Dissociation of Magnitude of Relaxation from cyclic AMP Levels in Rabbit Urinary Bladder Smooth Muscles

Takashi MORITA, Masao ANDO, Kazunori KIHARA, Hiroyuki OSHIMA, Shun KONDO* and Yotaroh TASHIMA*

Department of Urology, Tokyo Medical and Dental University School of Medicine, Tokyo 113, Japan
*Second Department of Biochemistry, Akita University School of Medicine, Akita 010, Japan

Abstract
The effects of forskolin and isoproterenol on contractile force and cyclic AMP levels were compared in smooth muscle strips from rabbit urinary bladder dome. Both of forskolin and isoproterenol produced the time- and the dose-dependent relaxation and the time- and the dose-dependent increases in cAMP levels. The relaxant response to forskolin caused more slowly than that to isoproterenol. The two agents caused almost the same relaxant responses at same concentration. However, cAMP levels induced by forskolin was much more than those induced by isoproterenol. These results that amounts of cAMP in urinary bladder don't correlate with the relaxation responses in urinary bladder smooth muscle strips suggest that some forms of functional compartmentalization of cAMP may exist in the urinary bladder.

Key words: Forskolin, Isoproterenol, cAMP levels, Magnitude of relaxation, Urinary bladder smooth muscle

Introduction
Forskolin, a diterpene derivative isolated from the indian plant coleus forskohlii, is a potent stimulant of adenylate cyclase and has been shown to increase adenosine 2':3'-cyclic monophosphate (cAMP) levels in a variety of tissues (Seamon et al., 1981). Forskolin also has been reported to produce relaxation in a variety of smooth muscle preparations (Muller and Baer, 1983; Dubey et al., 1981). On the other hand, it is also well known that beta-adrenoceptor agonists usually cause smooth muscle relaxation associated with an increase of cAMP levels (Bür, 1974). However, some studies (Scheid et al., 1979; Kroeger, 1979) suggest a good correlation between tissue cAMP levels and relaxant responses induced by beta-adrenoceptor stimulation, other studies (Honda et al., 1977; Meisher et al., 1978) do not suggest such correlations. There have been reported the cAMP elevation in urinary bladder which is responsible for the relaxation to forskolin (Morita et al., 1986) or beta-adrenoceptor agonists (Rohner and Hannigan, 1680; Morita et al., 1990). The purpose of the present study, therefore, is to compare the changes in cAMP levels with the magnitude of relaxation of urinary bladder...
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smooth muscle induced by forskolin or isoproterenol.

Materials and Methods

Measurement of contractile force: Twenty two male Japanese white rabbits weighing 2-2.5 kg were stunned and bled. Muscle strips, 2 mm wide and 10 mm long, were dissected from the urinary bladder dome and were mounted in a 3.0 ml bath containing modified Krebs solution of the following composition (mM): NaCl 133.6, KCl 4.7, CaCl₂ 2H₂O 1.9, MgCl₂ 6H₂O 1.2, glucose 8.3 and were gassed with 95% O₂ and 5% CO₂; pH 7.4 at 37°C. One end of the strips was attached with a 4-0 silk thread to a fixed hook in the bottom of the bath and the other end was similarly attached to a Statham UC-2 force transducer. A baseline force of 0.3 g was applied and maintained during the experiment.

Drugs used were forskolin 10⁻⁸-10⁻⁴ M (Carbiochem-Behring), dl-isoproterenol hydrochloride 10⁻⁸-10⁻⁴ M, N⁶, O²'-dibutyryl adenosine 3':5'-cyclic monophosphate (db-cAMP) 10⁻⁷-10⁻³ M (Sigma) and phentolamine 10⁻⁶ M (Ciba-Geigy). Forskolin, 10⁻² M, was dissolved in 95% ethanol, then diluted with distilled water. Other drugs were dissolved in distilled water. Varying concentrations of forskolin or isoproterenol were cumulatively added directly to the muscle bath. Effects of drugs were determined by calculating the change in peak contractile force (the active contractile force + the baseline force).

Measurement of cyclic AMP content: Muscle strips (60-100 mg) from the rabbit urinary bladder dome were equilibrated in modified Krebs solution for 40 min. The tissues were equilibrated in the presence of phentolamine, 10⁻⁶ M and 4-(3, 4-dibutoxybenzyl)-2-imidazolidinone (Ro 20-1274), 10⁻⁶ M for additional 20 min. After incubation with forskolin and/or isoproterenol, strips were rapidly frozen with liquid nitrogen. cAMP was extracted with perchloric acid, and its content was measured using a Yamasa cAMP assay Kit. Chemicals used were forskolin 10⁻⁶-10⁻⁵, isoproterenol 10⁻⁶-10⁻⁴ M, phentolamine 10⁻⁶ M, Ro20-1274 10⁻⁶ M (Roche). Forskolin 10⁻² M and Ro20-1274 10⁻² M were dissolved in 95% ethanol, then diluted in distilled water. Isoproterenol and phentolamine were dissolved in distilled water.

Statistical analysis of drug effect and differences between treatment groups was obtained with a two-way nested analysis of variance (ANOVA). The Scheffe test was used to determine what concentration of drug produced a significant effect and a non-paired t-test was used to determine significant between responses at any given drug concentration. All statistical analysis were performed on raw (absolute) data values.

Results

Forskolin, isoproterenol and db-cAMP, all caused a reversible relaxation of rabbit detrusor muscle strips. Fig.1 shows the time course of relaxant responses induced by these agents. Isoproterenol, 10⁻⁶ M, in the presence of phentolamine, 10⁻⁶ M, quickly relaxed rabbit detrusor muscles. It decreased 81% of maximum response in 1 min and 100% in 4 min, respectively. Forskolin, 10⁻⁶ M, decrease 13% of maximum response in 1 min, 75% in 5 min and 100% in 10 min, respectively. Db-cAMP, 10⁻³ M, slowly relaxed the rabbit detrusor muscles. It decreased 20% of maximum response in 5 min, 40% in 10 min and 100% in 30 min. Dose-response curves of maximum relaxant responses induced by these agents are shown in
Fig. 1. Time-dependent changes in the relaxant responses of rabbit detrusor smooth muscle strips to isoproterenol, 10^{-6} M (○) (n=5), forskolin, 10^{-6} M (□) (n=5) and db-cAMP, 10^{-3} M (△) (n=4).
Maximal relaxation was obtained in 4 min for isoproterenol, in 8 min for forskolin and in 30 min for db-cAMP. Data are shown as mean±SEM. Asterisks show significant relaxant responses from control (before drug administration) (**p<0.05, *p<0.01).

Fig. 2. Dose-response curves of rabbit detrusor muscle strips to isoproterenol (○) (n=5), forskolin (□) (n=5) and db-cAMP (△) (n=5).
Asterisks show significant differences from control (**p<0.05, *p<0.01). 100% relaxation was determined by administrating isoproterenol 10^{-4} M. Data are shown as mean±SEM. The response curve to isoproterenol is not significantly different from that to forskolin.
Table 1. Cyclic AMP contents induced by isoproterenol and forskolin in rabbit detrusor muscle. Results shown are expressed as mean±SEM of absolute values and as percent of basal levels in 6 experiments. Asterisks show significant difference from basal levels (**p<0.05, *p<0.01).

| Incubation time | 1 min | 2.5 min | 5 min | 10 min |
|-----------------|-------|---------|-------|--------|
| Basal           | 3.85 ± 0.40 | 3.85 ± 0.10 | 3.35 ± 0.20 | 3.00 ± 0.10 |
| Isoproterenol, 10^{-6} M | 6.00 ± 0.25* (156%) | 4.50 ± 0.15** (117%) | 5.10 ± 0.30* (152%) | 3.75 ± 0.15* (125%) |
| Forskolin, 10^{-6} M | 4.10 ± 0.15 (106%) | 4.81 ± 0.20*** (125%) | 4.81 ± 0.40* (144%) | 7.20 ± 0.80* (240%) |
| Isoproterenol, 10^{-5} M | 6.95 ± 0.45* (181%) | 5.55 ± 0.25* (144%) | 5.65 ± 0.30* (169%) | 5.30 ± 0.30* (177%) |
| Forskolin, 10^{-5} M | 4.30 ± 0.20 (112%) | 5.77 ± 0.30* (150%) | 7.53 ± 1.0* (225%) | 18.00 ± 1.40* (600%) |

Fig. 3. Comparison of the response to a combination of isoproterenol, 10^{-6} M and forskolin, 10^{-6} M (○) (n=6) with each drug alone (isoproterenol (○) (n=5), forskolin (○) (n=5)). 100% relaxation was determined by administering isoproterenol, 10^{-4} M. Data are shown as mean±SEM. Asterisks show significant differences from control (before drug administration) (**p<0.05, *p<0.01). The response to a combination of isoproterenol and forskolin is not significantly different from that to isoproterenol alone.

Fig. 2. The dose–response curve of forskolin was not significantly different from that of isoproterenol. Db-cAMP, even at 10^{-3} M, caused only 80% relaxant response of the responses induced by isoproterenol, 10^{-5} M.

Cyclic AMP levels produced by isoproterenol and forskolin were shown in table 1. When isoproterenol, 10^{-6} M was added to muscle strips, cAMP levels increased 56% after 1 min, 17%
Table 2. Addictive effect of isoproterenol and forskolin in cyclic AMP levels. Results are expressed as mean±SEM of absolute values and as percent of basal levels in 4 experiments. Asterisks show significant differences from basal levels (**p<0.05, *p<0.01).

| Incubation time | 2.5 min | 10 min |
|-----------------|---------|--------|
| Basal           | 4.05 ± 0.15 | 3.00 ± 0.17 |
| Isoproterenol, $10^{-5}$ M | $4.58 ± 0.32^{**}$ (113 %) | $3.97 ± 0.17^*$ (132 %) |
| Forskolin, $10^{-5}$ M | $5.61 ± 0.40^*$ (139 %) | $6.45 ± 0.34^*$ (215 %) |
| Isoproterenol, $10^{-4}$ M + Forskolin, $10^{-5}$ M | $12.51 ± 1.46^*$ (309 %) | $12.35 ± 0.76^*$ (412 %) |

after 2.5 min, 52% after 5 min and 25% after 10 min over basal levels, respectively. There was no significant increase in cAMP levels after 1 min with forskolin, $10^{-6}$ M, but at 2.5, 5 and 10 min there were 25%, 44% and 140% increases over basal levels, respectively. When isoproterenol, $10^{-5}$ M was added, cAMP levels increased 81% after 1 min, 44% after 2.5 min, 69% after 5 min and 77% after 10 min over basal levels, respectively. There was no significant increase in cAMP levels after 1 min with $10^{-5}$ M forskolin, but at 2.5, 5 and 10 min there were 50%, 125% and 500% increases over basal levels, respectively.

Fig. 3 shows the relaxant responses of detrusor muscles with the combination of forskolin, $10^{-6}$ M, and isoproterenol, $10^{-6}$ M, with forskolin, $10^{-6}$ M or with isoproterenol, $10^{-6}$ M. The relaxant response induced by the combination of forskolin and isoproterenol was not significantly different from the response induced by isoproterenol alone. The cAMP content was shown in table 2 when a combination of forskolin, $10^{-5}$ M and isoproterenol, $10^{-6}$ M was added to muscle strips. Cyclic AMP levels induced by the combination were much larger than the total amounts of cAMP levels induced by each of forskolin and isoproterenol.

Discussion

Forskolin caused an elevation of cAMP levels and a relaxation of rabbit detrusor muscle strips, which is consistent with the previous report (Morita et al., 1986). The cAMP elevation and the relaxation response were correlated with each other in both the time- and the dose-dependent manners. Isoproterenol also caused the time- and the dose-dependent increases in cAMP levels and the relaxation of the muscle strips. These data coupled with the finding that db-cAMP, a cAMP derivative, caused a relaxation in time- and dose-dependent manners, suggest that the relaxation induced by forskolin or isoproterenol may be mediated by cAMP.

The present study has also demonstrated that there are big differences between isoproterenol and forskolin in the time course of relaxant responses and in the amount of cAMP accumulation. Isoproterenol quickly relaxed detrusor muscle strips and quickly increased
cAMP levels to maximum in 1 min, whereas, forskolin slowly relaxed detrusor muscle strips and slowly increased cAMP levels to maximum in 10 min. This finding may support the previous reports (Seamon and Daly, 1981b; Green and Clark, 1982) that the activation of adenylate cyclase by forskolin lacks a functional coupling protein. The present study also has demonstrated a big difference in amounts of cAMP produced by the same concentration of isoproterenol and forskolin, although the agents cause a similar relaxation of detrusor muscle strips. For example, isoproterenol, $10^{-6}$ M and $10^{-5}$ M induced 56% and 81% increases of cAMP over basal level after 1 min, respectively, while forskolin, $10^{-6}$ M and $10^{-5}$ M, induced 140% and 500% increases of cAMP over basal level after 10 min, respectively. The relaxant response induced by isoproterenol, $10^{-6}$ M was not significantly different from that by forskolin, $10^{-6}$ M, nor the relaxant response by isoproterenol, $10^{-5}$ M was either different from that by forskolin, $10^{-5}$ M. The reason why the cAMP levels induced by forskolin is different from those by isoproterenol is unclear, although the maximum relaxation induced by the two agents is similar. Marshall and Fain (1985) reported that isoproterenol produces much smaller elevation of cAMP than does forskolin, but completely relaxes the rabbit and rat uterine muscle. Litosch (1982) reported that $10^{-6}$ M forskolin increases cAMP levels 4-fold with little stimulation of lipolysis whereas a similar elevation of cAMP by isoproterenol is accompanied by maximum activation of lipolysis in rat adenocytes. Vegesna and Diamond (1983) reported that $10^{-7}$ M forskolin increases cAMP levels 5.5-fold but does not relax the bovine coronary artery. In the rabbit papillary muscle, forskolin causes a much greater increase in cAMP levels than did isoproterenol at the same level of function (Rodger and Shahid, 1984). In spite of the absence of a functional response to forskolin in the guinea pig soleus muscle, the cAMP level increases more than 10 times after forskolin (Bowman et al., 1985). The data of rabbit detrusor presented in this paper are basically analogous with the previous reports (Marshall and Fain, 1985; Litosch et al., 1982; Vegesna and Diamond, 1983; Rodger and Shahid, 1984; Bowman et al., 1985).

The dissociation between relaxant responses and cAMP contents could be explained in several ways. If it is assumed that cAMP is responsible for the relaxation of detrusor smooth muscle caused by forskolin and isoproterenol, then isoproterenol may increase cAMP in a small intracellular compartment (Hayes and Brunton, 1982; Seamon and Daly, 1983) linked to physiological function or forskolin may also increase cAMP in cells which are not necessarily relevant physiological functions. The agonist-related differences in cAMP contents which are shown in this experiment on the urinary bladder have been reported in canine tracheal smooth muscle (Zhou et al., 1992), which supports subcellular compartments of cAMP cascade. Forskolin, which appears to directly activate adenylate cyclase in a wide variety of functionally different cells (Seamon and Daly, 1981a), may increase cAMP levels in all cell types, whereas isoproterenol, which acts through beta-adrenoceptors, may more selectively activate adenylate cyclase in detrusor smooth muscle cells (Morita et al., 1990; Levin et al., 1986). A final possibility that must be considered is that cAMP is not directly responsible for the smooth muscle relaxation caused by forskolin or isoproterenol. This possibility, however, may be denied since db-cAMP, a cAMP derivative, relaxes the detrusor smooth muscle. Anyway, our findings suggest that only small amount of cAMP in the cell may be necessary to produce the
physiological function in urinary bladder smooth muscles. In the present experiment, when forskolin and isoproterenol are added in combination, the cAMP levels was greater than the sum of the cAMP levels produced by each agent alone. This finding seems to show another effect of forskolin that is forskolin activates some hormone activity, which has been suggested Seamon and Daly (1983). However, the relaxant responses induced by the combination of forskolin and isoproterenol was not significantly different from the relaxation induced by isoproterenol alone. This finding also suggests that the small amount of cAMP may play a role on detrusor smooth muscle relaxation.

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