EFFECTS OF PAPAVERINE, BENACTYZINE AND D600 ON Ca^{2+} EFFLUX, DETERMINED BY MEANS OF A Ca^{2+} -SELECTIVE ELECTRODE, FROM GUINEA PIG TAENIA COLI INTO A Ca^{2+} -FREE PHYSIOLOGICAL SOLUTION

Toshiharu SHIBA, Tsutomu URUNO, Kazuhiko KUBOTA
and Keijiro TAKAGI

Department of Pharmacology, Faculty of Pharmaceutical Sciences,
Science University of Tokyo, Ichigaya, Shinjuku-ku, Tokyo 162, Japan

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Abstract—The effects of papaverine, benactyzine and D600 on Ca^{2+} efflux from the taenia coli of guinea pig into a Ca^{2+} -free physiological solution were studied. The effluxed free Ca^{2+} concentrations in the solution were continuously monitored during 30 min by means of a Ca^{2+} -selective electrode. Papaverine (5×10^{-5} and 10^{-4} M) increased the amount of effluxed Ca^{2+} after a 10-min efflux time. The increase in the rate of Ca^{2+} efflux by papaverine was observed after a 4-min efflux time and lasted for the following 15–20 min. Dibutyryl cyclic AMP induced the increase in Ca^{2+} efflux in a similar manner as papaverine. Benactyzine at a concentration of 10^{-4} M, which exerts a non-specific antispasmodic action, produced an increase in the amount of effluxed Ca^{2+} during a 30-min efflux period, and the increase in the rate of Ca^{2+} efflux was observed only 1 min after the efflux initiation. D600 (10^{-6} M) had no effect on Ca^{2+} efflux and the rate of Ca^{2+} efflux, while an increase in the concentration of D600 to 10^{-5} M produced an enhancement of Ca^{2+} efflux and an increased rate of efflux, during the 30-min efflux period. These results suggest that smooth muscle relaxants, papaverine, benactyzine and D600, may increase Ca^{2+} efflux from the guinea pig taenia coli into a Ca^{2+} -free physiological solution, in a different manner(s).

Contraction of the smooth muscle may be dependent upon an increase in the influx of extracellular Ca and/or in the release of intracellularly stored Ca, while its relaxation may be dependent upon the increased Ca efflux from the cell, the rebinding of cytoplasmic Ca^{2+} to the intracellular storage sites, a decrease in the Ca influx or the combination of these processes. Spasmolytics may, therefore, elicit relaxation of the smooth muscle by affecting, at least, one of these Ca translocation processes in some manners.

Non-specific antispasmodics such as papa-

verine and benactyzine have been divided into two groups according to their mechanisms of action; potent, basic compounds or synthetic antispasmodics such as benactyzine hydrochloride and 1,1-diphenyl-3-piperidinobutanol hydrochloride (Aspaminol), and weak basic compounds such as papaverine (1–4). It has been reported that papaverine inhibits the supply of Ca to the contractile elements at the cell membrane (5), and that benactyzine and Aspaminol, synthetic antispasmodics, compete with Ca^{2+} at the surface sites of the muscle cell

membrane related to Ca uptake process, thus decreasing the supply of Ca to the contractile elements (6). Moreover, it has been reported that papaverine strongly inhibits phosphodiesterase and increases the intracellular level of cyclic 3',5' adenosine monophosphate (cyclic AMP), while benactyzine and Aspaminol have no effect upon the cyclic AMP level (3, 4, 7). Thus, it is generally accepted that papaverine and benactyzine have different mechanisms of action.

On the other hand, it has been documented that specific Ca^{2+} antagonists such as D600, a methoxy derivative of verapamil, induce relaxation by inhibiting Ca^{2+} influx through Ca^{2+} channels in the cell membrane (8) and block Ca influx in various smooth muscles (9–11).

In the present studies, we investigated the effects of papaverine, benactyzine and D600 on Ca^{2+} efflux from the taenia coli of guinea pig into a Ca^{2+} -free physiological solution by means of a Ca^{2+} -selective electrode, and compared their actions on the Ca^{2+} efflux. In addition, the effect of papaverine on the Ca^{2+} efflux was compared with that of N^6, O^2' -dibutyryl-3',5'-cyclic adenosine monophosphate (dibutyryl cyclic AMP).

MATERIALS AND METHODS

Apparatus and calibration of the electrode:

The potentiometric set-up for Ca^{2+} determination consisted of an F2112Ca Calcium Selectrode, a K401 saturated calomel electrode, a PHM64 pH meter (Radiometer A/S, Copenhagen, Denmark) and a recorder. An amplifier (ME Commercial, Tokyo, Japan) with an offset voltage controller (12) was inserted in series between the pH meter output and the recorder input.

Ca^{2+} concentrations were measured in the mV-range. A Ca^{2+} electrode was calibrated by successive addition of 5 n moles Ca^{2+}

with a concentrated CaCl_2 stock solution and a calibration curve for the Ca^{2+} electrode was obtained by plotting changes in potential (Δ mV) against the logarithm to the added Ca^{2+} concentrations. Since the potential decreases with increasing Ca^{2+} concentration in the solution, changes in the potential were expressed in terms of absolute values. Under the conditions used here, calibration curves became linear within the range of Ca^{2+} concentrations from 5×10^{-6} to 2.5×10^{-5} M.

Tissue preparation: Pieces of taenia coli (50 mm long *in situ*) were dissected from the caecum of male guinea pig weighing 400–500 g. Before determination of Ca^{2+} effluxes, the tissues were allowed to equilibrate for 120 min in a normal Tris-solution, bubbled with 100 % O_2 , at 26°C. The normal Tris-solution used was the following composition (mM): NaCl 137; KCl 2.7; CaCl_2 1.4; glucose 8.0 and tris (hydroxymethyl) aminomethane (Tris) 5.0. The Tris-solution was adjusted to pH 7.5 with 6 N HCl.

Measurement of Ca^{2+} efflux: After equilibration, the tissue was rinsed in a Ca^{2+} -free Tris-solution (5 ml) for 1 min in order to remove free Ca^{2+} in the extracellular space of the tissue. The Ca^{2+} -free Tris-solution was prepared by omitting CaCl_2 from the normal Tris-solution. The tissue was, then, blotted on ashless filter paper (Whatman No. 42) and placed in a vessel containing the Ca^{2+} -free Tris-solution (5 ml). Free Ca^{2+} ions effluxed from the tissue into the Ca^{2+} -free Tris-solution were continuously measured during 30-min efflux period by using the Ca^{2+} electrode. Measurement of Ca^{2+} efflux was performed at 26°C to slow the rate of Ca^{2+} efflux and to minimize evaporation of the efflux solution. Under the present experimental conditions, the Ca^{2+} concentrations effluxed from the tissues into the Ca^{2+} -free Tris-solution (5 ml) for 30 min were approximately 6×10^{-6} – 10^{-5} M. Stirring of the

efflux solution was accomplished by the bubbling of 100% O_2 .

To examine the effects of relaxants on Ca^{2+} efflux, the Ca^{2+} efflux into the Ca^{2+} -free Tris-solution containing a relaxant was measured and in this case the calibration for the Ca^{2+} electrode was performed in the presence of the relaxant.

After determination of Ca^{2+} efflux, the tissue was gently blotted on ashless filter paper, transferred into a quartz tube, dried at 100°C for 12 hr and weighed to obtain its dry weight. The dry weights of the tissues were approximately 7–10 mg. Ca^{2+} concentrations effluxed into the medium were expressed as m moles Ca^{2+} /kg dry wt and the rate of Ca^{2+} efflux as μ moles Ca^{2+} /kg dry wt·min.

Water and drugs: All solutions were made using reagent-grade water obtained through Milli-Q systems (Millipore Corp., Bedford). The drugs used were as follows: papaverine

hydrochloride (Sigma, St. Louis), benactyzine hydrochloride (Tokyo-Kasei Co. Tokyo), D600 hydrochloride (Knoll, A.G., Ludwigshafen/RH) and $\text{N}^6, \text{O}^{2'}$ -dibutyryl-3',5'-cyclic adenosine monophosphate (dibutyryl cyclic AMP) (Sigma, St. Louis). The other reagents used here were of the highest purity available. Concentrations given in this paper represented final vessel concentrations in terms of free base.

Statistical analysis: The results were expressed as the mean $\pm$ S.E. Differences between experiments were compared with Student's *t*-test for paired samples. *P* values of 0.05 or less were considered to be significant.

RESULTS

Figure 1 shows the effect of papaverine on Ca^{2+} efflux from the taenia coli of guinea pig into the Ca^{2+} -free Tris-solution. Papaverine at a concentration of 5×10^{-5} M,

Fig. 1. Effects of papaverine on Ca^{2+} efflux (A) and the rate of Ca^{2+} efflux (B) from guinea pig taenia coli into Ca^{2+} -free Tris-solution. $\circ$, control; $\bullet$, 5×10^{-5} M papaverine; $\triangle$, 10^{-4} M papaverine. (A): The curves represent the average of 6 experiments. An upward movement in the Ca^{2+} trace indicates an increase in Ca^{2+} concentrations in the solution. Each point represents the mean value at each efflux time and the S.E. of mean is given only when it is larger than the size of symbols. Significant difference from control: (*) $p < 0.005$. (B): The rate of Ca^{2+} efflux is calculated on the basis of the data in (A) and is represented at intervals of 1 min except for the first 1-min efflux time. The rate of Ca^{2+} efflux at the first 1-min efflux time is referred to the amount of effluxed Ca^{2+} at 1-min efflux time in (A). Each point represents the mean value of 6 experiments.

which suppressed the carbachol (10^{-5} M)-induced contraction by 50% in the normal Tris-solution, significantly increased the amount of effluxed Ca^{2+} after an efflux time of 10 min (Fig. 1, A). The papaverine (5×10^{-5} M)-induced increase in the rate of Ca^{2+} efflux was observed at a 4-min efflux time and this increase lasted for the following 15–20 min (Fig. 1, B). The increases in the amount of effluxed Ca^{2+} and the rate of Ca^{2+} efflux were also observed at a concentration of 10^{-4} M papaverine, which completely abolished the carbachol (10^{-5} M)-induced contraction. Although papaverine at both concentrations of 5×10^{-5} and 10^{-4} M induced the increase in Ca^{2+} efflux from the tissue in a similar manner, the dose-dependent relationship was not observed (Fig. 1).

It has been reported that papaverine strongly inhibits phosphodiesterase, thus increasing the intracellular level of cyclic AMP (3, 4, 13) and that dibutyl cyclic AMP has effects similar to papaverine (5, 14). Therefore, we investigated the effect of dibutyl cyclic AMP on Ca^{2+} efflux from the guinea pig taenia coli into the Ca^{2+} -free Tris-solution. Dibutyl cyclic AMP (10^{-4} M)

increased the amount of effluxed Ca^{2+} (Fig. 2) in a similar time course as papaverine (Fig. 1). The increase in the rate of Ca^{2+} efflux induced by dibutyl cyclic AMP was observed at a 2-min efflux time and lasted for the following 10 min (Fig. 2, B).

It is well known that benactyzine has, at least, two pharmacological actions (1). At a concentration below 10^{-5} M, benactyzine showed an antiacetylcholine action, while it exerted a non-specific antispasmodic one at concentrations above 3×10^{-5} M (data not shown). Benactyzine at a concentration of 1.6×10^{-7} M, which suppressed the carbachol (10^{-5} M)-induced contraction by 50%, did not affect Ca^{2+} efflux (data not shown). Benactyzine (5×10^{-6} M), which abolished the carbachol (10^{-5} M)-induced contraction, increased the amount of effluxed Ca^{2+} at 10, 15 and 20-min efflux times as shown in Fig. 3, A. At a concentration of 10^{-4} M, benactyzine which showed a non-specific antispasmodic action, increased the amount of effluxed Ca^{2+} at all efflux times (Fig. 3, A). The significant increase in the rate of Ca^{2+} efflux induced by benactyzine (10^{-4} M) was, however, observed during the first 1-min

Fig. 2. Effects of dibutyl cyclic AMP on Ca^{2+} efflux (A) and the rate of Ca^{2+} efflux (B). $\bigcirc$, control; $\bullet$, 10^{-4} M dibutyl cyclic AMP. The data is the average of 5 experiments. In (A), significant difference from control: (*) $p < 0.05$; (**) $p < 0.01$. The other expressions are the same as described for Fig. 1.

Fig. 3. Effects of benactyzine on Ca^{2+} efflux (A) and the rate of Ca^{2+} efflux (B). $\circ$, control; $\bullet$, 5×10^{-6} M benactyzine; $\triangle$, 10^{-4} M benactyzine. The data is the average of 4 experiments. In (A), significant difference from control: (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.005$. The other expressions are the same as described for Fig. 1.

Fig. 4. Effects of D600 on Ca^{2+} efflux (A) and the rate of Ca^{2+} efflux (B). $\circ$, control; $\bullet$, 4×10^{-6} M D600; $\triangle$, 10^{-5} M D600. The data is the average of 4 experiments. In (A), significant difference from control: (*) $p < 0.05$; (**) $p < 0.005$. The other expressions are the same as described for Fig. 1.

efflux period ($p < 0.05$) (Fig. 3, B).

Figure 4 shows the effect of D600 on Ca^{2+} efflux from the taenia coli of guinea pig into the Ca^{2+} -free Tris-solution. D600 at a concentration of 10^{-5} M induced no increase in Ca^{2+} efflux at all efflux times (data not shown). In contrast with papaverine, D600 at concentrations of 4×10^{-6} and 10^{-5} M

dose-dependently increased Ca^{2+} efflux after a 1-min efflux time (Fig. 4, A). D600 at a concentration of 4×10^{-6} M increased the rate of Ca^{2+} efflux only during the first 1-min efflux period, while during 30-min efflux period, 10^{-5} M D600 increased the efflux rates.

DISCUSSION

Intracellular organelles such as sarcoplasmic reticulum and mitochondria, cytoplasm and plasma membrane have been usually accepted as Ca locations in smooth muscle cells (15–17). Moreover, plasma membrane Ca fractions have been suggested to be present in two forms, differing either in site of binding or in binding energy: "superficial" and "sequestered (or less superficial)" (18, 19). On the other hand, Karaki et al. (20) have divided cellular Ca into two parts using the results of ^{45}Ca uptake (4 min wash) experiments, in which the fractions which did and did not exchange within 4 min have been termed the "loosely" and the "tightly bound fraction", respectively.

We have reported that a Radiometer Ca^{2+} -selective electrode can accurately and continuously monitor free Ca^{2+} ions effluxed from smooth muscles into a Ca^{2+} -free physiological solution, at micromolar levels (21, 22). It has been, however, reported that in various types of smooth muscle, the effluxed ^{45}Ca is much greater into a medium containing ^{40}Ca than that into a ^{40}Ca -free medium, due to the presence of a ^{40}Ca - ^{45}Ca exchange mechanism at the binding sites of the cell surface (23, 24). In previous work (22), we observed in the taenia coli of guinea pig and the thoracic aorta of rabbit that after incubation in a Ca^{2+} -free medium for different periods, the tissue, in which the residual Ca gradually decreased with increasing the time of incubation, maintained superficially-bound Ca which is readily displaced by La^{3+} . These results suggested that superficially-bound Ca may not be readily omitted by incubation of the tissues in a Ca^{2+} -free medium for the given period. Therefore, under the present experimental conditions, it could be assumed that the drugs which interact with Ca at the superficial Ca binding sites of the plasma membrane increase Ca^{2+}

efflux in the initial phase of efflux periods, and that the drugs which influence some Ca sinks in the cytoplasm increase Ca^{2+} efflux in the later phase of efflux periods.

Tomiyama et al. (14) reported that papaverine increases ^{45}Ca efflux from the guinea pig taenia coli into a medium containing ^{40}Ca , while Banerjee and Lewis (25) reported that papaverine decreases ^{47}Ca efflux from the guinea pig ileum. Thus, there are discrepancies regarding the effect of papaverine on Ca efflux. In this study, the significant increase in the amount of Ca^{2+} effluxed into the Ca^{2+} -free Tris-solution induced by papaverine (5×10^{-5} and 10^{-4} M) was observed after an efflux time of 10 min, although the increase in the rate of Ca^{2+} efflux was observed after 4-min efflux periods (Fig. 1). These results suggest that papaverine may not directly influence the superficial Ca binding sites of the plasma membrane. Since it has been reported that papaverine exists for the most part as non-ionized molecule in a physiological solution (1) this drug may intracellularly exert its action on Ca^{2+} efflux of the taenia coli into a Ca^{2+} -free medium. It is also suggested that the increased Ca^{2+} efflux induced by papaverine might be derived from intracellular origin and/or from the superficial binding sites into the medium by translocation of Ca from the "sequestered" sites to the superficial sites of the plasma membrane.

Papaverine did not increase Ca^{2+} efflux in a dose-dependent manner (Fig. 1, A). This might be related to the stimulatory effect of papaverine on Ca uptake by microsomal fraction (6) and/or mitochondrial fraction (26) of smooth muscles and to the inhibitory effect of papaverine on Ca release by microsomal fraction (27).

Papaverine strongly inhibits phosphodiesterase thus increasing the intracellular level of cyclic AMP (3, 4, 7, 13) and dibutyryl cyclic AMP as well as papaverine induces

the increase in ^{45}Ca efflux into a medium containing ^{40}Ca (14). Dibutyl cyclic AMP (10^{-4} M), as shown in Fig. 2, increased the amount of effluxed Ca^{2+} and the rate of Ca^{2+} efflux in a similar time course as papaverine, although the increase in the rate of Ca^{2+} efflux induced by the former did not last so long as that induced by the latter. From the present results, it might be assumed that the increase in the Ca^{2+} efflux into the Ca^{2+} -free Tris-solution induced by papaverine is partly mediated through an increase of the intracellular level of cyclic AMP as the result of the inhibition of phosphodiesterase.

Since synthetic antispasmodics such as benactyzine and Aspaminol exist mainly in an ionized form in a physiological solution, most of these antispasmodic molecules fail to penetrate a cell (1). These drugs have no effect upon the cyclic AMP level (2–4,7). Takayanagi et al. (6) reported that benactyzine and Aspaminol compete with Ca^{2+} at the surface sites of the muscle membrane concerned with Ca uptake process, thus decreasing the supply of Ca^{2+} to the contractile elements. In the present experiments, benactyzine (10^{-4} M) increased Ca^{2+} efflux from the guinea pig taenia coli into the Ca^{2+} -free Tris-solution (Fig. 3, A). In contrast with papaverine, benactyzine (10^{-4} M) significantly increased the amount of effluxed Ca^{2+} at an efflux time of 1 min ($p < 0.05$) and the significant increase in the rate of Ca^{2+} efflux was observed only during the first 1-min efflux period ($p < 0.05$). Benactyzine at a concentration of 3×10^{-5} M had an effect similar to that of 10^{-4} M benactyzine and a dose-dependent relationship was observed (data not shown). These results suggest that the antispasmodic action of benactyzine may be mainly related to an interaction with Ca at the superficial binding sites of the plasma membrane and consequently benactyzine may increase Ca^{2+} release at the initial phase of the efflux. Our

results support the suggestion proposed by Takayanagi et al. (6).

It has been reported that D600 inhibits ^{45}Ca influx in smooth muscles (9–11). D600 at a concentration of 4×10^{-6} M, significantly increased the amount of effluxed Ca^{2+} at 1.5 and 10-min efflux times, while significant increase in the rate of Ca^{2+} efflux was observed only during the first 1-min efflux period (Fig. 4). The effects of D600 at this concentration are quite similar to those of benactyzine (10^{-4} M) (Fig. 3). These results suggest the possibility that D600 might increase Ca^{2+} efflux from the guinea pig taenia coli into a Ca^{2+} -free medium in a manner similar to benactyzine. However, D600 at a higher concentration of 10^{-5} M increased the amount of effluxed Ca^{2+} and the rate of Ca^{2+} efflux at all the efflux times. It is thus impossible to explain the effects of D600 on Ca^{2+} efflux from the taenia coli into the Ca^{2+} -free medium only on the basis of inhibition of backinflux of Ca^{2+} from the external Ca^{2+} -deficient medium. Church and Zsotér (28) reported that Ca^{2+} antagonists such as nifedipine have intracellular actions because nifedipine increases ^{45}Ca efflux from membrane-bound and intracellular sites of the rabbit mesenteric veins into a Ca^{2+} -free medium. From these results, the increased Ca^{2+} efflux by D600 at a higher concentration of 10^{-5} M might be exerted by its effect on the intracellular sites and/or the binding sites of the plasma membrane.

In summary, we could differentiate the effects of three antispasmodics, papaverine, benactyzine and D600, on Ca^{2+} efflux from the taenia coli of guinea pig into a Ca^{2+} -free physiological solution by means of a Ca^{2+} -selective electrode. In addition, our results suggest that these three relaxants may have different mechanisms of action on Ca^{2+} efflux of the tissue into a Ca^{2+} -free physiological solution.

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