured in our assay might have been partly due to contamination by other kinases. This possibility seems unlikely, in view of our observation that the PKA-specific inhibitor was able to inhibit about 97% of the kinase activity measured in this tissue. In addition, it has been shown that kemptide, the kinase substrate used in this study, is remarkably specific for PKA (2, 18).

In agreement with a previous report (15), we found that db-cAMP decreased the magnitude of the PE-induced contractions in rat vas deferens by more than 87%, whereas 8-Br-cAMP had no significant effect on the contractions. Both analogs however, were capable of significantly activating soluble PKA in the vas deferens, and the magnitude of the kinase activation was actually greater with 8-Br-cAMP, even though it did not inhibit contractions in these preparations. Moreover, the activation of particulate PKA was significant only with 8-Br-cAMP. Previous studies have shown that 8-Br-cAMP is a better activator of PKA in broken cell preparations than cAMP itself and that db-cAMP does not bind and is, thus, unable to activate PKA (14, 24). The smaller activation of PKA by db-cAMP may indeed be produced by mb-cAMP, which is formed by intracellular degradation of db-cAMP and which is probably the biologically active form (14). Application of mb-cAMP in our experiment did inhibit the PE-induced contraction of vas deferens to a certain extent but, surprisingly, the effect was much less than with db-cAMP. It is possible that the monobutyryl derivative does not penetrate cell membranes as readily as the dibutyryl form. It is also possible that db-cAMP inhibits the PE-induced contractions in vas deferens by the release of the butyrate ion of db-cAMP (13). However, preliminary experiments in our laboratory indicate that 10 $\mu$M sodium butyrate does not inhibit the PE-induced contractions, suggesting that this is not an important factor (data not shown).

The vas deferens preparations used in this study contain other cell types, in addition to smooth muscle cells, and it is possible that non-muscle cells contribute to the kinase activity measured in our experiments. However, smooth muscle cells constitute the major cell type in the vas deferens and would, therefore, be the major contributor to the kinase activation observed in preparations exposed to the cAMP analogs for 30 min. Additionally, we have no evidence in our preparations for differential activation of specific subcellular compartments or pools of PKA, although such compartmentalization has been reported in other tissues (25, 26). Therefore, it seems unlikely that db-cAMP and 8-Br-cAMP are activating different pools of PKA, leading to differential effects on the tension in rat vas deferens. Because db-cAMP was unable to activate particulate PKA and was a weaker activator of soluble PKA than was 8-Br-cAMP, it does not appear that activation of PKA is responsible for the ability of db-cAMP to inhibit the contraction of rat vas deferens.

It should be noted that considerable evidence exists in the literature that is not consistent with the general hypothesis that cAMP is a mediator of smooth muscle relaxation (27, 28). For example, prostaglandin $E_1$ was able to increase cAMP and activate PKA in rabbit aorta, but this was accompanied by contraction rather than relaxation (29). Prostaglandin $E_1$-contracted muscles could then be relaxed by isoproterenol with no further elevation of cAMP (30). When intracellular $Ca^{2+}$ concentrations were examined, the effects of cAMP were variable; it either decreased or increased intracellular $Ca^{2+}$ concentrations in resting muscle (31–33). Furthermore, even in bovine coronary artery, where a good correlation between PKA activation and relaxation has been reported (12), direct application of cAMP analogs was not effective in relaxing this muscle (34). These observations, and our own results as described above, are difficult to reconcile with the hypothesis that activation of PKA is responsible for the smooth muscle-relaxing effects of cAMP-elevating agents.

If PKA does not mediate the inhibition by db-cAMP of contraction in vas deferens, the question remains as to how cAMP or cAMP-elevating agents exert their relaxant effects. Recent studies have shown that cAMP can exert biological effects directly. There have been reports that cAMP directly depolarized neurons (35), caused short and long term enhancement of synaptic transmission (36), and reduced $K^+$ current of neurons (37). However, direct effects of cAMP on muscle contraction have not yet been demonstrated in smooth muscles. Interestingly, several recent studies have presented data showing that agents that increase cellular cAMP levels may be able to activate PKG (33, 38). It was suggested that forskolin and isoproterenol reduce angiotensin II-induced increases in intracellular $Ca^{2+}$ in cultured vascular smooth muscle cells via the activation of PKG, rather than PKA (38). Studies using a large number of cyclic nucleotide analogs also demonstrated that the potencies of these analogs as smooth muscle relaxants were better correlated with their abilities to activate PKG than with their abilities to activate PKA (39). In addition, Felbel *et al.*

(32) showed that PKG was very effective in decreasing elevated intracellular $Ca^{2+}$ levels, whereas the effect of PKA was variable. These studies suggest that the relaxant effect of cAMP in some smooth muscles may indeed be the result of PKG activation. It is, therefore, very tempting to propose that the inhibitory effect of db-cAMP on the contraction of rat vas deferens results from the activation of PKG instead of PKA and that the failure of 8-Br-cAMP to inhibit vas deferens contraction is due to its inability to activate PKG. Although this is supported by an earlier report that 8-Br-cAMP was ineffective in activating PKG (24), other recent studies have reported that 8-Br-cAMP is a much better activator of purified PKG than is db-cAMP or mb-cAMP (39, 40). Thus, our data do not seem to be consistent with a role for either of the cyclic nucleotide-dependent protein kinases in mediating the inhibitory effects of db-cAMP on the contraction of rat vas deferens. The mechanism by which db-cAMP relaxes these preparations is unclear. It may act via some other cAMP-dependent mechanism that is not dependent on activation of the kinase. However, it may also be possible, as noted above, that cAMP *per se* is not responsible for the relaxation of smooth muscle caused by cAMP analogs or even by other cAMP-elevating agents. Further research along these lines is, therefore, required before a definite conclusion can be made.

In summary, there appears to be a dissociation between PKA activation and the inhibition of rat vas deferens contraction by cAMP analogs under our experimental conditions, suggesting that activation of PKA may not mediate relaxation in all smooth muscle. In view of this and previous results in the literature, additional mechanisms must be sought in order to account for the relaxing effects of some cAMP-elevating agents in smooth muscles.

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