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Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle

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The sarcoplasmic reticulum of skeletal muscle is a specialized form of endoplasmic reticulum¹ that controls myoplasmic calcium concentration and, therefore, the contraction–relaxation cycle². Ultrastructural studies³ have shown that the sarcoplasmic reticulum is a continuous but heterogeneous membranous network composed of longitudinal tubules that surround myofibrils and terminal cisternae. These cisternae are junctionally associated, via bridging structures called 'feet'⁴, with sarcolemmal invaginations (the transverse tubules) to form the triadic junction⁴. Following transverse tubule depolarization, a signal, transmitted along the triadic junction, triggers Ca^{2+} release from terminal cisternae^{5,6}, but the mechanism of this coupling is still unknown⁷. Inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) has recently been shown to mobilize Ca^{2+} from intracellular stores, referable to endoplasmic reticulum, in a variety of cell types (see ref. 8 for review), including smooth muscle cells of the porcine coronary artery⁹ and canine cardiac muscle cells¹⁰. Here we show that $\text{Ins}(1,4,5)\text{P}_3$ (1) releases Ca^{2+} from isolated, purified sarcoplasmic reticulum fractions of rabbit fast-twitch skeletal muscle, the effect being more pronounced on a fraction of terminal cisternae that contains morphologically intact feet structures¹¹; and (2) elicits isometric force development in chemically skinned muscle fibres.

Ca^{2+} uptake by and Ca^{2+} release from isolated sarcoplasmic reticulum (SR) fractions were measured with a Ca^{2+} -sensitive electrode. Figure 1a shows that terminal cisternae (TC) accumulated 43 nmol of Ca^{2+} per mg of protein and then (dashed line) reached a slightly lower steady state¹². Addition of 25 μM $\text{Ins}(1,4,5)\text{P}_3$ (continuous line) promoted Ca^{2+} release, and a new, much lower steady state was attained. Following a biphasic pattern, approximately 50% of the accumulated Ca^{2+} was released within 5 min and then slowly re-accumulated. The effect of $\text{Ins}(1,4,5)\text{P}_3$ was specific since neither inositol 1,4-bisphosphate ($\text{Ins}(1,4)\text{P}_2$) nor inositol 4,5-bisphosphate ($\text{Ins}(4,5)\text{P}_2$) caused Ca^{2+} release from either TC fractions or fractions of longitudinal tubules (LSR), when tested at 25 μM (Table 1).

Figure 1b shows the relationship between the extent of Ca^{2+} release and $\text{Ins}(1,4,5)\text{P}_3$ concentration. Half-maximal stimulation of Ca^{2+} release was obtained at 4–5 μM $\text{Ins}(1,4,5)\text{P}_3$ for both TC and LSR fractions. $\text{Ins}(1,4,5)\text{P}_3$ was more effective on TC than on LSR, where only 20% of the accumulated Ca^{2+} was released. The partial sensitivity of LSR to $\text{Ins}(1,4,5)\text{P}_3$ is due, at least in part, to the contamination of this fraction by about 20% TC vesicles (compare with ref. 11), as indicated by the effect of ruthenium red, an inhibitor of Ca^{2+} release from TC^{13–15} but not from LSR^{15,16} fractions. Ruthenium red at 20 μM almost completely blocked $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from TC, but produced a decrease of only 50% in release from LSR

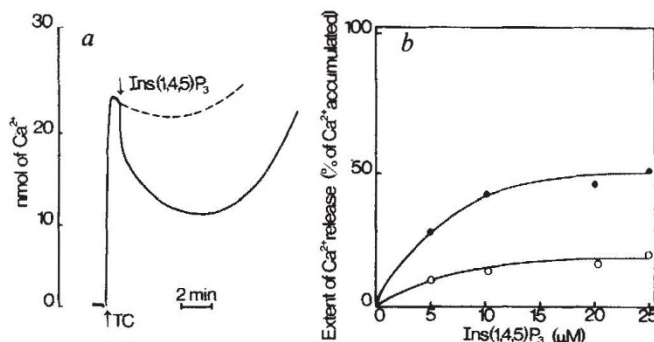


Fig. 1 $\text{Ins}(1,4,5)\text{P}_3$ promotes Ca^{2+} release from isolated SR fractions. *a*, After peak Ca^{2+} accumulation, there was a small, basal Ca^{2+} release (dashed line). Addition of 25 μM $\text{Ins}(1,4,5)\text{P}_3$ (continuous line) promoted a large Ca^{2+} release from TC, and a new lower steady state was attained. The rate of Ca^{2+} release induced by $\text{Ins}(1,4,5)\text{P}_3$ (fast phase) could not be determined accurately because of the inherent slow response of the Ca^{2+} electrode. As a lower limit, we calculated a rate of 1 nmol of Ca^{2+} per s per mg of TC protein, that is, three orders of magnitude lower than that estimated *in vivo*³¹. $\text{Ins}(1,4,5)\text{P}_3$ by itself produced only a small increase of medium Ca^{2+} that accounted for about 4% of the effect shown. *b*, A summary of data obtained from several experiments as shown in *a*, using either LSR (○) or TC (●) fractions. The extent of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was calculated as the difference between total (+ $\text{Ins}(1,4,5)\text{P}_3$) and basal Ca^{2+} release at steady state, and was expressed as percentage of Ca^{2+} accumulated by the SR. Similar results were obtained using three different SR preparations.

Methods. SR fractions were isolated from rabbit fast-twitch skeletal muscles and purified by differential and density gradient centrifugation^{11,32}. R2 (LSR) and R4 (TC) fractions were resuspended in 0.3 M sucrose, 5 mM imidazole pH 7.4 and stored at -70°C until used. Protein concentration was determined by the Lowry method³³, using bovine serum albumin as a standard. $\text{Ins}(1,4,5)\text{P}_3$ was prepared by incubating human erythrocyte ghosts at 37°C for 15 min with 2 mM CaCl_2 followed by Dowex-formate column separation, salt removal with 2 M LiCl and three consecutive extractions with ethanol^{24,34}. Ca^{2+} uptake and Ca^{2+} release were continuously monitored with a Ca^{2+} -sensitive electrode (G. Moller, Zurich). The assay was carried out at room temperature ($22\text{--}24^\circ\text{C}$) in a medium containing, in a final volume of 4 ml, 20 mM Tris-Cl pH 6.8, 0.1 M KCl, 5 mM MgSO_4 , 2.5 mM ATP, 5 mM $\text{Na}_2\text{-phosphocreatine}$ and 80 μg creatine kinase. Several pulses of 10 nmol CaCl_2 were sequentially added to calibrate the response of the Ca^{2+} electrode, and the reaction was started with either 600 μg of TC or 300 μg of LSR protein, when medium-free Ca^{2+} was 4 μM LSR and TC fractions accumulated 102 ± 9 and 40 ± 5 nmol of Ca^{2+} per mg of protein, respectively (mean \pm s.d.; $n = 5$).

(Table 1). The small ruthenium red-insensitive $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release appears to be a LSR characteristic (Table 1). Thus, our results suggest that $\text{Ins}(1,4,5)\text{P}_3$ acts preferentially on junctional SR (TC) fractions. Interestingly, Berridge and Irvine⁸ have postulated a subspecialization for endoplasmic reticulum of other cell types in which only regions lying close to the plasma membrane might be sensitive to $\text{Ins}(1,4,5)\text{P}_3$.

The ability of $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} from the SR was further investigated using a chemically skinned fibre preparation¹⁷. Ca^{2+} release from the SR was measured indirectly by monitoring isometric force development¹⁷. Segments of single fibres (average mean diameter 50 μm) were exposed to Ca^{2+} loading solution (free $\text{Ca}^{2+} = 0.40 \mu\text{M}$) for 30 s, so that the SR could accumulate Ca^{2+} , rinsed in EGTA-free solution and then challenged with $\text{Ins}(1,4,5)\text{P}_3$ that elicited transient force development (Fig. 2a). At low $\text{Ins}(1,4,5)\text{P}_3$ concentrations (1 μM , upper trace in Fig. 2a), the time course of force development was biphasic, that is, a slow phase turned into a faster one after approximately 30 s. An S-shaped time course suggests that $\Delta[\text{Ca}^{2+}]/\Delta t$ in the intermyofibrillar space was very small¹⁸ after stimulation. At higher $\text{Ins}(1,4,5)\text{P}_3$ concentrations, (20 μM , lower trace in Fig. 2a), force development was monophasic. These results imply that $\text{Ins}(1,4,5)\text{P}_3$ was able to release Ca^{2+}

from TC, which are the only Ca^{2+} storage compartments of the SR of skeletal muscle fibres^{6,19}. At 20 μM $\text{Ins}(1,4,5)\text{P}_3$, $t_{1/2}$ to peak tension was 3 s and rate of tension rise, normalized against that obtained in the presence of 10 mM caffeine, a drug known to cause Ca^{2+} release from TC of isolated fractions²⁰ and of intact²¹ and skinned²² fibres, was 0.58. The slow rate of force development might be determined by the rate of $\text{Ins}(1,4,5)\text{P}_3$ diffusion throughout the cross-section of the fibre, and by the extent of $\text{Ins}(1,4,5)\text{P}_3$ hydrolysis (see below). Figure 2b shows a plot of relative rate of tension rise versus $\text{Ins}(1,4,5)\text{P}_3$ concentration. Half-maximal rate was obtained at 3 μM . $\text{Ins}(1,4,5)\text{P}_3$ action depended critically on Ca^{2+} loading by the SR of skinned fibres. The effect of 10 μM $\text{Ins}(1,4,5)\text{P}_3$ on the rate of tension rise was decreased by 70% when loading time was reduced from 30 to 15 s or was abolished if the free Ca^{2+} of the loading solution was decreased from 0.4 to 0.16 μM .

As with isolated SR fractions, the ability to release Ca^{2+} appears to be specific for $\text{Ins}(1,4,5)\text{P}_3$, since 20 μM of either $\text{Ins}(1,4)\text{P}_2$ or $\text{Ins}(4,5)\text{P}_2$ had a minimal effect on force development in skinned fibres, that is 3–6% of that obtained with the same amount of $\text{Ins}(1,4,5)\text{P}_3$ (not shown). Furthermore, $\text{Ins}(1,4,5)\text{P}_3$ -induced force development was completely abolished by 20 μM ruthenium red (not shown). Ruthenium red inhibits Ca^{2+} release from the SR of chemically skinned fibres (G.S. and P.V., in preparation) as well as from isolated TC fractions.

The proposed role of $\text{Ins}(1,4,5)\text{P}_3$ as a second messenger for releasing Ca^{2+} from intracellular stores⁸ requires: first, substrates (phosphatidylinositol 4,5-bisphosphate, $\text{PtdIns}(4,5)\text{P}_2$), specific hydrolases ($\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase or phospholipase C) at the surface membrane level, and inactivating enzymes (inositol trisphosphatase); second, specificity for endoplasmic reticulum receptors; and third, a polyphosphoinositide breakdown rate compatible with cellular response times.

The data reported here are consistent with the possibility that $\text{Ins}(1,4,5)\text{P}_3$ is the specific chemical transmitter in the triadic junction where signal transduction for muscle activation occurs. In fact: (1) $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was preferentially displayed by TC fractions, which are enriched in junctional SR membranes, and was antagonized by ruthenium red, a blocker of TC Ca^{2+} channels¹³; (2) $\text{Ins}(1,4,5)\text{P}_3$ evoked Ca^{2+} release from TC of skinned muscle fibres; and (3) $\text{Ins}(1,4,5)\text{P}_3$ was preferred over $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(4,5)\text{P}_2$. A simplified model for transverse tubule (TT)–TC coupling may be outlined as follows. After TT depolarization, $\text{Ins}(1,4,5)\text{P}_3$ is produced at the myoplasmic leaflet of the TT membrane and released into the triadic junction; $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} channels, localized in junctional SR (TC) membranes, open and myoplasmic free Ca^{2+} rises. Alternatively, $\text{Ins}(1,4,5)\text{P}_3$ may evoke a small Ca^{2+}

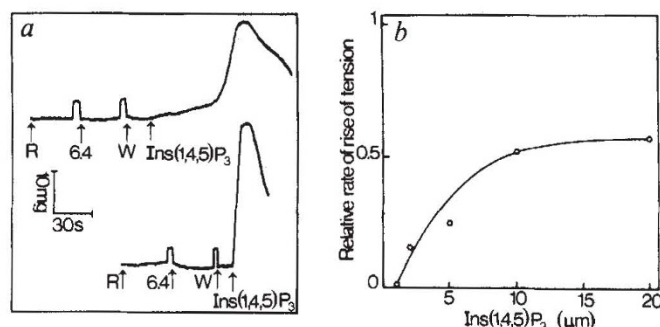


Fig. 2 $\text{Ins}(1,4,5)\text{P}_3$ elicits isometric force development in chemically skinned muscle fibres. **a**, Upper and lower traces show the effect of 1 and 20 μM $\text{Ins}(1,4,5)\text{P}_3$, respectively. **b**, A summary of data from several experiments as shown in **a**. Rate of tension rise, at specified $\text{Ins}(1,4,5)\text{P}_3$ concentration, was normalized against that obtained with 10 mM caffeine. When rates were biphasic, faster rates were considered. Variability was observed in the response to 20 μM $\text{Ins}(1,4,5)\text{P}_3$: 10 fibres were fully responsive, 4 fibres were partially responsive ($\sim 20\%$ of the effect shown in **b**) and 3 fibres were not responsive at all. Such differences can be tentatively ascribed to variations in (1) $\text{Ins}(1,4,5)\text{P}_3$ hydrolysis (which should be already low at 0.1 mM free Mg^{2+})^{23,24}; (2) Ca^{2+} loading by the fibres and (3) fibre diameter and relative diffusion times into the fibre. In some experiments, we used $\text{Ins}(1,4,5)\text{P}_3$ derived from ox brain $\text{PtdIns}(4,5)\text{P}_2$ (ref. 30) and results were comparable.

Methods. Rabbit adductor muscle bundles were chemically skinned^{17,35} and stored at -20°C in a solution containing 50% glycerol, 5 mM $\text{K}_2\text{-EGTA}$, 0.17 M K-propionate pH 7.0, 2.5 mM $\text{K}_2\text{-Na}_2\text{-ATP}$, 2.5 mM Mg-propionate and 10 mM imidazole propionate. For isometric force measurements, segments of single fibres were positioned between two clamps, one of them attached to a strain gauge, and stretched to 130% of the slack length³⁵. After 30–45 s in solution R (5 mM $\text{K}_2\text{-EGTA}$, 0.17 M K-propionate , 2.5 mM Mg-propionate , 5 mM $\text{K}_2\text{-Na}_2\text{-ATP}$ and 10 mM imidazole propionate pH 7.0) fibres were allowed to accumulate Ca^{2+} for 30 s in a pCa 6.4 loading solution (0.17 M K-propionate , 2.5 mM Mg-propionate , 5 mM $\text{K}_2\text{-Na}_2\text{-ATP}$, 2.5 mM CaCl_2 , 5 mM $\text{K}_2\text{-EGTA}$ and 10 mM imidazole propionate pH 7.0), rinsed for 20–30 s in solution W (solution R without EGTA) and then challenged with $\text{Ins}(1,4,5)\text{P}_3$. Free Mg^{2+} (kept constant at 0.09 mM) and free Ca^{2+} (0.40 μM) were determined using association constants given in Orentlicher *et al.*³⁶. All experiments were carried out at room temperature ($22\text{--}24^\circ\text{C}$) and ionic strength 0.2 M.

efflux which, in turn, triggers a massive Ca^{2+} release by opening TC Ca^{2+} -gated Ca^{2+} channels¹³. The relevance of this model to *in vivo* Ca^{2+} release could not be assessed fully in the skinned fibre preparation because of an experimental constraint that cannot be overcome, that is, exogenous $\text{Ins}(1,4,5)\text{P}_3$ must be added to the bathing solution from where it diffuses into the fibre. Interestingly, $\text{Ins}(1,4,5)\text{P}_3$ triggered Ca^{2+} release from the SR of mechanically skinned frog skeletal muscle fibres²³, and $\text{Ins}(1,4,5)\text{P}_3$ was more effective²³ if its hydrolysis was inhibited by either low free Mg^{2+} (ref. 24) or 2, 3-diphospho-D-glycerate²⁴.

The proposed model is still highly speculative and several crucial questions remain unanswered: does $\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase exist in TT membranes and, more importantly, does polyphosphoinositide breakdown take place during muscle activation? In this respect we note that Novotny *et al.*²⁵ have shown that K^+ depolarization increased ^{32}P -labelling of phosphatidylinositol in frog sartorius muscles, and that the phosphatidylinositol response is caused by K^+ depolarization and not by the ensuing transient Ca^{2+} release from the SR. Moreover, preliminary data obtained by us indicate that water-soluble inositol phosphates could be extracted from rat diaphragm muscle and that ^3H -myo-inositol incorporation into membrane phospholipids increased following a 5-s tetanus. Finally, in frog sartorius and semitendinosus muscles, a short-pulse tetanus followed by rapid freezing²⁶ increased the level of phosphatidylinositol, $\text{Ins}(4,5)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$ to levels three to four times those in controls (J. Vergava and R. Y. Tsien, personal communication). Taken together, these observations suggest that

Table 1 Effect of ruthenium red on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from isolated SR fractions, and effect of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(4,5)\text{P}_2$

Additions	Extent of Ca^{2+} release (% of Ca^{2+} accumulated)	
	LSR	TC
$\text{Ins}(1,4,5)\text{P}_3$ (25 μM)	17	47
$\text{Ins}(1,4,5)\text{P}_3$ (25 μM) plus ruthenium red (20 μM)	7	8
$\text{Ins}(1,4)\text{P}_2$ (25 μM)	2	1
$\text{Ins}(4,5)\text{P}_2$ (25 μM)	3	1

Ca^{2+} uptake and Ca^{2+} release were measured as described in Fig. 1 legend. $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(4,5)\text{P}_2$ were prepared by alkaline hydrolysis of ox brain $\text{PtdIns}(4,5)\text{P}_2$ (ref. 30). With no additions there was a basal Ca^{2+} release that corresponded to the slightly lower steady state attained after peak Ca^{2+} accumulation (compare Fig. 1a, dashed line), and accounted for approximately 4% and 8% of the Ca^{2+} accumulated in LSR and TC fractions, respectively. The extent of $\text{Ins}(1,4,5)\text{P}_3$ induced Ca^{2+} release was calculated as the difference between total and basal Ca^{2+} release (see Fig. 1). Data are from a typical experiment in which duplicate determinations were performed.

polyphosphoinositide metabolism may be causally related to muscle activation.

Another fundamental question is whether the phosphoinositide breakdown rate is fast enough (in the millisecond range) to exert a physiological role in skeletal muscle contraction. This question was not directly addressed. However, the recent findings^{27,28} that $\text{PtdIns}(4,5)\text{P}_2$ breakdown is involved in phototransduction, a quite complex and rapid event²⁹, encourage us not to rule out *a priori* such a mechanism for muscle activation.

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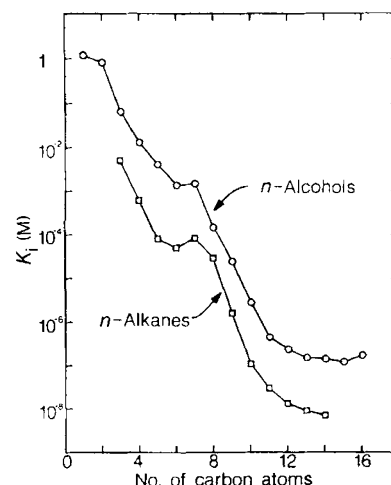


Fig. 1 Inhibition constants K_i for the homologous series of *n*-alcohols and *n*-alkanes acting on the enzyme luciferase. Firefly luciferase was purified, its activity assayed and the inhibition constants for competition with the natural substrate luciferin determined as described previously¹⁴. Standard errors in the K_i values were $\leq 20\%$. As before, the data were analysed using a simple binding model¹⁴ in which *N* anaesthetic molecules bind to the enzyme with equal affinities but only one molecule is sufficient to cause inhibition. We find that the break between *N* = 2 and *N* = 1 occurs for the alcohols between *C*₇ and *C*₈. For the alkanes, we confirmed that this break occurs between *C*₆ and *C*₁₀. However, the low activities of the intervening members meant that the exact point could not be ascertained; we took it as being the same as for the alcohols. For the largest molecules with *N* = 2, it seems likely that the two molecules bind with different affinities, although our data were not sufficiently precise to detect this. In such cases, the K_i values we have determined represent weighted averages. The alcohols *C*₁₁ → *C*₁₆ were added as ethanolic solutions, such that the final ethanol concentration never exceeded 5% of the K_i for ethanol; the controls nonetheless contained the appropriate concentration of ethanol. Solutions containing the gaseous alkanes *C*₃ and *C*₄ were prepared by bubbling the gases through scintered glass; results obtained after 1 day were identical to those obtained after 2 days of bubbling. The final concentrations were calculated using the mole fraction solubilities given in ref. 18. Saturated solutions of alkanes *C*₅ → *C*₁₃ were prepared by prolonged and vigorous shaking with small (~20 ml) volumes of buffer. In addition, alkanes *C*₈ → *C*₁₄ were added as ethanolic solutions such that their final concentrations were only about five times in excess of their saturated solutions. Results obtained using these two methods gave excellent agreement. Values for the saturated aqueous solutions of these alkanes were calculated using the straight line relationship shown in Fig. 2b. For each anaesthetic, the concentration of enzyme was always kept well below the lowest concentration of anaesthetic used. The alcohols and alkanes were the purest grades available from Sigma or BDH.

Mapping of general anaesthetic target sites provides a molecular basis for cutoff effects

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A longstanding and unresolved problem in general anaesthesia is the so-called 'cutoff' effect; as one ascends a homologous series of anaesthetic agents, the potencies progressively increase with anaesthetic size but then, rather suddenly, anaesthetic potency disappears¹⁻⁵. Curiously, this cutoff in potency occurs at very different points in different series. Various explanations have been offered^{5,6}, usually based on the notion that lipid bilayers are the primary target sites in general anaesthesia^{3,4,7}. However, accumulating evidence now suggests that proteins are the primary sites of action⁸⁻¹⁴. Here we demonstrate cutoff effects for the anaesthetic inhibition of a soluble protein (firefly luciferase) which mirror those found for general anaesthesia, and we describe how the molecular architecture of the binding site accounts for the different cutoffs in the different homologous series. We show that this

behaviour is a natural consequence of anaesthetics binding to an amphiphilic protein pocket of circumscribed dimensions. When general anaesthetic target sites in animals and the luciferase protein are mapped out using the fine details of the potency data, remarkable similarities are revealed. Our results thus suggest that the target sites in general anaesthesia are amphiphilic pockets on proteins.

The best-characterized examples of homologous series which display cutoffs in anaesthetic potency are the *n*-alcohols and *n*-alkanes. General anaesthetic activity disappears at different points in these two series; the alcohols^{1,4} cut off at about *C*₁₃ while the alkanes^{2,5} cut off between *C*₆ and *C*₁₀, the exact point being species-dependent. To elucidate the molecular basis for these observations, we have determined the inhibition constants for homologous series of both alcohols and alkanes acting on a pure soluble protein. This protein, firefly luciferase, is competitively inhibited by a wide range of general anaesthetics at concentrations which are essentially identical to those which produce general anaesthesia in animals¹⁴.

The inhibition constants K_i for the homologous series of