INFLUENCES OF AMINOPHYLLINE AND REDUCTION IN EXTERNAL Na ON THE ANTISPASMODIC ACTION OF ISOPROTERENOL IN THE ISOLATED RAT RECTUM

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Accepted August 25, 1976

Abstract—Effects of aminophylline and the reduction in external Na on the antispasmodic action of isoproterenol were investigated in relation to the mobilization of Ca in the isolated rat rectum. The antispasmodic action of isoproterenol on the phasic contractions by acetylcholine and K both in Ca-free and in Ca-free and Na-poor media was potentiated by treatment with aminophylline, however the antispasmodic action was attenuated by reducing Na in the Ca-free medium. Dibutyryl cyclic AMP inhibited acetylcholine- and K-induced phasic contractions in Ca-free and in Ca-free and Na-poor media and the inhibitory action was also potentiated by treatment with aminophylline, while the inhibitory action of dibutyril cyclic AMP was attenuated by reducing Na in the Ca-free medium. From these findings, it appears that isoproterenol inhibits the release of Ca from storage sites induced by acetylcholine and K via the increase of intracellular cyclic AMP content and that the external Na may play an important role in the Ca release-inhibiting effect of cyclic AMP.

It is generally accepted (1-4) that the action of adrenergic β-stimulator, isoproterenol (Iso), increases cyclic AMP content of the cell by stimulation of adenyl cyclase activity and thereby produces the relaxation of smooth muscle. On the other hand, it has been reported that the relaxation induced by Iso in intestine and uterus is potentiated by methylxanthine derivatives, theophylline (3, 5, 6) and caffeine (4-6), which inhibit the phosphodiesterase that inactivates cyclic AMP.

It has been proposed (7, 8) that Iso accelerates the uptake of Ca by storage sites in smooth muscle. Andersson et al. (1) reported that Iso increased cyclic AMP content, thereby facilitating the binding of intracellular Ca by storage sites in rabbit intestinal smooth muscle. However, little information is available as to how the external Na participates in action of Iso or cyclic AMP in relation to the mobilization of Ca. It has been described (9) that Iso-induced inhibitory action on the contraction by the cholinergic receptor stimulant, ACh, was potentiated by the combination with aminophylline and that this action was attenuated by replacing external NaCl with LiCl.

In the present study, the antispasmodic action of Iso on the phasic contractions by cholinergic receptor stimulant, ACh, and non-receptor stimulant, K, was investigated in relation to cyclic AMP using the isolated rat rectum. Furthermore, data concerning the influence of reduction of external Na on the action by means of replacing external NaCl

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MATERIALS AND METHODS

Mature rats were sacrificed by a blow on the neck and the rectum was dissected. Strips of the rectum, approx. 1 cm long, were fixed vertically in the organ bath containing 30 ml of nutrient solution (Locke's solution: NaCl, $9 \times 10^{-3}$; KCl, $4.2 \times 10^{-4}$; CaCl$_2$, $2.4 \times 10^{-4}$; glucose, $10^{-3}$; NaHCO$_3$, $4 \times 10^{-4}$ (g/ml)). Isotonic contractions were recorded on a smoked paper with a magnification of approx. 10 times. The solution was maintained at $29 \pm 1^\circ$C and continuously bubbled with a mixture of 95% O$_2$ and 5% CO$_2$.

Ca-free medium was prepared in the same fashion as Locke's solution except that CaCl$_2$ was omitted and 0.1 mM EDTA was added. Ca-free and Na-poor medium was prepared in the same way as the Ca-free medium except that NaCl was substituted by LiCl or choline Cl for the isotonic adjustment.

Effect of aminophylline (Am) on the antispasmodic actions of isoproterenol (Iso) and dibutryl cyclic AMP (db-cAMP) was determined according to the following procedures. The magnitude of phasic contraction by $5 \times 10^{-5}$ g/ml acetylcholine (ACh) or $3 \times 10^{-3}$ g/ml K after incubation for 10 min in Ca-free medium (a), that after incubation for 10 min in Ca-free medium containing $10^{-9}$ g/ml Iso or $10^{-4}$ g/ml db-cAMP (b) and that after incubation for 10 min in Ca-free medium containing $10^{-9}$ g/ml Iso with $10^{-5}$ g/ml Am or $10^{-4}$ g/ml db-cAMP with $10^{-5}$ g/ml Am (c) were determined in the same preparation. A typical example of this series of experiments is shown in Fig. 1. After the procedure (a), the preparation was transferred to the normal medium and when the magnitude of phasic contraction by ACh or K was restored to the initial magnitude in normal medium, the subsequent procedure (b) was carried out. After the procedure (b), a similar experiment was performed, thereafter the procedure (c) was carried out. The experimental procedures employed in Ca-free and Na-poor medium were as those described above, except that the Ca-free medium was substituted by Ca-free and Na-poor medium. The concentration-action curve of Iso

![Fig. 1. A typical example of experiments concerning effect of aminophylline (Am) on the antispasmodic action of isoproterenol (Iso). Concentrations used were $5 \times 10^{-8}$ g/ml acetylcholine (ACh), $10^{-5}$ g/ml Iso and $10^{-2}$ g/ml Am.](image-url)
and that of Iso together with Am were determined by plotting the mean percentage inhibition of ACh- or K-induced phasic contraction at each log concentration of Iso. The percentage inhibition to Iso or db-cAMP and to Iso together with Am or db-cAMP together with Am were determined according to the following formulae:

\[
\text{percentage inhibition to Iso or db-cAMP} = \frac{(a) - (b)}{(a)} \times 100
\]

\[
\text{percentage inhibition to Iso together with Am or db-cAMP together with Am} = \frac{(a) - (c)}{(a)} \times 100
\]

Effect of reduced Na from Ca-free medium on the actions of db-cAMP and Iso was investigated according to the following procedures. The magnitude of phasic contraction by 5 \times 10^{-8} \text{ g/ml ACh} or 3 \times 10^{-3} \text{ g/ml K} after incubation for 10 min in Ca-free medium (A), that after incubation for 10 min in Ca-free medium containing 10^{-4} \text{ g/ml db-cAMP} or 10^{-3} \text{ g/ml Iso} (B), that after incubation for 10 min in Ca-free and Na-poor medium (C) and that after incubation for 10 min in Ca-free and Na-poor medium containing 10^{-4} \text{ g/ml db-cAMP} or 10^{-8} \text{ g/ml Iso} (D) were determined in the same preparation. This series of experiments was the same as that of "effect of Am on the anti-spasmodic actions of Iso and db-cAMP". After the procedure (A), the procedure (B) and the procedure (C), the magnitude of phasic contraction by ACh or K was restored to the initial height by transferring to the normal medium, respectively, thereafter the subsequent procedure was performed. The log concentration-action curves of Iso in Ca-free and in Ca-free and Na-poor media were constructed by plotting the mean percentage inhibition of ACh- or K-induced contraction at each log concentration of Iso. The percentage inhibition to Iso was determined according to the following formulae;

\[
\text{percentage inhibition in Ca-free medium} = \frac{(A) - (B)}{(A)} \times 100
\]

\[
\text{percentage inhibition in Ca-free and Na-poor medium} = \frac{(C) - (D)}{(C)} \times 100
\]

Drug solutions were added directly to the medium. Concentrations of drugs are expressed in term of g/ml of the salts. Drugs employed were as follows: acetylcholine chloride (Tokyokasei), isoproterenol hydrochloride (protornol-L, Nikken Chemicals), aminophylline (neophylline, Eisai Co. Ltd) and N\textsubscript{6},O\textsubscript{3}-dibutyryl adenosine 3'5' cyclic monophosphoric acid (Sigma Chem. Co.) (dibutyryl cyclic AMP).

RESULTS

Effect of aminophylline on the antispasmodic actions of isoproterenol and dibutryl cyclic AMP

Concentrations used were 5 \times 10^{-8} \text{ g/ml ACh} and 3 \times 10^{-3} \text{ g/ml K}, which were obtained as approximately ED60, and 10^{-9} \text{ g/ml Iso}, 10^{-4} \text{ g/ml db-cAMP} and 10^{-8} \text{ g/ml Am}. Am 10^{-5} \text{ g/ml did not influence the phasic contractions by these stimulants either in Ca-free or in Ca-free and Na-poor media. Phasic contractions by ACh and K in Ca-free medium were inhibited after the treatment with Iso, whereas after the application of Iso together
### Table 1. Effect of aminophylline (Am) on inhibitory actions of isoproterenol (Iso) and dibutyryl cyclic AMP (db-c AMP)

|                      | against ACh (5 × 10⁻⁶ g/ml) contraction | against K (3 × 10⁻⁶ g/ml) contraction |
|----------------------|----------------------------------------|--------------------------------------|
|                      | Pretreatment with Am (10⁻⁵ g/ml)       | Pretreatment with Am (10⁻⁵ g/ml)     |
|                      | without with | Significance | without with | Significance |
| Iso (10⁻⁵ g/ml)      | 30.1 ± 3.1 (7)  | 45.1 ± 3.2 (7) | 0.01         | 49.9 ± 3.2 (11) | 65.8 ± 3.2 (11) | 0.01 |
| db-c AMP (10⁻⁴ g/ml) | 37.8 ± 4.7 (5)  | 66.7 ± 8.3 (5) | 0.01         | 46.9 ± 5.2 (5)  | 77.4 ± 3.9 (5)  | 0.01 |

Percentage inhibition against the magnitude of acetylcholine (ACh)- and K-induced contraction was measured. Each value is given as means ± S.E. Figures in parentheses indicate number of experiments.

### Table 2. Effect of reduced external Na on inhibitory actions of isoproterenol (Iso) and dibutyryl cyclic AMP (db-c AMP)

|                      | against ACh (5 × 10⁻⁶ g/ml) contraction | against K (3 × 10⁻⁶ g/ml) contraction |
|----------------------|----------------------------------------|--------------------------------------|
|                      | in Ca-free medium | in Ca-free and Na-poor medium | Significance | in Ca-free medium | in Ca-free and Na-poor medium | Significance |
| Iso (10⁻⁵ g/ml)      | 72.6 ± 3.6 (7)  | 35.0 ± 4.2 (7)  | 0.01         | 77.0 ± 3.0 (10) | 45.7 ± 5.4 (10) | 0.01 |
| db-c AMP (10⁻⁴ g/ml) | 41.7 ± 3.8 (7)  | 24.5 ± 2.2 (7)  | 0.01         | 49.7 ± 2.5 (7)  | 23.1 ± 3.2 (7)  | 0.01 |

Percentage inhibition against the magnitude of acetylcholine (ACh)- and K-induced contraction was measured. Each value is given as means ± S.E. Figures in parentheses indicate number of experiments.
with Am they were further inhibited (Table 1). On the other hand, phasic contractions by ACh and K were reduced after the treatment with db-cAMP, whereas after the application of db-cAMP together with Am they were further inhibited (Table 1). Thus the inhibitory actions of Iso and db-cAMP were significantly potentiated by the combination with Am. Ten min after replacing external NaCl by LiCl, phasic contractions by ACh and K were reduced by 5.7±2.2% (N=10) and by 3.8±1.8% (N=11), respectively, as compared with those in Ca-free medium. In Ca-free and Na-poor medium replaced external NaCl by LiCl, phasic contraction by ACh was not modified and that by K was reduced by 10.6±3.6% (N=7) after the treatment with Iso, whereas after the application of Iso together with Am they were inhibited by 9.0±2.8% (N=6) and by 30.1±1.3% (N=7), respectively. When NaCl was replaced by choline Cl in the presence of atropine, similar effects of Iso and Am to those obtained in the medium replaced by LiCl were observed. Thus, like that in Ca-free medium, the inhibitory action of Iso on the phasic contractions by ACh and K was significantly potentiated by the combination with Am. The log concentration-action curve of Iso was shifted to the left by 10−5 g/ml Am both in Ca-free medium and in Ca-free and Na-poor medium (Fig. 2).

\[ \begin{align*}
\text{Fig. 2. Effect of aminophylline 10−3 g/ml on concentration-action curves of isoproterenol (Iso) in Ca-free (a) and in Ca-free and Na-poor (b) media. These concentration-action curves were determined by plotting the mean percentage inhibition of } \\
3\times10^{-9} \text{ g/ml K-induced contraction. } \bullet - \bullet : \text{ control, } \bullet - \bullet - \bullet : \text{ combination with aminophylline. Vertical bars represent S.E. Preparations used, 7–11.}
\end{align*} \]

Effect of reduced Na on the actions of dibutyryl cyclic AMP and isoproterenol

Concentrations used were 5×10−8 g/ml ACh and 3×10−3 g/ml K. Db-cAMP (10−4 g/ml) inhibited ACh- and K-induced phasic contractions in Ca-free and Na-poor medium as well as in Ca-free medium, however the inhibitory action was significantly attenuated by reducing external Na, as compared with that in Ca-free medium (Table 2). Iso (10−8 g/ml)-induced inhibitory action was significantly decreased by reducing external Na, as compared with that in Ca-free medium (Table 2). When NaCl was replaced with choline Cl, similar results were obtained.
As shown in Fig. 3, the log concentration-action curve of Iso was shifted to the right by reducing Na from bath medium.

DISCUSSION

As also observed in previous studies (10), the phasic contractions by ACh and K in the Ca-free and in the Ca-free and Na-poor media were found to be caused by the release of Ca from storage sites. In these media, the phasic contractions by ACh and K were inhibited by Iso. This inhibitory action of Iso was potentiated by the combination with Am. Furthermore, the log concentration-action curve of Iso shifted to the left by the combination with Am. These findings parallel the current suggestions that cyclic AMP is a mediator of relaxation induced by stimulation of adrenergic β-receptor responsible for the action of Iso (1-4) and that Am inhibits phosphodiesterase activity which inactivates cyclic AMP (3-6, 11), thereby potentiating the action of Iso. Thus, it is suggested that Iso and Am inhibit the release of Ca from storage sites responsible for the smooth muscle contraction and that these inhibitory actions may be attributed to the increase of intracellular cyclic AMP. This suggestion is further supported by the observations that db-cyclic AMP inhibited the ACh- and K-induced contractions and that the inhibitory action was significantly potentiated by application of Am. It has been reported that db-cyclic AMP penetrates the cells (12) and that it acts by mimicking cyclic AMP at its site of action (2).

When external NaCl was replaced by LiCl or choline Cl, the inhibitory actions of Iso on ACh- and K-induced contractions were attenuated. Furthermore, the log concentration-action curve of Iso was shifted to the right by reducing external Na. Li ion has been shown to inhibit the catecholamine-induced increase in cyclic AMP level of brain slices (13). In the present study, we did not determine whether or not Li ion inhibits the increase in cyclic AMP level. However, when external NaCl was replaced by choline Cl, similar results to...
those obtained by substitution of LiCl were produced. In addition, the action of db-cyclic AMP was also inhibited by reducing external Na. Hence, the attenuation of Iso-induced inhibitory action may be due to reduction in external Na ion, but not to the effect of Li ion.

It has been reported that Ca ion is required for the agonist-receptor interactions (14) and that Ca ion plays an important role in the action of adenyl cyclase (15). In the present study, the β-receptor stimulant, Iso, inhibited the phasic contraction by the cholinergic receptor stimulant, ACh, as well as that by non-receptor stimulant, K, in the absence of external Ca. Furthermore, the effect of Iso was similar to that of db-cyclic AMP. Thus, Iso can induce an inhibitory action on the contractions by ACh and K in a Ca-free medium. However, reduced external Na in addition to the absence of external Ca may inhibit the antispasmodic effects of Iso and db-cyclic AMP at their sites of action.

Reduced external Na decreases the Na influx—Ca efflux coupling system, as suggested in mesenteric artery and aorta (16), vascular smooth muscle (17), cardiac muscle (18) and giant axon (19). Suppression of the coupling system would then induce an increase in the intracellular Ca level, and such may attenuate the inhibitory action of Iso. On the other hand, it has been proposed that reduction or removal of external Na induced a depolarization of smooth muscle cell membrane (20–22). The actions of Iso and db-cyclic AMP may be attenuated by the depolarization of cell membrane. Furthermore, it has been reported that the binding of intracellular Ca by storage sites was depressed by reducing external Na in isolated rat ileum (23) and rectum (24) and rabbit pulmonary artery (25). Since the reduced external Na may change the function of Ca storage sites presumably attached to muscle cell membrane, the attenuation of Iso-induced inhibitory action by the reduction in external Na may be due to the decrease in binding of intracellular Ca by storage sites.

It has been proposed that the antispasmodic mechanism of Iso may be related to inhibition of the transmembrane influx of Ca as well as release of Ca (7). From the present study, it would appear, that as a mechanism of its antispasmodic action, Iso increases the intracellular cyclic AMP, resulting in inhibition of release of Ca from storage sites induced by ACh and K. In addition, the inhibitory ability of Iso and cyclic AMP would depend on the presence of external Na.

Acknowledgement: We wish to thank Mrs. T. Kosuda for technical assistance.

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