EFFECT OF CALCIUM IONS AND PHOSPHOLIPIDS ON ANTIACETYLCHOLINE ACTION OF ACETONE AND PAPAVERINE

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Dikstein and Sulman (1) reported that the treatment of the rectus abdominis muscle of Bufo viridis toad with 3.4 M (25%) acetone for 2 minutes eliminated the response of the muscle to acetylcholine (ACh), but 5 to 20 μg/ml of phospholipids such as phosphatidylserine could restore the response of the treated muscle to ACh. They also observed similar effects of phospholipids on the rabbit uterus treated with acetone and restoration of the response of the ileum to norepinephrine after the treatment with phospholipids, and postulated that the phospholipids of receptors were extracted by acetone and could be replaced by those from an exogenous source.

In a recent report, however, Ehrenpreis, Hazra and Bigo-Gullino (2) presented evidence that smooth muscles of various organs treated with 3.4 M (25%) acetone were not restored in their responses to agonists by application of phospholipids, and the effect of acetone was concerned with the denaturation of the receptor protein by acetone, but not with lipid extraction from the receptor.

We have found in this paper that the mode of action of acetone is very similar to that of papaverine. The aims of this paper are to study the mechanism of antiacetylcholine action of acetone and papaverine, and to test the restoration of the response of the papaverine- or acetone-treated ileum to ACh by phospholipids.

Also in our experiments, as described by Ehrenpreis, Hazra and Bigo-Gullino (2), 3.4 M (25%) acetone applied for 2 minutes at 32°C abolished the response of ileum to ACh, and even large doses of phospholipids could not restore the responsiveness of the ileum after the acetone treatment. In an ileum maintaining a moderate response to ACh after the acetone treatment under milder conditions, however, various phospholipids could restore distinctly the responsiveness of the ileum.

MATERIALS AND METHODS

After killing an adult male guinea pig by a blow on the head, small intestine was isolated and the ileum approximately 3 cm length was suspended in a 30 ml organ bath...
filled with Locke Ringer solution, kept at 32°C and bubbled with air through the bath fluid. The tension on the muscle was approximately 0.5 g and the contraction was recorded isotonically.

Synthetic β, γ-dipalmitoyl-DL-α-lecithin was obtained from Sigma Chemical Company, phosphatidylserine and phosphatidylethanolamine from Tokyo Kasei Kogyo Co. Ltd. Ovo-lecithin was obtained from Tokyo Kasei Kogyo Co. Ltd. and purified by the method of Pangborn (3). Phospholipids were dispersed in water by either sonication or dialysis (4).

In order to test the effect of phospholipids, 0.3 ml of each phospholipid dispersion was given to the 30 ml organ bath in which the isolated ileum was suspended. Acetone was dissolved in Locke Ringer solution. The Locke Ringer solution containing acetone was called “acetone-Locke Ringer solution” in this paper.

Locke Ringer solution in the organ bath was replaced by acetone-Locke Ringer solution to test the antiacetylcholine action of acetone. Locke Ringer solution had the following composition (g/l): NaCl 9.0, KCl 0.42, CaCl₂ 0.11, MgCl₂ 0.20, NaHCO₃ 0.20, glucose 0.50. Isotonic KCl Locke Ringer solution was obtained by replacing NaCl of the Locke Ringer solution with the same moles of KCl.

In order to examine the effects of drugs on the membrane activity of the smooth muscle, experiments were done on strips of isolated taenia caecum (or taenia coli) of the male guinea pig. A piece of the isolated taenia caecum was dissected free from the underlying tissue and mounted in a sucrose gap apparatus which was originally described by Bulbring and Burnstock (5). Temperature of Locke Ringer solution was kept at 32°C. In this apparatus a mechano-electrotransducer (RCA-5734) was also used to record isotonic contraction of the smooth muscle.

The results in this paper were presented as the means of at least 6 experiments.

RESULTS

Effects of acetone on the response of the isolated ileum

A segment of ileum was suspended in the organ bath filled with Locke Ringer solution, kept at 32°C, and was repeatedly applied ACh (3×10⁻⁴ g/ml) until a constant contraction was obtained. The Locke Ringer solution in the organ bath was replaced by 2.1 M (15.5%), 2.3 M (17.0%), 2.4 M (17.7%) or 2.5 M (18.4%) acetone Locke Ringer solution previously kept at 32°C. After 2 minutes incubation with an acetone-Locke Ringer solution, the ileum was washed with Locke Ringer solution 4 times during subsequent 15 minutes and received ACh (3×10⁻⁴ g/ml) at each washing. Every 15 minutes thereafter ACh (3×10⁻⁴ g/ml) was applied to the ileum. Each contraction height produced by ACh was compared with the control contraction height obtained before the acetone treatment. The results are presented in Fig. 1. Similarly 3.4 M (25%) acetone was applied to an ileum for 2 minutes, and the ileum was tested with ACh (3×10⁻⁴ g/ml), but any response had never been observed even after exhaustive washings of the ileum for 2 hours. On the other hand, 4.1 M (30%) acetone applied for 4 minutes at 0°C could not eliminate
FIG. 1. Antagonistic relation between acetone and ACh.

The isolated ileum of the guinea pig was suspended for 2 minutes in 2.1 to 2.5M acetone-Locke Ringer solutions kept at 32°C. After washing, ACh $3 \times 10^{-4}$ g/ml was applied to the ileum every 15 minutes and each contraction was compared with ACh response before acetone-treatment. mean ± S.E.

- 2.1M (15.5%) acetone
- 2.3M (17.0%) acetone
- 2.4M (17.7%) acetone
- 2.5M (18.4%) acetone

completely the response of the ileum to ACh. A 45 seconds incubation with 2.7M (20%) acetone exerted nearly same effects on the ileum as a 2 minutes incubation with 2.4M (17.7%) acetone-Locke Ringer solution. After the 2 minutes treatment of the ileum with 2.4M (17.7%) acetone was observed not only small excitatory response to ACh but also small contracture by KCl Locke Ringer solution (Fig. 2).

As indicated in Fig. 1, the inhibition of the ACh-induced contraction by the treatment of the ileum with 2.3M (17.0%) or lower concentrations of acetone was too small and that by the treatment with 2.5M (18.4%) or higher concentrations of acetone was too large. The inhibition of the ACh-induced contraction by the treatment with 2.4M (17.7%) acetone was most suitable to test effect of the phospholipids on the inhibitory action of acetone, so 2.4M (17.7%) acetone was used in most of the following experiments.

Effects of phospholipids on the response of the ileum treated with acetone solution

An isolated guinea pig ileum was placed for 2 minutes at 32°C in 2.4M acetone-Locke Ringer solution, washed and received ACh every 15 minutes. Concentration action curve of ACh was cumulatively drawn to examine the response to ACh. After the 2 minutes incubation of the ileum with 2.4M (17.7%) acetone, concentration action curve of ACh was shifted to the higher concentrations of ACh, besides depression of the maximum
FIG. 2. Contractions of the isolated ileum of the guinea pig induced by ACh and KCl-Locke Ringer solution after acetone-treatment. The bath fluid was kept at 32°C and time indicated is minutes after acetone-treatment.

After 2 min incubation with acetone (2.4 M), washed out thoroughly.

Most ileum preparations came to show practically a definite small excitatory response to ACh in less than 60 minutes after the treatment with acetone. Phospholipids were applied to the ileum in this state for 5 to 10 minutes. After the incubation with phospholipid, the ileum was washed once with Locke Ringer solution for 1 minute and tested with ACh, and the responses to ACh before and after the application of phospholipid were compared with each other. By this method the maximum response to ACh was restored but the effective concentrations were not (Fig. 3). This phenomenon was observed in the experiments with ove-lecithin (400 µg/ml) (Fig. 4), phosphatidylserine (300 µg/ml) and phosphatidylethanolamine (200 µg/ml). Both dispersions of phospholipids prepared either by sonication or dialysis were effective. The phospholipids were less effective on the ileum whose response to ACh was strongly inhibited by acetone than on the ileum whose response was not strongly.

Antagonism between phospholipids and acetone or papaverine

The concentration action curve of ACh was shifted to the higher concentrations of ACh in the presence of 0.37 M (2.7%) acetone or papaverine (3 x 10^{-8} g/ml), besides de-
Fig. 3. Effect of phosphatidylethanolamine on the ACh response of the isolated ileum of the guinea pig after acetone-treatment.

Upper: registrogram. Time indicated is minutes after acetone-treatment.
Lower: concentration action curves.
Effect of ovo-lecithin on the ACh response of the isolated ileum of the guinea pig after acetone-treatment. Time indicated is minutes after acetone-treatment.

The inhibition of the maximum response of the curve. This inhibitory effect of acetone or papaverine disappeared immediately after washing the ileum with Locke Ringer solution. The inhibition of the maximum response of the curve of ACh was antagonized by phospholipids (300 μg/ml) such as ovo-lecithin, phosphatidylethanolamine, phosphatidylyserine etc. But the shift of the curve was not affected. The experiment with ovo-lecithin and acetone was demonstrated in Fig. 5 and that with ovo-lecithin and papaverine in Fig. 6.

Effect of calcium ions on the inhibitory action of acetone

As described previously, 2.4 M (17.7%) acetone exerted inhibitory effects on the response of the ileum to ACh and phospholipids could antagonize this effect of acetone. The high concentrations of CaCl₂ (2.2 to 5.5×10⁻³ g/ml or 20 to 50 mM) could also restore the reduced ACh response of the ileum which had received a 2.4 M (17.7%) acetone treatment and washed, and the effect of calcium ions disappeared by washing of the ileum. The maximum contraction by ACh in the presence of low concentration (0.37 M) of acetone was converted to the normal level of contraction by an application of CaCl₂ (2.2×10⁻³ g/ml or 20 mM) (Fig. 7). The effect of the increase of CaCl₂ in the bath fluid is similar to that of phospholipids.
Fig. 5. Effect of ovo-lecithin on the ACh response of the isolated ileum of the guinea pig in the presence of acetone.

upper: registrogram, A: acetone (0.37M or 2.7%)  
B: ovo-lecithin (300 µg/ml)

lower: concentration action curves.
Effect of calcium ions and ovo-lecithin on the actions of hexanoycholine, 5-hydroxytryptamine and acetylcholine.

The maximum response to hexanoylcholine, the cholinergic partial agonist was potentiated by an increase of calcium ions \((2.2 \times 10^{-3} \text{g/ml})\) in the bath fluid or by ovo-lecithin \((300 \mu\text{g/ml})\). On the other hand, the maximum responses to acetylcholine and to 5-hydroxytryptamine were slightly affected by them. These phenomena were demonstrated in Fig. 8.

Membrane activity

Acetone \((0.41 \text{m or 3.0\%})\) and papaverine \((3 \times 10^{-4} \text{g/ml})\) hyperpolarized the membrane and abolished the spontaneous spike. These phenomena were accompanied by a fall in tension. ACh still depolarized the membrane in the presence of acetone or papaverine but spike was not observed. Tension was not observed with the phenomena. However, in the presence of high concentration of CaCl\(_2\) \((2.8 \times 10^{-3} \text{g/ml})\) and acetone or papaverine, ACh depolarized the membrane and spike was also observed. Tension of the smooth muscle developed accordingly. These phenomena were demonstrated in Figs. 9 and 10. These results indicate that acetone and papaverine inhibit supply of...
FIG. 7. Effect of calcium ions on the ACh response of the isolated ileum of the guinea pig in the presence of acetone.

upper: registrogram, A: acetone (0.37M or 2.7%)

B: CaCl₂ (2.2 x 10⁻³ g/ml or 20 mm)

lower: concentration action curves.
FIG. 8. Effects of calcium ions and ovo-lecithin on the responses of the guinea pig taenia caecum to ACh, hexanoylcholine (HexCh) and 5-hydroxytryptamine (5-HT).

upper: effect of calcium ions, 
HIGH Ca$^+$: CaCl$_2$ $2.2 \times 10^{-3}$ g/ml or 20 mM
lower: effect of ovo-lecithin (300 $\mu$g/ml).
FIG. 9. Records of electrical and mechanical changes in the taenia caecum from the guinea pig—effect of calcium ions on the ACh response in the presence of acetone. 
upper: electrical activity, 
lower: mechanical activity.

A

B

acetone 0.41 M

\[ \text{Ach} \times 10^{-6} \text{ g/ml} \]

C

acetone 0.41 M

\[ \text{Ach} \times 10^{-6} \text{ g/ml} \]

\[ + \text{CaCl}_2 \times 2.8 \times 10^{-3} \text{ g/ml} \]

Fig. 9. Records of electrical and mechanical changes in the taenia caecum from the guinea pig—effect of calcium ions on the ACh response in the presence of acetone. 
upper: electrical activity, 
lower: mechanical activity.

FIG. 10. Records of electrical and mechanical changes in the taenia caecum from the guinea pig—effect of calcium ions on the ACh response in the presence of papaverine. 
upper: electrical activity, 
lower: mechanical activity.

A

B

acetone 0.41 M

\[ \text{Ach} \times 10^{-6} \text{ g/ml} \]

\[ + \text{CaCl}_2 \times 2.8 \times 10^{-3} \text{ g/ml} \]

C

\[ \text{papaverine} \times 10^{-5} \text{ g/ml} \]

\[ \text{Ach} \times 10^{-7} \text{ g/ml} \]

5' in B and C: 5 minutes after application of the drug(s).
calcium ions to the contractile elements of the smooth muscle and that an increase of calcium ions in the bath fluid accelerates supply of calcium ions.  

**Effect of the extract obtained from the small intestine**

A guinea pig weighing 450 g or a rabbit weighing 3 kg was killed by depletion of blood, and each small intestine was isolated. After washing out the contents of the intestine, both ends of the intestine were ligated with thread. Intestines were extracted with 3.4 M (25%) acetone solutions (200 ml for guinea pig, 2000 ml for rabbit) for 30 minutes at 32°C with slow rocking over nitrogen. Acetone solutions were filtered and evaporated to dryness under reduced pressure, and the residues were extracted with chloroform-methanol (2:1). The evaporation of the solvents gave approximately 150 mg of the residue from the rabbit intestine and 20 mg from the guinea pig intestine. Each extract was dispersed in water by sonication and applied to the isolated guinea pig ileum which was treated with 2.4 M acetone-Locke Ringer solution, in order to observe the effects on the response of the ileum to ACh. As shown in Fig. 11, these extracts produced rather inhibitory effects on ACh contracture of the ileum.
DISCUSSION

Dikstein and Sulman (1) did not refer to the effect of the temperature of the bath fluid in the treatment of the organ with acetone. In our experiments using isolated guinea pig ileum, however, it was shown that the effect of acetone on the response of the ileum to ACh was highly dependent upon the incubation temperature in addition to the incubation time and the concentration of acetone. For example, the 2 minutes incubation with 3.4 M (25%) acetone at 32°C eliminated the response of the ileum to ACh, while the 4 minutes incubation at 0°C with 4.1 M (30%) acetone left still considerable responses to ACh.

All sorts of phospholipids employed in our experiments were effective to restore the response of the acetone-treated ileum to ACh. However, even large doses of phospholipids could not restore the response of the ileum to ACh which was completely abolished by acetone. So a severe condition such as 2 minutes incubation with 3.4 M (25%) acetone at 32°C, was inadequate to test the effects of phospholipids, and one of the best condition for this purpose seemed to be the 2 minutes incubation of the preparation with 2.4 M (17.7%) acetone at 32°C.

It was shown by Dikstein and Sulman (1) that lower concentrations (5 to 20 µg/ml) of phospholipids could restore the ACh response of the ileum treated with 3.4 M (25%) acetone and phospholipid solutions prepared by sonication were ineffective even in high concentrations. In our experiments, however, such low concentrations of phospholipids could not restore the response of the acetone-treated ileum to ACh.

According to Chapman et al. (6), the activity of dispersed phospholipids on interaction of protein is concerned with the increased surface area of the lipid rather than the production of a special phase in particles. It is known that the mean weight of phospholipid micelle obtained by sonication is approximately 2.10^5 (8) and the mean weight by dialysis is 5.10^6 to 10^6 (7).

Dikstein and Sulman (1) postulated that the inhibitory effect of acetone was due to the extraction of phospholipids from the organ, but Ehrenpreis, Hazra and Bigo-Gullino (2) suggested that acetone produced the denaturation of receptor proteins. According to the results in this paper, acetone induced an irreversible inhibition of the response of an ileum to ACh by the 2 minutes incubation with 2.4 M (17.7%) acetone-Locke Ringer solution or a reversible inhibition under the existence of 0.41 M (3%) solution. The 2.4 M (17.7%) acetone exerted inhibitory effects on not only the ACh response but also the potassium contracture of the ileum. This action of acetone was antagonized by phospholipids. Extracts obtained from small intestines by the extraction with 3.4 M (25%) acetone were unable to restore the ACh response of the ileum treated with 2.4 M (17.7%) acetone.

It has been suggested that the mechanism of action of papaverine is concerned with the inhibition of supply of calcium ions to the contractile element of the smooth muscle (9–11). Sunano and Miyazaki (12) have presented evidence that acetone may induce the change in permeability of calcium ions through the cell membrane of the guinea pig taenia caccum (or taenia coli).
CALCIUM IONS AND PHOSPHOLIPIDS

From these considerations and the present work, it is conceivable that the effect of acetone treatment is attributed to some reversible and/or irreversible changes of the muscle cell membrane of an ileum and to the subsequent inhibition of supply of calcium ions to the contractile element, and phospholipids may promote supply of calcium ions to the contractile element.

It has been reported by Takagi and Takayanagi (9-11) that as the partial agonists in their high concentrations has the non-specific inhibitory action or papaverine like action, the maximum responses of the taenia caecum to the partial agonists is smaller than that to the full agonist. The same authors have also shown that on the other hand, 5-hydroxytryptamine has not the non-specific inhibitory action, though the maximum response of the ganglion free taenia caecum to it is smaller than that to acetylcholine or the full agonist. Only the maximum response to the partial agonist or hexanoylcholine is potentiated by an increase of calcium ions in the bath fluid and a phospholipid or ovo-lecithin. This fact supports our previous reports (9-11) that the cholinergic partial agonists have the non-specific inhibitory action or papaverine like action in their high concentrations.

SUMMARY

The response of the isolated guinea pig ileum to acetylcholine and KCl were irreversibly inhibited by the 2 minutes pretreatment with acetone (2.5 m or 18.4%) or by the presence of the low concentrations of acetone or papaverine. The ileum treated with acetone or papaverine was restored in the response to the agonists by the incubation with phospholipids such as synthetic lecithin, ovo-lecithin, phosphatidylerine and phosphatidylethanolamine. However, the extracts obtained from the small intestine of the guinea pig and of the rabbit with 3.4 m (25%) acetone did not restore the response of the acetone-treated ileum to acetylcholine. High concentrations of Ca ions could also restore the response of the ileum to acetylcholine which had been treated with 2.4 m (17.7%) acetone. These results suggest the possibilities that inhibitory action of acetone and papaverine is attributed to some changes of the cell membrane of the intestinal smooth muscle, so that the supply of Ca ions to the contractile elements may be depressed by acetone and that phospholipids may promote supply of Ca ions.

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