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THE MECHANISM OF ACTION OF TWO BRADYKININ-POTENTIATING PEPTIDES ON ISOLATED SMOOTH MUSCLE

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Bradykinin-induced contractions in the guinea-pig ileum were potentiated by the peptides A-VI-5 (Val-Glu-Ser-Ser-Lys) and BPP_{5a} (Pyr-Lys-Trp-Ala-Pro), while the contractions induced by other agonists were not affected. Neither peptide added alone caused any response. Previous addition of the peptides shortened the latent period following the addition of bradykinin to a value corresponding to the contraction height with an equivalent dose of bradykinin added alone. Bradykinin in contact with a piece of ileum was inactivated at a relatively slow rate. This inactivation was not inhibited by either A-VI-5 or BPP_{5a} in doses causing potentiation. Suppression of the cholinergic activity by cooling, atropine, morphine or tetrodotoxin did not influence the potentiating activity. Addition of the peptides at the moment a submaximal contraction due to bradykinin had been fully established, increased the contraction height within seconds. The two peptides caused a parallel shift to the left of the dose-effect curve of bradykinin, whereas the maximum bradykinin effect remained unchanged. It is concluded that sensitization of bradykinin receptors due to an increased affinity of the receptor for bradykinin is the hypothesis which best fits the experimental findings.

Smooth muscle preparations Bradykinin A-VI-5 BPP_{5a}
Bradykinin potentiation

1. Introduction

In a previous investigation (Ufkes et al., 1976) two bradykinin-potentiating peptides, A-VI-5 (Val-Glu-Ser-Ser-Lys) and BPP_{5a} (Pyr-Lys-Trp-Ala-Pro), were compared with respect to their potentiation of a number of different effects of bradykinin on six isolated smooth muscle preparations. Apart from the considerable difference in effective concentrations their effect was identical. Furthermore, the maximum potentiation was identical for both peptides and was not increased by combination of the two peptides. It was concluded that no indication could be found for the existence of a different mechanism of action in spite of a completely different structure. However, this investigation left unanswered the question of how this highly specific potentiation could be achieved.

According to several authors, the effect of bradykinin on isolated smooth muscle may theoretically be potentiated by a number of different mechanisms: (1) inhibition of kininase activity (Ferreira and Rocha e Silva, 1965; Hamberg et al., 1969); (2) facilitation of acetylcholine release from nerve endings (Potter and Walaszek, 1972); (3) prevention of the binding of bradykinin to "silent receptors" (Faber and van der Meer, 1973); (4) accelerated penetration of bradykinin (Tewksbury, 1968; Faber and van der Meer, 1973); (5) increase in the number of available bradykinin receptors by cleavage of peptide bonds (Edery, 1965) or disulfide bonds (Cîrstea, 1965); (6) sensitization of the bradykinin

receptor (Camargo and Ferreira, 1971); (7) sensitization of smooth muscle myofilaments (Cîrstea, 1965).

In order to exclude some of the above hypotheses, a number of experiments was done using various pharmacological techniques. On the basis of the results obtained, the most plausible mechanism of action explaining the peptide-induced bradykinin potentiation is discussed.

2. Materials and methods

A 6 cm piece of guinea-pig ileaum was prepared and suspended in a 5 ml organ bath containing Krebs–Ringer bicarbonate solution at 35°C (Ufkes et al., 1976).

2.1. Potentiating activity

In order to test the bradykinin-potentiating activity of the two peptides, the contractions obtained were compared with dose–response curves for bradykinin (BRS 640, kindly supplied by Sandoz, Basel, Switzerland), in which both the contraction height and the latent period were determined. The potentiating factor, Pf, for contraction as well as for the latent period was defined as:

Pf = (bradykinin-equivalent of bradykinin plus potentiating peptide)/(actual dose of bradykinin added)

Pf values were expressed as the geometric mean and its standard deviation (S.D.).

2.2. Peptides

A-VI-5 was synthesized according to the solid-phase method (Stewart and Young, 1969) and purified by ion-exchange chromatography (AG 50W-X4) and gel filtration (Sephadex G-10). Thin layer chromatography as well as electrophoresis was used as a test for homogeneity of A-VI-5. The amino acid sequence was confirmed by mass spectrometry *. BPP_{5a} was obtained from Spectrum Medical Industries Inc. (Los Angeles, U.S.A.). In most of the experiments the peptides were added to the bath fluid 30 sec prior to the bradykinin addition.

2.3. Latent period

The latent period was defined as the period of time elapsing between the addition of bradykinin and the start of the contraction, determined using a displacement transducer (7DCDT-1000, Hewlett Packard, California, U.S.A.) and a fast running recorder (Kipp Micrograph BD9, Delft, Holland).

2.4. Kininase activity

In order to test the kininase activity a 6 cm piece of guinea pig ileum was suspended in a 5 ml organ-bath to which bradykinin was added in a concentration of 40 ng/ml. At various intervals the bath fluid was transferred to another bath containing a second piece of ileum so as to estimate the bradykinin concentration in this fluid; the contractions due to this fluid were compared with those caused by standard bradykinin doses.

2.5. Temperature studies

Some experiments were conducted to determine the effects of cooling on the ability of the peptides to potentiate the bradykinin effect. The bath temperature was lowered from 35 to 22°C and after a 30 min equilibration period the potentiating activity of the peptides was determined.

2.6. Studies with agents suppressing cholinergic activity

Atropine sulfate (Brocades, Amsterdam, Holland), 10^{-7}—10^{-6} g/ml, morphine hydro-

* Mass spectrometry was performed by Dr. H.A.H. Craenen, Chemical Laboratory TNO, Lange Kleiweg 137, Rijswijk, The Netherlands.

chloride (O.P.G., Utrecht, Holland), 10^{-7}—10^{-6} g/ml, and tetrodotoxin (Schuchardt, München, Germany), 10^{-8}—10^{-7} g/ml, were used to determine the influence of inhibition of the cholinergic activity on the ability of the peptides to potentiate the bradykinin effect. The agents were added to the bath fluid 2.5 min before bradykinin addition in the presence or absence of the potentiating peptides.

2.7. Maximum response assay

According to the method of Tewksbury (1968) the potentiating peptides were added to the bath fluid at the moment a submaximal contraction due to bradykinin had been fully established.

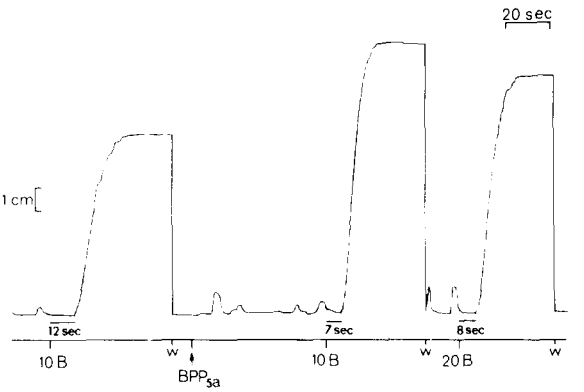

Fig. 1. Guinea-pig ileum. Contractions and latent periods (in sec) induced by bradykinin (B, in ng/ml bath fluid) alone or in the presence of BPP_{5a} (0.04 μg/ml).

3. Results

3.1. Effect of the peptides on contraction and latent period

Using the guinea-pig ileum, bradykinin in concentrations ranging from 2 to 80 ng/ml caused dose-dependent contractions with a relatively long latent period (4—30 sec). There was an inverse relation between the height of the contraction and the latent period. Previous addition of A-VI-5 or BPP_{5a} increased the contraction height and also shortened the latent period (see fig. 1). Added alone, neither peptide affected the guinea-pig ileum. Pf values were calculated separately for contraction and for latent period. From the results summarized in table 1 it can be concluded that previous addition of the potentiating peptides shortened the latent period to a value corresponding to the contraction height with an equivalent dose of bradykinin added without peptide.

3.2. Specificity of the potentiating activity

The effect of the peptides on contracting substances other than bradykinin was studied. A-VI-5 in concentrations up to 220 μg/ml as well as BPP_{5a} in concentrations up to 0.2 μg/ml were tested on the effects of acetylcholine, histamine, 5-hydroxytryptamine,

TABLE 1

The effect of A-VI-5 and BPP_{5a} on the contraction height and the latent period induced by bradykinin on the guinea-pig ileum. Pf values are calculated for the contraction as well as for the latent period.

Peptide	Conc. (μg/ml)	Potentiating activity					
		Contraction			Latent period		
		Pf	S.D.	(n)	Pf	S.D.	(n)
A-VI-5	27.5	1.5	0.2	(16)	1.6	0.3	(16)
A-VI-5	55	2.3	0.4	(8)	2.2	0.5	(8)
BPP_{5a}	0.04	2.1	0.4	(17)	2.0	0.4	(17)
BPP_{5a}	0.08	2.8	0.4	(4)	3.0	0.8	(4)

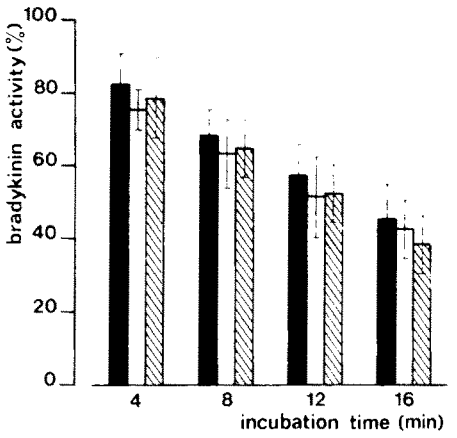

Fig. 2. Column diagram of the inactivation of bradykinin by an isolated piece of guinea-pig ileum at various incubation times in the presence and absence of A-VI-5 (55 μg/ml) or BPP_{5a} (0.04 μg/ml). The bradykinin activity is expressed as percentage of the original concentration of 40 ng/ml bath fluid, ± S.D. (n = 6). ■, Bradykinin; ▨, bradykinin + A-VI-5; ▩, bradykinin + BPP_{5a}.

angiotensin II and eledoisin. None of these agonists were potentiated by either A-VI-5 or BPP_{5a} in the concentrations used.

3.3. Effect of the peptides on the kininase activity

In order to test kininase activity, bradykinin (40 ng/ml) was added to an isolated guinea-pig ileum. After 4, 8, 12 and 16 min intervals the content of the organ bath was tested for bradykinin activity by means of a second ileum. Fig. 2 shows that bradykinin was inactivated with a half-time of 12–16 min. These experiments were also performed in the presence of A-VI-5 (55 μg/ml) and BPP_{5a} (0.04 μg/ml) in concentrations causing bradykinin potentiation (Pf values 2.4 and 2.0 respectively). From fig. 2 it can be concluded that the inactivation of bradykinin was not inhibited by either A-VI-5 or BPP_{5a}.

3.4. Influence of temperature and agents suppressing cholinergic activity

Lowering of the bath temperature from 35 to 22°C did not affect the height of the bradykinin-induced contractions. Only the latent period appeared to be prolonged, while the small spontaneous activity of the ileum

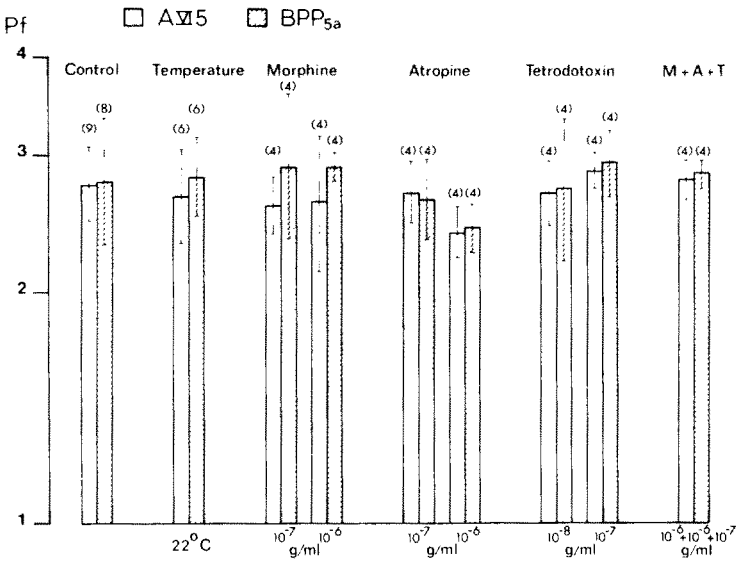

Fig. 3. Column diagram of the potentiating activity (Pf) of A-VI-5 (50 μg/ml) and BPP_{5a} (0.07 μg/ml) in relation to several factors suppressing cholinergic activity. Pf values (log scale) are expressed as the geometric mean ± S.D. Number of experiments in parentheses.

was slowed down. Fig. 3 shows that the potentiating activity of A-VI-5 or BPP_{5a} was not influenced by lowering the temperature.

Previous addition of atropine, morphine, tetrodotoxin alone or in combination, in concentrations more than sufficient to suppress cholinergic activity, did not affect the potentiating activity of either peptide (see fig. 3). Moreover, these agents hardly influenced the height of bradykinin-induced contractions but completely inhibited any spontaneous activity which might have been present.

3.5. Maximum response assay

Using the maximum response assay it was demonstrated that addition of A-VI-5 (55 μg/ml) or BPP_{5a} (0.04 μg/ml) at the moment a submaximal contraction due to bradykinin had been fully established, increased the contraction height within seconds. Fig. 4 shows that the effect of a combination of a single bradykinin dose with one of the peptides was equal to the effect of two combined single bradykinin doses or one double bradykinin dose.

3.6. Effect of the peptides on the dose—response curve of bradykinin

In previous experiments (Ufkes et al., 1976) the Pf values appeared to be independent of the bradykinin concentration at which the test was performed, provided the response to bradykinin was submaximal. This suggests that the dose—response curve of bradykinin would shift parallel to the left. To confirm this, a series of experiments was performed to investigate the influence of a fixed concentration of the bradykinin potentiating peptides on the dose-response curve to bradykinin. The results, summarized in fig. 5, show that previous addition of A-VI-5 (50 μg/ml) and BPP_{5a} (0.05 μg/ml) caused a parallel shift to the left of the dose—response curve to bradykinin, whereas the maximum response remained unchanged. Besides neither peptide, in concentrations up to 220 and 0.2 μg/ml respectively, affected the maximum response to bradykinin either added as a single dose or in a cumulative dose range.

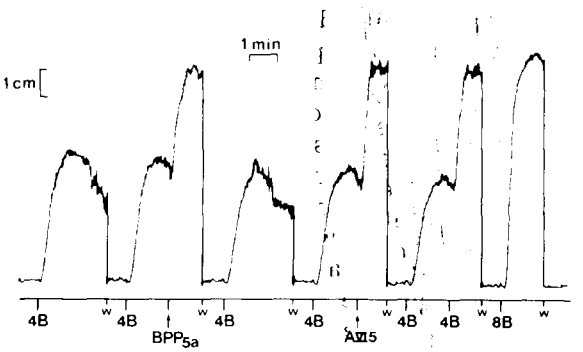

Fig. 4. Guinea-pig ileum. Contractions induced by bradykinin (B, in ng/ml bath fluid) alone or with BPP_{5a} (0.04 μg/ml) or A-VI-5 (55 μg/ml) added at the moment the contraction had been fully established.

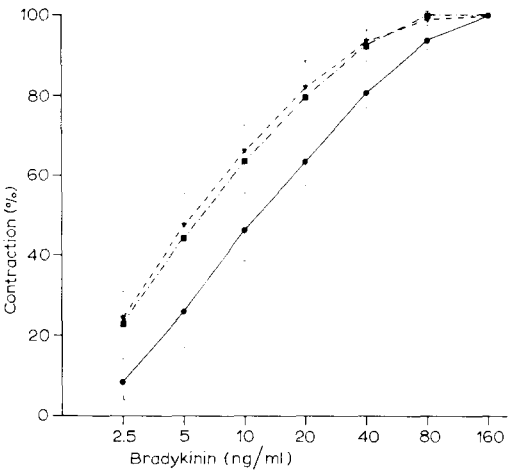

Fig. 5. Log dose—response curves with bradykinin alone and in combination with previously added A-VI-5 (50 μg/ml) and BPP_{5a} (0.05 μg/ml). ●———●, Bradykinin alone ± S.D. (n = 8); ▼- - - - -▼; bradykinin with A-VI-5 ± S.D. (n = 4); ■—·—·—■, bradykinin with BPP_{5a} ± S.D. (n = 4).

4. Discussion

The extent to which each of the hypotheses mentioned in the Introduction might explain the peptide-induced potentiation of

the bradykinin effect on isolated smooth muscle will be discussed on the basis of the experimental findings.

4.1. Inhibition of kininase activity

This is not a very likely explanation since it was demonstrated that neither peptide in concentrations causing a marked potentiation could inhibit the inactivation of bradykinin. Since bradykinin is inactivated at a relatively slow rate, at least under the conditions used which can be considered as relatively crude, it seems that kininase activity is of little importance in isolated smooth muscle preparations. This is in accordance with the observations of Cîrstea (1965), Auerswald and Doleschel (1967) and Faber and van der Meer (1973).

The fact that potentiation is also obtained when the maximum response assay is used, demonstrates according to Tewksbury (1968) that this phenomenon cannot be explained by kininase inhibition, since the interaction between bradykinin and the smooth muscle is completed before the potentiating agent is added. However, if the kininase were very active and would therefore keep up a concentration gradient of bradykinin across the longitudinal muscle layer, instantaneous kininase inhibition would result in a rapid increase in contraction height (Faber and van der Meer, 1973). Since the kininase activity in the guinea-pig ileum was shown to be rather low, this explanation seems unlikely. Furthermore, in the rabbit ileum, only the bradykinin-induced contractions are potentiated and not the earlier relaxation Ufkes et al., 1976). This may be used as further evidence against kininase inhibition as the cause of the potentiation. This has also been suggested by Camargo and Ferreira (1971) on the basis of their observations with isolated rat intestine. However, it must be noted in this respect that in the guinea-pig ileum, bradykinin-induced contractions as well as relaxations (after a previous addition of carbachol) were potentiated by both peptides to about the same extent (Ufkes et al., 1976).

4.2. Facilitation of acetylcholine release from nerve endings

Since the observations of Potter and Walaszek (1972) suggested that the bradykinin potentiating activity of some sulfhydryl compounds was due to a facilitation of acetylcholine release from nerve endings, similar experiments were conducted. In our hands however, suppression of cholinergic activity by cooling and by addition of atropine, morphine or tetrodotoxin did not affect the potentiating activity of either of the peptides. This hypothesis can therefore not be considered as a plausible explanation of the peptide-induced bradykinin potentiation.

4.3. Prevention of the binding of bradykinin to "silent receptors"

Substances may become attached to indifferent sites of binding, the so-called "silent receptors" or "sites of loss" (see Ariëns, 1964). It is well known that many drugs have common binding sites on plasma proteins and one drug may be displaced by a second. As a result there is an increase in the unbound concentration and thus an increase in the pharmacological response. A similar mechanism might exist in isolated preparations as well. "Silent receptors" envisaged in this way should be regarded as moieties of the tissue proteins. In this context however, Rocha e Silva (1976) made the point that "silent receptors", if they exist at all, would not participate in the general computation of the affinity of the agonist toward the specific pharmacological receptors, Besides it is extremely difficult to detect their existence and to distinguish them from active receptors.

In our case, it can be assumed that a certain fraction of the bradykinin added was bound aspecifically and was thus not available to induce a response. Potentiating substances added previously might occupy a part of these binding sites with the consequence that a higher fraction of the bradykinin added would be available at the active receptor sites.

This would result in a superimposed response. If the same dose of potentiating substance is combined with a higher dose of bradykinin, the absolute amount of aspecific binding sites already blocked by the potentiating substance should be equal, but the relative bradykinin fraction being displaced should be decreased. This would result in a superimposed response which would also be relatively smaller compared with those recorded after lower bradykinin doses. As a consequence, the potentiation expressed as Pf value being a relative magnitude would decrease as well. This conflicts with the observations that the Pf values were independent on the bradykinin dose at which the test was performed, as can also be seen from the parallel shift to the left of the dose—response curve for bradykinin in the presence of the potentiating peptides (fig. 5).

The observation that, in the rabbit ileum, only the contractions were potentiated and not the relaxations (Ufkes et al., 1976) may be another indication that diplacement from "silent receptors" is not the explanation for the potentiation.

4.4. Accelerated penetration of bradykinin

By "opening up" barriers, an increase in the penetration or permeation might be achieved with the consequence that more bradykinin reaches the receptors within a shorter time. Such a mechanism of action presumes that the rate at which receptor occupation takes place, and not the concentration, is the most important factor determining the magnitude of a response due to an agonist (Paton, 1961). Accelerated penetration may cause a shortening of the latent period. However, it does not explain why the latent periods corresponded exactly to those due to equivalent bradykinin doses in the absence of peptides. This might indicate that, in the presence of the peptides, the penetration of bradykinin to the bradykinin receptors exactly mimics the situation following the addition of a higher dose of bradykinin. This might be explained better by an inhibition of kininase or by prevention of the binding of bradykinin to "silent receptors" rather than by an accelerated bradykinin penetration.

The maximum response assay assumes that at the moment the contraction is completed the bradykinin penetration is finished. An increase in penetration during that stage of the excitation—contraction process would not have any additional effect. This is not in agreement with our findings that under these conditions the fully developed contraction is rapidly increased after addition of the potentiating peptides.

The bradykinin-induced relaxation in the guinea-pig ileum (after a previous addition of carbachol) appears within 1 or 2 sec (Ufkes et al., 1976), which shows that no important penetration barriers exist for the relaxation. Therefore, the peptide-induced potentiation of the relaxation cannot be considered to result from an accelerated penetration.

4.5. Increase in the number of available bradykinin receptors by cleavage of peptide or disulfide bonds

It has been suggested by Edery (1965) that chymotrypsin may act as a potentiating substance by selectively uncovering specific bradykinin receptors by splitting peptide bonds in the smooth muscle membranes. A similar suggestion was made by Cîrstea (1965) using sulfhydryl compounds as potentiating substances. In that case, the potentiating activity might be based on an increase in the amount of bradykinin receptors following the unfolding of protein complexes by the rupture of disulfide bridges. This hypothesis appears to be quite consistent with the experimental findings so far mentioned. However, if peptide bonds are split, a more prolonged potentiating effect can be expected as was found with chymotrypsin (Edery, 1965). In the present study, the potentiation induced by the peptides was quickly reversible: after washing, the normal response after addition of bradykinin without peptide was obtained

immediately. It is hard to suppose that these potentiating peptides, whose structure is relatively simple (pentapeptides), should elicit a proteolytic activity comparable to that of chymotrypsin. This also holds for an eventual rupture of disulfide bridges. In the potentiating peptides used, there is no chemical group or configuration with disulfide-reducing properties comparable to those of the sulfhydryl compounds.

4.6. Sensitization of the bradykinin receptor

Theoretically, sensitization of a receptor might detected as an increase in affinity of the receptor for an agonist and as an increase in intrinsic activity. If the affinity of the receptor were increased, a parallel shift to the left of the dose—response curve could be expected, while the maximum effect would remain unchanged (see Ariens, 1964). On the other hand if the intrinsic activity were influenced, the maximum effect of the agonist would increase as well. Our findings show that the peptides induce a parallel shift to the left of the dose—response curve to bradykinin, whereas the maximum bradykinin effect remained unchanged. This points to a mechanism of action mainly based on an effect on the affinity of the bradykinin receptor. A similar mechanism was also suggested by Camargo and Ferreira (1971), who assumed an allosteric transition of the bradykinin receptor.

The other experimental findings are mostly consistent with this hypothesis. This also holds for the proportional shortening of the latent period which does not exclusively imply an increase in the effective amount of agonist in the vicinity of the receptor. If the potentiation were due to a sensitization of the receptor, a similar response could be caused at a lower bradykinin concentration. Since lower bradykinin concentrations reach the receptor sites in an earlier stage of the diffusion process, a proportional shortening of the latent period could be expected, provided the latent period were mainly determined by and therefore proportional with the diffusion rate. An indication of the latter is given by the prolongation of the latent period when the temperature is lowered.

The observation that, in the rabbit ileum, only the bradykinin-induced contraction is potentiated and not the relaxation may mean that two different receptor sites are involved as was suggested earlier (Ufkes et al., 1976). It must be assumed that in this preparation only the receptors inducing contractions are sensitized.

4.7. Sensitization of smooth muscle myofilaments

It must be emphasized that, under the same conditions in which bradykinin was potentiated, the effects of other contracting substances such as acetylcholine, histamine, 5-hydroxytryptamine, angiotensin II and eledoisin were not potentiated by the peptides. These findings show that the potentiation is specific for bradykinin. If the potentiation were caused by a sensitization of the smooth muscle myofilaments, the potentiation would be nonspecific and the response due to any other stimulating substance would be potentiated to a similar extent.

4.8. Conclusion

Evaluating the several theoretically possible mechanisms of action underlying the peptide-induced potentiation of the bradykinin effect in isolated smooth muscle it can be concluded that the hypothesis of a sensitization of bradykinin receptors due to an increased affinity of the receptor for bradykinin, is the one which appears to best fit the experimental findings. Final proof of this hypothesis must await further experimentation.

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