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COMPARISON OF THE EFFECTS OF CAFFEINE AND A 2-ALKYL-1,2,3-BENZOTRIAZINIUM IODIDE ON FROG RECTUS ABDOMINIS

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1 The mode of action of 2-*n*-propyl-4-*p*-tolylamino-1,2,3-benzotriazinium iodide (TnPBI), which induced contractures of frog rectus abdominis muscle, was investigated.

2 TnPBI caused contractures of frog rectus abdominis when the muscle had been depolarized with potassium chloride.

3 Experiments with TnPBI and caffeine in calcium-free Ringer suggested that both compounds produce contractures by releasing intracellular bound calcium.

4 It is suggested that at least two calcium stores are involved, one of which is sensitive to caffeine while both are sensitive to TnPBI.

Introduction

Cull & Scott (1973) described the contractile effects on the frog rectus abdominis and chick biventer cervicis muscles of four series of 2-alkyl-1,2,3-benzotriazinium iodides. The mode of action of these compounds did not appear to involve acetylcholine receptors, since (+)-tubocurarine did not antagonize the contractures. Since quinidine and quinine produce similar effects, it was suggested that a common mode of action is involved. More recent work has suggested that the benzotriaziniums may produce their contractile effects by interfering with intracellular calcium binding sites in a manner similar to quinidine (Bondani & Karler, 1970) or caffeine (Axelsson & Thesleff, 1958).

This paper suggests that 2-*n*-propyl-4-*p*-tolylamino-1,2,3-benzotriazinium iodide (TnPBI, chosen as a representative of the benzotriaziniums) owes its contractile effects on the frog rectus to displacement of calcium ions from one or, more probably, two sites of intracellular binding.

Methods

The rectus abdominis muscle from *Rana pipiens* was used in all experiments. The muscles were divided longitudinally and each half was mounted separately, under a resting tension of 0.5 g in a solution of the following composition (mM): NaCl 128, KCl 1.85, $CaCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.108 ('Starling Ringer') at room temperature (20–22°C) in a 10 ml bath gassed with atmospheric air. To assist relaxation after induced contractures, an additional tension of 1.0 g was applied to the tissue and removed 2 min before the next drug addition. The calcium-free Ringer solution was identical with Starling Ringer solution except for the absence of calcium chloride, and the addition of 0.1 mM ethylene-glycol-bis (β-aminoethyl-ether)-*N*,*N*^{1}-tetraacetic acid. Depolarizing Ringer solution was identical with Starling Ringer except that 128 mM sodium chloride was replaced by 128 mM potassium chloride. Contractures of the muscles were recorded on Washington Oscillographs, model 400 MD/2, using isotonic displacement transducers made in the section by Mr A. McK. French. In all experiments (except those performed in the presence of depolarizing Ringer) the tissues were initially set up in Starling Ringer and control contractures obtained to TnPBI (0.1 mM) and caffeine (4.0 mM). The Starling Ringer was then replaced by calcium-free Ringer (in which the tissues remained for the rest of the experiment), and the tissues left for 30 min, during which time they were washed several times with the calcium-free Ringer. Preliminary experiments showed that the muscles contracted slowly on exposure to calcium-free Ringer, and that these contractures reached a maximum within 15 to 20 minutes. When the additional tension was then applied, the tissues returned to their initial resting length within 5 minutes. In all further experiments, therefore, the additional tension was applied immediately after exposure to calcium-free Ringer, and the tissues were left under tension for 30 min, by which time all the tissues had relaxed to their original length.

Initial experiments showed that repeated additions

of TnPBI (0.1 mM) to the tissues in calcium-free Ringer resulted in a progressive reduction of the contractile response until no response could be produced on further drug additions. Repeated additions of caffeine (4 mM) also resulted in progressive reduction to zero of the contractile responses as would be expected due to its calcium releasing properties. Four different procedures were then carried out:

(1) Control responses were obtained in Starling Ringer to 0.1 mM TnPBI. After the tissues had relaxed to their resting length under the additional tension, the Starling Ringer was replaced with depolarizing Ringer, in which the tissues remained for the rest of the experiment. This procedure resulted in a slowly developing contracture: under the influence of the additional tension, the tissues slowly relaxed to their original length after approximately 2 h: at this point another dose of 0.1 mM TnPBI was added.

(2) After obtaining control responses to 0.1 mM TnPBI and 4 mM caffeine in Starling Ringer, the tissues were bathed in calcium-free Ringer for the remainder of the experiment. The tissues were then dosed repeatedly with 4 mM caffeine until no further responses could be elicited. One dose of 0.1 mM TnPBI was then added to the tissues.

(3) In these experiments, after the control responses to TnPBI and caffeine in Starling Ringer had been obtained, the tissues were bathed in calcium-free Ringer (as in (2) above) and repeated doses of 0.1 mM TnPBI added until no further response could be elicited. One dose of 4 mM caffeine was then added to the tissues.

(4) Finally the effect, in calcium-free Ringer, of a single dose of TnPBI on a following dose of either TnPBI or caffeine, and the effect of a single dose of caffeine on a subsequent dose of TnPBI or caffeine was investigated.

Due to the differences in the time course of the action of TnPBI and caffeine, different time cycles were employed as follows: for TnPBI, a 10 min drug contact time every 45 min; for caffeine, a 5 min drug contact time every 15 minutes.

Results

Procedure 1

There was no significant difference between the contractile responses of the rectus muscle to 0.1 mM TnPBI in normal and depolarizing Ringer: the response of the depolarized tissue was 108.7% (s.e. 15.2, $n=9$) of the control responses. This suggests that membrane depolarization is not the mechanism by which TnPBI initiates the contractile process of this muscle.

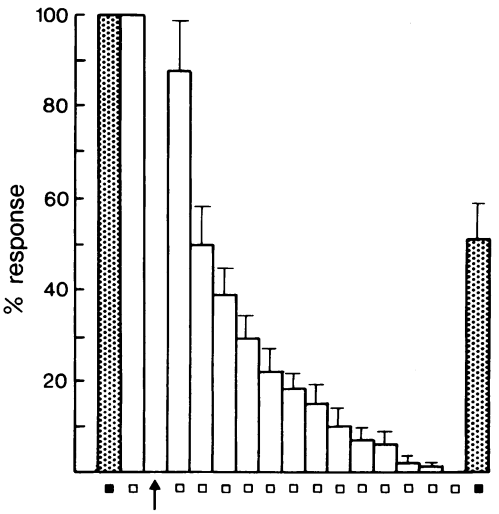

Figure 1 The effect of repeated doses of caffeine 4 mM (□) to the frog rectus abdominis in calcium-free Ringer solution. The first two columns represent the control responses in Starling Ringer to 2-*n*-propyl-4-*p*-tolylamino-1,2,3-benzotriazinium iodide (TnBPI) 0.1 mM (stippled column, ■) and to caffeine 4 mM (open column, □). All other columns represent the mean responses (with s.e. mean represented by vertical bars) of six tissues, each individual value being calculated as a percentage of its own control response. The final testing dose of TnPBI was added when responses to caffeine had been abolished. Starling Ringer replaced by calcium-free Ringer at arrow.

Procedure 2

Figure 1 illustrates the control responses to TnPBI and caffeine in Starling Ringer, and the decline to zero of the responses to caffeine on repeated dosage in calcium-free Ringer. The testing doses of 0.1 mM TnPBI produced responses which averaged 51.1% (s.e. 8.3, $n=6$) of the TnPBI control responses. This value is not significantly different from the first response of the tissue to 0.1 mM TnPBI (51.6%, s.e. 7.8, $n=6$) in calcium-free Ringer.

Procedure 3

Figure 2 illustrates mean control responses to TnPBI and caffeine as in Figure 1, and the decline to zero of the responses to repeated doses of 0.1 mM TnPBI in calcium-free Ringer. The response to the testing dose of 4 mM caffeine was totally abolished in every experiment ($n=6$).

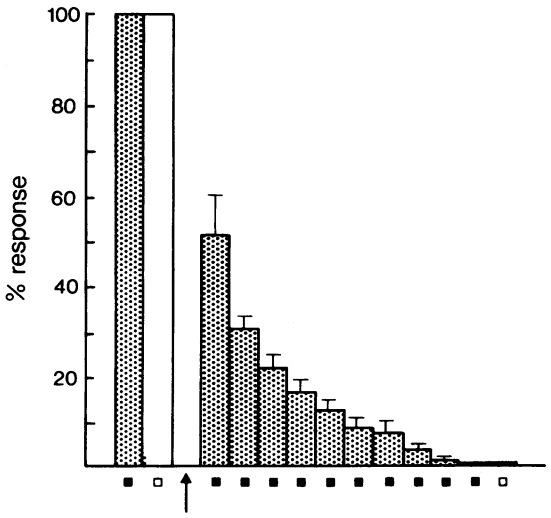

Figure 2 The effect of repeated doses of 2-*n*-propyl-4-*p*-tolylamino-1,2,3-benzotriazinium iodide (TnPBI) 0.1 mM to the frog rectus abdominis in calcium-free Ringer solution. Control columns shading and symbols as in Figure 1. The values are also the mean of results from six tissues: the final testing dose of caffeine produced no response after responses to TnPBI had declined to zero.

Procedure 4

The administration of one dose of TnPBI had a greater depressant effect on a subsequent dose of TnPBI than it did on a subsequent dose of caffeine. In ten experiments, the response to a subsequent dose of TnPBI was reduced to 25.5% (s.e. 3.4) of the control value, while in four experiments, the response to a subsequent dose of caffeine was 51.3% (s.e. 8.9). These values are significantly different ($P < 0.01$). The addition of one dose of caffeine reduced the effect of a subsequent dose of caffeine to 48.6% (s.e 9.0, $n=6$). This is not significantly different from the effect of a single dose of TnPBI on a subsequent dose of caffeine (51.3% as above). However, one dose of caffeine did not depress a following dose of TnPBI at all, the mean responses being 62.5% (s.e. 2.8, $n=4$) of the control value. This is not significantly different from the initial response to TnPBI in calcium-free Ringer (51.6%, s.e. 7.8, $n=6$), nor is it significantly different from the TnPBI responses obtained with procedure 2 after repeated caffeine dosage had resulted in complete extinction of the responses: the responses to the testing dose of TnPBI in this case being 51.1% (s.e. 8.3, $n=6$).

Discussion

Contractions of muscle tissue appear to be brought about by an increase in the free calcium ion concentration in the sarcoplasm (Heilbrunn & Wiercinski, 1947; Davis, 1963). When an isolated skeletal muscle is placed in a calcium-free medium, contractions may be elicited by compounds which release calcium ions from stores within the muscle. Caffeine (Frank, 1962), quinine (Benoit, Carpeni & Przybyslawski, 1964), and quazodine (Nott & Winslow, 1973) are examples of drugs which appear to cause contraction by a direct releasing action on calcium stores without causing any significant membrane depolarization. It seems probable that TnPBI causes muscle contractions in the frog rectus via a direct releasing effect on calcium stores, since potassium-depolarized tissue contracts in the presence of TnPBI, and also since contractions can be produced in both frog and chick biventer muscle in the presence of high concentrations of tubocurarine (Cull & Scott, 1973).

From the results of procedures 2 and 3, it may be concluded that TnPBI does release intracellular calcium, but that its mechanism of action is not identical to that of caffeine. There are two possible explanations of these results: firstly that only one calcium store is involved, and that, at the concentrations used, TnPBI is more effective than caffeine at releasing calcium ions, and so can release a greater percentage of this store than caffeine. This would enable TnPBI to produce contractions in calcium-free Ringer when caffeine is no longer effective. The alternative, more probable suggestion is that at least two sites of calcium storage exist and that TnPBI can release calcium from both stores while caffeine can release calcium ions from only one of them. It would appear from the results of procedure 4 that TnPBI is not more effective at releasing calcium ions from a single calcium store than is caffeine, since caffeine-induced responses are less affected than TnPBI-induced responses by the administration of a prior dose of TnPBI.

The second hypothesis of two binding stores therefore seems more probable, and suggests that TnPBI releases calcium from both stores while caffeine releases calcium from only one. Also continued dosage with caffeine had no significant effect on the response to a testing dose of TnPBI even when caffeine itself was no longer capable of eliciting a contracture (procedure 2). Benoit *et al.* (1964) reported that quinine could still produce a contracture of frog skeletal muscle in calcium-free Ringer when contractures to caffeine had been abolished by repeated administration of the latter. Batra (1974) has shown that two calcium stores exist, one associated with mitochondria and the other with sarcoplasmic reticulum. Quinidine preferentially released calcium from mitochondria while caffeine preferentially

released calcium from fractionated sarcoplasmic reticulum. It would be interesting to investigate the effects of TnPBI upon these cell fractions.

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