Sulphydryl Reagents and cAMP-dependent Kinase Increase the Sensitivity of the Inositol 1,4,5-Trisphosphate Receptor in Hepatocytes*

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Sulphydryl reagents such as tert-butyl hydroperoxide (TBHP) have been shown to increase cytosolic Ca2+ concentration ([Ca2+]i) in rat hepatocytes in a way that resembles responses to Ca2+-mobilizing hormones (Sai-kada, I., Thomas, A. P., and Farber, J. L. (1991) J. Biol. Chem. 266, 717–722; Rooney, T. A., Renard, D. C., Sass, E. J., and Thomas, A. P. (1991) J. Biol. Chem. 266, 12272–12282) and to increase the amount of Ca2+ released by inositol 1,4,5-trisphosphate ((1,4,5)IP3) from permeabilized guinea pig hepatocytes (Rooney et al., 1991, op. cit.; Missiaen, L., Taylor, C. W., and Berridge, M. J. (1991) Nature 352, 241–244; Renard, D. C., Seitz, M. B., and Thomas, A. P. (1992) Biochem. J. 284, 507–512). The effects of sulphydryl reagents were studied in fura-2-injected rat and guinea pig hepatocytes and compared with the actions of cAMP (Burgess, G. M., Bird, G. St. J., Obie, J. F., and Putney, J. W., Jr. (1991) J. Biol. Chem. 266, 4772–4781). In rat liver cells, the increases in [Ca2+]i, induced by TBHP and thimerosal were prevented by microinjection of the cells with the (1,4,5)IP3 receptor antagonist heparin. In guinea pig hepatocytes, TBHP was not able to increase [Ca2+]i unless the cells were pretreated with angiotensin II to raise endogenous levels of (1,4,5)IP3 or were first injected with a sub-threshold concentration of inositol 2,4,5-trisphosphate ((2,4,5)IP3). The responses to TBHP in (2,4,5)IP3-injected guinea pig cells were also blocked by heparin. In many respects, the actions of TBHP appeared to be similar to those of cAMP, which has previously been shown to increase sensitivity to (1,4,5)IP3 in intact guinea pig hepatocytes (Burgess et al., 1991, op. cit.). TBHP also mimicked the effect of cAMP-dependent kinase (PKA) in permeabilized guinea pig hepatocytes by increasing the amount of Ca2+ released by (1,4,5)IP3. The responses to TBHP and cAMP in (2,4,5)IP3-injected guinea pig hepatocytes differed, however, in that the increase in [Ca2+]i evoked by elevating intracellular cAMP was greatly reduced by Wiptide, an inhibitor of PKA, while Wiptide had no effect on the Ca2+ transients induced by TBHP. This provides evidence that the sensitizing effect of TBHP is not mediated by PKA and is more likely to be a direct effect on the inositol trisphosphate receptor. It is possible, however, that the sulphydryl reagents and PKA act on a common regulatory site on the receptor protein.

TBHP1 is an organic hydroperoxide that causes oxidative stress. Application of TBHP to cells results in activation of glutathione peroxidase and the conversion of glutathione to glutathione disulfide. Glutathione disulfide can form mixed disulfides with protein thiol groups, and this has been associated with inhibition of intracellular Ca2+ pumping and altered Ca2+ homeostasis (Bellomo and Orrenius, 1985). More recently, it has been shown that TBHP is able to produce oscillatory increases in cytosolic calcium concentration [Ca2+]i in rat hepatocytes (Sakaida et al., 1991; Rooney et al., 1991) and a number of other cell types including leukocytes, oocytes, HeLa cells, and pancreatic acinar cells (Hatzelmann et al., 1990; Carroll and Swann, 1992; Bootman et al., 1992; and Thorn et al., 1992). These oscillations, which are very similar to those produced by Ca2+-mobilizing hormones, are generated in the absence of any changes in inositol phosphates and can be blocked by dithiothreitol. It has also been demonstrated that TBHP and oxidized glutathione increase the sensitivity of permeabilized rat liver cells to exogenously applied (1,4,5)IP3 and that this too can be prevented by dithiothreitol (Missiaen et al., 1991; Renard et al., 1992). As the (1,4,5)IP3 receptor possesses several thiol groups thought to be important for activity (Yang and Lee, 1989), it is possible that reduction of these groups increases the sensitivity of the receptor to (1,4,5)IP3. It is likely, therefore, that increased sensitivity to (1,4,5)IP3 mediates the effect of TBHP on [Ca2+] in hepatocytes, but this has yet to be demonstrated directly in intact liver cells.

It has been known for some time that hormones that activate adenylyl cyclase can potentiate the effects of hormones that mobilize Ca2+ in hepatocytes (Jenkins and Koller, 1977; Burgess et al., 1986; and Poggioli et al., 1986). We have previously shown that cAMP, acting through PKA, increases the sensitivity of guinea pig hepatocytes to low concentrations of Ca2+-mobilizing hormones and to microinjected (2,4,5)IP3. PKA can also potentiate the ability of (1,4,5)IP3 to release Ca2+ from permeable hepatocytes (Burgess et al., 1991). As the (1,4,5)IP3 receptor is a major substrate for cAMP-dependent phosphorylation (Walaas et al., 1986; Supattapone et al., 1988; and Furuiich et al., 1989), it was suggested that a PKA-mediated phosphorylation might increase its sensitivity for (1,4,5)IP3 thus allowing lower concentrations of (1,4,5)IP3 to induce Ca2+ release.

It appears, therefore, that there are striking parallels between the effects of PKA and of the sulphydryl reagents in

1 The abbreviations used are: TBHP, tert-butyl hydroperoxide; (1,4,5)IP3, inositol 1,4,5-trisphosphate; (2,4,5)IP3, inositol 2,4,5-trisphosphate; PKA, cAMP-dependent kinase; IP3, inositol trisphosphate, DPPD, N,N'-diphenylphenylenediamine.
their ability to potentiate the effects of exogenous (1,4,5)IP₃ in permeable hepatocytes. In the present study, we have sought to extend this comparison and to characterize further the mechanism of action of TBHP in intact hepatocytes by microinjection of the (1,4,5)IP₃ receptor blocker heparin and the PKA inhibitor Wiptide. We have also investigated the effects of TBHP on Ca²⁺ homeostasis in lacrimal cells, which lack the mechanism by which cAMP potentiates the effect of (1,4,5)IP₃.

EXPERIMENTAL PROCEDURES

All values given under "Results" represent means ± S.E. of at least four independent determinations (n values are given under "Results"). Statistical significance was assessed by means of the Student's t test. An asterisk indicates a significant difference from control values (as shown in Fig. 7).

Preparation of Hepatocytes and Lacrimal Cells—Hepatocytes were prepared by collagenase perfusion of either rat or guinea pig livers as described previously (Burgess et al., 1983). The cells were suspended in Eagle's solution with 2% bovine serum albumin at pH 7.4 and stored at 0 °C in a sealed flask flushed with 5% CO₂ in O₂ until required. Mouse lacrimal cells were prepared as described previously (Parod and Putney, 1986; Bird et al., 1991). After isolation, they were resuspended in sterile Dulbecco's minimum essential medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 units/ml streptomycin.

Measurement of Ca²⁺ Release in Single Cells with Fura-2—[Ca²⁺], in intact cells was measured as described previously (Bird et al., 1991; Burgess et al., 1991). Hepatocytes were plated onto polylysine-coated glass coverslips and incubated for 10–20 min at 37 °C. The lacrimal cells were allowed to attach to glass polylysine-coated coverslips for at least 3 h at 37 °C before use. A coverslip was then mounted in a Teflon chamber, immersed in 0.5 ml of Hepes-buffered Krebs-Ringer medium containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 1.2 mM NaHCO₃, 25 mM NaH₂PO₄, 8 mM Na₂HPO₄, 26 mM KH₂PO₄, and 10 mM fura-2, pH 7.3, attached to a WPI PV830 Picopump. In some experiments, the pipette also contained (2,4,5)IP₃ (100 μM), heparin (200 mg/ml), or Wiptide (10 mM). We previously estimated that the dilution into the cytoplasm from these injections is approximately 100-fold (Bird et al., 1991).

The fluorescence of the fura-2-loaded cells was measured at room temperature with a photomultiplier-based system as described previously (Parod and Putney, 1986; Bird et al., 1991). The cells were suspended in Eagle's solution with 2% bovine serum albumin at pH 7.4 and stored at 0 °C in a sealed flask flushed with 5% CO₂ in O₂ until required. Mouse lacrimal cells were prepared as described previously (Parod and Putney, 1986; Bird et al., 1991). After isolation, they were resuspended in sterile Dulbecco's minimum essential medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 units/ml streptomycin.

Measurement of Ca²⁺ Release from Permeabilized Guinea Pig Hepatocytes with Fura-2—Release of Ca²⁺ from permeabilized guinea pig hepatocytes was measured as described previously (Burgess et al., 1991). The cells (20 mg of protein/ml) were incubated at 37 °C in the course of a Delta-Scan fluorometer in a cuvette type medium containing 1 μM fura-2, 1.5 mM ATP, and an ATP regenerating system in the presence or absence of TBHP (200 μM) and DPPD (1 μM) for 2 min. Saponin (50 μg/ml) was then added to permeabilize the cells, and 10–20 min later when the Ca²⁺ in the medium had reached steady state, (1,4,5)IP₃ and other agents were added. Ca²⁺ uptake into the permeable cells was measured as described by Burgess et al. (1983).

Materials—Fura-2 was obtained from Molecular Probes. (1,4,5)IP₃ and (2,4,5)IP₃ were obtained from Boehringer Mannheim. Thapsigargin was purchased from L. C. Services, agonistin II and Wiptide from Peninsula Laboratories, and other chemicals (including the catalytic subunit of cAMP-dependent kinase) from Sigma.

RESULTS

The Effect of Heparin on the Increase in [Ca²⁺], Induced by TBHP and Thimerosal in Intact Rat Hepatocytes—It has previously been shown that TBHP causes [Ca²⁺], to rise in isolated rat hepatocytes (Saikada et al., 1991; Rooney et al., 1991). We have confirmed this result for TBHP and, in addition, obtained similar responses with thimerosal (Fig. 1), which has been shown to increase [Ca²⁺], in a number of other cell types (e.g., Hatzelmann et al., 1990; Carroll and Swann, 1992; and Bootman et al., 1992). TBHP (200 μM) induced responses in 13 out of the 16 cells tested, and thimerosal (100 μM) caused [Ca²⁺], spikes in the six cells to which it was applied. The responses to both compounds varied from cell to cell and included single spikes, oscillatory responses, and sustained increases in [Ca²⁺]. The mean amplitude of the first spike was 674 ± 172 nM (n = 13) for TBHP and 735 ± 191 nM (n = 6) for thimerosal. A notable feature of the Ca²⁺ transients induced by both sulfhydryl reagents was the long delay before the onset of the rise in [Ca²⁺],; 205 ± 41 s for TBHP and 266 ± 46 s for thimerosal (see Fig. 1 for examples).

To investigate the possibility that the effect of TBHP and thimerosal was mediated by endogenous (1,4,5)IP₃, rat cells were injected with heparin at a concentration (200 mg/ml in the pipette) that has been shown to inhibit responses to high concentrations of Ca²⁺-mobilizing hormones (Burgess et al., 1991). Preinjection with heparin prevented the rises in [Ca²⁺], induced by TBHP (n = 11) and thimerosal (n = 4) in all cells tested (see Fig. 2 for an example). The heparin-injected cells failed to respond even when the concentration of TBHP was increased from 200 to 500 μM. Heparin did not compromise the ability of the cells to respond to thapsigargin, which induces a rise in [Ca²⁺] independently of (1,4,5)IP₃ by inhibiting the Ca²⁺ pump of the (1,4,5)IP₃-sensitive Ca²⁺ store.

Although a high percentage (81%) of the rat hepatocytes responded to TBHP with an increase in [Ca²⁺], none of the cells tested (n = 6) responded to either the adenyly cyclase activating hormone isoprenaline (up to 10 μM) or 1 mM dibutyryl cAMP (see Fig. 3A). If, however, the rat liver cells were microinjected with a subthreshold concentration of (2,4,5)IP₃ (100 μM in the pipette), application of both isopren-
Microinjection of rat hepatocytes with heparin blocked the response to TBHP. In the example, a cell was microinjected with heparin (200 mg/ml in the pipette) simultaneously with fura-2. The cell failed to respond to TBHP, although thapsigargin (2 μM) caused an increase in [Ca²⁺]. Of 11 cells that were injected with heparin, none responded to TBHP. Heparin also blocked the response to thimerosal (n = 4).

The Effect of TBHP on [Ca²⁺] in Single Guinea Pig Hepatocytes—In contrast to the rat liver cells, none of the guinea pig hepatocytes tested (12 cells) responded to TBHP (from 200 μM up to 1 mM). If, however, the guinea pig hepatocytes were pre-treated with a low or sub-threshold concentration of the Ca²⁺-mobilizing hormone angiotensin II, a subsequent addition of TBHP evoked a rise in [Ca²⁺]; in 15 out of the 22 (68%) cells examined (Fig. 4, B and C). A similar percentage (70%) of the angiotensin II-treated cells responded to 50 nM isoprenaline (n = 10). The protocol for all these experiments was to apply a low concentration of angiotensin II (usually 5 nM), allow the response (if any) to return to base line, and then add TBHP (or isoprenaline) in the continued presence of angiotensin II. Under these conditions, endogenous levels of (1,4,5)IP₃ are about twice background, and previous studies have shown that this is an effective method for demonstrating the sensitizing effect of cAMP (Burgess et al., 1991). In the cell shown in Fig. 4B, 5 nM angiotensin II, applied alone, did not increase [Ca²⁺], but when TBHP was added 10 min later it generated two Ca²⁺ transients. In the other cell shown (Fig. 4C), 5 nM angiotensin II itself induced two base-line spikes, and subsequent applications of TBHP and of isoprenaline caused [Ca²⁺] to rise.

Microinjection of (2,4,5)IP₃ into Guinea Pig Hepatocytes—It is possible to raise the intracellular concentration of IP₃ independently of Ca²⁺-mobilizing hormones by microinjecting it into cells. To this end, (2,4,5)IP₃ was microinjected into guinea pig cells to investigate whether TBHP required the presence of an inositol trisphosphate to generate an increase [Ca²⁺] in the guinea pig cells. The concentration of (2,4,5)IP₃ that was chosen (100 μM in the pipette) had previously been
shown to be ineffective on its own but capable of increasing \([\text{Ca}^{2+}]\); in the presence of a sensitizing concentration of cAMP (Burgess et al., 1991). Following a microinjection of this concentration of \((2,4,5)\text{IP}_3\), 87% of the cells (27 out of 31) responded to TBHP with an increase in \([\text{Ca}^{2+}]\) (see Fig. 5A). The proportion of \((2,4,5)\text{IP}_3\)-injected cells that responded to TBHP was similar to the proportion of the \((2,4,5)\text{IP}_3\)-injected cells that responded to 5 nM isoprenaline (86%; \(n = 52\)) in these experiments. In general, the amplitudes of the responses to TBHP were smaller than for isoprenaline, and the average latency between the application of TBHP and the onset of the \([\text{Ca}^{2+}]\) transients was longer than for isoprenaline (see Table 1).

### Table 1: Effects of Wiptide on the Response to TBHP and Isoprenaline in Guinea Pig Hepatocytes

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Amplitude (nM/(\mu m))</th>
<th>Latency (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Wiptide</td>
</tr>
<tr>
<td>ISO</td>
<td>540 ± 80</td>
<td>69 ± 17.7***</td>
</tr>
<tr>
<td>TBHP</td>
<td>382 ± 44.5</td>
<td>312.3 ± 55</td>
</tr>
<tr>
<td>AII</td>
<td>607 ± 72</td>
<td>792 ± 153</td>
</tr>
</tbody>
</table>

\*significantly different from control \((p = 0.0001)\); ***significantly different from control \((p < 0.05)\).

### Effect of TBHP Pretreatment on \((1,4,5)\text{IP}_3\)-Induced \([\text{Ca}^{2+}]\) Release in Permeable Guinea Pig Hepatocytes

Guinea pig hepatocytes were microinjected with either fura-2 and \((2,4,5)\text{IP}_3\) as described for Fig. 5 or fura-2, \((2,4,5)\text{IP}_3\), and Wiptide (10 nm in the pipette). The cells were then challenged with either isoprenaline (ISO, 5 nm), TBHP (up to 500 \(\mu m\)), or angiotensin II (AII, 1 \(\mu m\)). It did not prevent the response to thapsigargin (TG, 2 \(\mu m\)).

**Fig. 5.** Effect of TBHP on guinea pig hepatocytes injected with \((2,4,5)\text{IP}_3\). A. TBHP (200 \(\mu m\)) induced an increase in \([\text{Ca}^{2+}]\), in a guinea pig hepatocyte that had been injected with \((2,4,5)\text{IP}_3\), (100 \(\mu m\) in the pipette). Out of 22 cells injected with \((2,4,5)\text{IP}_3\), 16 responded to TBHP. The \((2,4,5)\text{IP}_3\)-injected cells also responded to isoprenaline (ISO) (see text). B. Heparin (200 mg/ml in the pipette) injected with \((2,4,5)\text{IP}_3\) gave a response to TBHP (500 \(\mu m\)). It also blocked the responses to isoprenaline (50 nm) and angiotensin II (AII, 1 \(\mu m\)). It did not prevent the response to thapsigargin (TG, 2 \(\mu m\)).

**Fig. 6.** Dithiothreitol blocked the response to TBHP in \((2,4,5)\text{IP}_3\)-injected guinea pig hepatocytes. TBHP (200 \(\mu m\)) did not increase \([\text{Ca}^{2+}]\), in a \((2,4,5)\text{IP}_3\)-injected guinea pig hepatocyte (100 \(\mu m\) \((2,4,5)\text{IP}_3\), in the pipette) that had been pretreated with 1 nm dithiothreitol (added at least 2 min before the start of the trace). Dithiothreitol did not block the response to isoprenaline (ISO, 50 nm) or angiotensin II (AII, 1 \(\mu m\)).
providing evidence that the inhibition of the response to cells. The data in the table confirm that the responses to tensin I1 or TBHP. Data obtained from several such experiments are shown in Table I, and it can be seen that the effects of TBHP pretreatment and PKA were not additive. The results have been expressed as a percentage of the calcium released (data not shown). The results in Fig. 7 also demonstrate that the effects of TBHP and of the catalytic subunit of PKA were not additive, even though more Ca2+ could be released by 2 μM thapsigargin (TG). The bars represent S.E. of between 5 and 8 observations. The asterisks indicate results that are significantly different from control (p < 0.05).

(200 μM) for 2 min prior to permeabilization than from control cells. The degree of sensitization obtained with the catalytic subunit of PKA (100 units/ml) also potentiated the response to (1,4,5)IP3. The effects of TBHP pretreatment and PKA were not additive. The results have been expressed as a percentage of the calcium released by 2 μM thapsigargin (TG). The bars represent S.E. of between 5 and 8 observations. The asterisks indicate results that are significantly different from control (p < 0.05).

The Effect of Wiptide on the Responses to Isoprenaline and TBHP in Guinea Pig Hepatocytes—Because of similarities in the actions of TBHP and isoprenaline in both intact and permeable hepatocytes, the possibility that TBHP was able to activate PKA was investigated with the PKA inhibitor Wiptide. Initial experiments were designed to show whether Wiptide, microinjected into the hepatocytes at the same time as fur-2 and (2,4,5)IP3, could inhibit the effect of isoprenaline. Wiptide is a peptide and likely to undergo rapid degradation inside the cells, so a high concentration (10 mM in the pipette) was used. Fig. 8a shows the response of a control, (2,4,5)IP3-injected, cell to 50 nM isoprenaline. In contrast, isoprenaline failed to elicit an increase in [Ca2+]i in a cell that had also been injected with Wiptide (Fig. 8b). Fig. 8 also shows that the Wiptide did not block the response to angiotensin II or TBHP. Data obtained from several such experiments are shown in Table I, and it can be seen that the amplitude of the response to isoprenaline was reduced by 87% and the latency increased by 20.5 s in the Wiptide-injected cells. The data in the table confirm that the responses to angiotensin II were not impaired in Wiptide-injected cells, providing evidence that the inhibition of the response to isoprenaline was not a nonspecific effect of microinjecting a high concentration of peptide. The lack of effect of Wiptide on the angiotensin II response also suggests that there was no effect of basal PKA activity on (1,4,5)IP3 in these cells. Wiptide had no effect on the amplitude of the response to TBHP, and its latency, which was longer than for isoprenaline, was not significantly increased by the kinase inhibitor.

**DISCUSSION**

Heparin Can Block the Rise in [Ca2+]i Induced by Sulfhydryl Reagents—One of the most important aspects of this work is the direct demonstration that the Ca2+ spikes induced by sulfhydryl reagents in intact hepatocytes are mediated by increased sensitivity to IP3. This has been shown in two ways. First, in guinea pig hepatocytes, TBHP was unable to increase [Ca2+]i unless (1,4,5)IP3 levels had already been increased to a subthreshold level. This was achieved either with a low concentration of a Ca2+-mobilizing hormone (angiotensin II) or by microinjecting (2,4,5)IP3, a poorly metabolized analogue of (1,4,5)IP3, into the cells. In experiments in which angiotensin II was used to raise endogenous (1,4,5)IP3, TBHP was added at a time when the level of (1,4,5)IP3 was still about double the control value, but [Ca2+]i, had returned to resting level (see Burgess et al., 1991). In the experiments with (2,4,5)IP3, a concentration was selected that had previously been shown to be ineffective on its own, although capable of inducing a rise in [Ca2+]i; in the presence of elevated cAMP (Burgess et al., 1991). Second, and more importantly, the Ca2+ transients induced by the sulfhydryl reagents could be prevented by injecting the cells with heparin. In the rat, where both TBHP and thimerosal could increase [Ca2+]i, in naive hepatocytes, the blocking effect of heparin provides convincing evidence that the Ca2+ spikes induced by the two sulfhydryl agents were mediated by an increase in the sensitivity of the IP3 receptor to basal levels of (1,4,5)IP3. In guinea pig hepatocytes, where IP3 had first to be elevated for TBHP to be effective, the inhibition by heparin indicates that the injected IP3 was directly responsible for the TBHP-induced increase in [Ca2+]i. The ability of heparin to block the effect of thimerosal in rat hepatocytes is in contrast to findings in mouse oocytes (Carroll and Swann, 1992), where heparin prevented a Ca2+ spike induced by (1,4,5)IP3 but had no effect on the response to thimerosal. A recent report of experiments...
FIG. 8. Wiptide inhibited the response to isoprenaline but not TBHP in (2,4,5)IP3-injected guinea pig hepatocytes. A, C, and E, control responses to 50 nM isoprenaline (ISO), 1 μM angiotensin II (AII), and 200 μM TBHP, respectively. In the experiments with isoprenaline and TBHP, the guinea pig hepatocytes were injected with (2,4,5)IP3 (100 μM in the pipette) and fura-2. B, D, and F, responses to isoprenaline, angiotensin II, and TBHP, respectively, in cells that had also been microinjected with Wiptide (10 mM in the pipette).

in pancreatic acinar cells, however (Thorn et al., 1992), showed blockage of thimerosal-induced Ca2+-activated chloride currents by heparin. Furthermore, a blocking antibody, which recognizes an epitope close to the Ca2+ channel of the inositol triphosphate receptor protein, has been shown to prevent thimerosal-induced Ca2+ spikes in hamster eggs (Miyazaki et al., 1992).

The ability of TBHP to increase [Ca2+]i in naive rat hepatocytes but not in the guinea pig cells is intriguing. One possibility is that the basal level of (1,4,5)IP3 is higher in rat hepatocytes than in guinea pig hepatocytes. Alternatively, it is possible that TBHP is more effective at sensitizing the IP3 receptor in the rat cells than in guinea pig cells. Our finding that cAMP, unlike TBHP, was able to increase [Ca2+]i in the rat cells only when the concentration of IP3 had been increased above control levels is in agreement with results reported by Cobbold et al. (1991) and tends to favor the latter possibility. Other investigators (Combettes et al., 1986; Kraus-Friedmann, 1986; and Capiod et al., 1991) have, however, reported that cAMP-dependent hormones can raise [Ca2+]i in naive rat hepatocytes, and this would suggest that the rat cells have higher resting levels of (1,4,5)IP3.

Comparison of the Effects of TBHP and of cAMP—The conditions necessary for TBHP to increase [Ca2+]i in the guinea pig hepatocytes appear to be the same as those for isoprenaline. Neither was effective on its own, but both evoked
Ca\(^{2+}\) transients in approximately the same percentage of cells if intracellular IP\(_3\) was raised to subthreshold levels. In addition, the responses to TBHP and to the cAMP-dependent hormone isoproterenol were both blocked by heparin. In other respects, the responses differed. The latency for TBHP was about three times longer than for isoproterenol, and the response to TBHP, but not to isoproterenol, could be blocked by dithiothreitol, implying that it was mediated by an effect on protein thiol groups. In rat hepatocytes, TBHP was able to generate Ca\(^{2+}\) transients in untreated cells, whereas even very high concentrations of isoproterenol and dibutyril cAMP had no effect in rat hepatocytes unless intracellular IP\(_3\) had already been raised. This implies that, in rat liver cells, TBHP is more effective at increasing the sensitivity to IP\(_3\) than cAMP. As the concentrations of isoproterenol and dibutyril cAMP used were sufficiently high to cause maximal activation of cAMP-dependent kinase (cAMP-activated protein kinase (PKA)), this suggests that the mechanism of action of TBHP does not involve activation of PKA.

**The Effect of Sulfhydryl Reagents in Other Cell Types**—The experiments with Wiptide confirmed that PKA was not a key factor in the sensitizing actions of TBHP. Microinjection of hepatocytes with Wiptide caused a large (87\%) reduction in the size of the responses to isoproterenol, which was accompanied by a tripling of the latency. In contrast, using the same protocol, Wiptide had no significant effect on the responses to TBHP.

The possibility that the Ca\(^{2+}\) spikes induced by TBHP were a result of an effect on Ca\(^{2+}\) pumping (Bellomo and Orrenius, 1985) can be ruled out on several counts. We found no significant effect of 200 µM TBHP on steady-state Ca\(^{2+}\) levels or Ca\(^{2+}\) reuptake in permeabilized guinea pig hepatocytes, suggesting that the Ca\(^{2+}\) pump was not inhibited, and, in intact hepatocytes, thapsigargin, which does inhibit Ca\(^{2+}\)-ATPases, did not cause Ca\(^{2+}\) spikes but rather caused a sustained, heparin-insensitive increase in [Ca\(^{2+}\)].

It would appear, therefore, that the ability of the sulfhydryl reagents to increase the sensitivity of the IP\(_3\) receptor to low levels of agonist, although similar in some respects to the sensitizing effect of cAMP, is not mediated by PKA but likely to be a direct effect on thiol groups of the IP\(_3\) receptor (see Yang and Lee, 1989). The finding that the potentiating effects of TBHP and the catalytic subunit of PKA in permeable hepatocytes were not additive suggests that they may have a similar action on the (1,4,5)IP\(_3\) receptor channel protein.

**The Effect of Sulfhydryl Reagents in Other Cell Types**—There are several reports that sulfhydryl reagents can cause Ca\(^{2+}\) spikes in other cells including mouse oocytes (Carroll and Swann, 1992), HeLa cells (Bootman et al., 1992), and pancreatic acinar cells (Thorn et al., 1992). The inability of TBHP or thimerosal to generate Ca\(^{2+}\) spikes in naive lacrimal cells and the extremely modest potentiation of the response to a low concentration of methacholine with thimerosal (TBHP had no effect) imply that sulfhydryl reagents are not equally effective in sensitizing the IP\(_3\) receptor in all cell types. This could be because the redox potential of the thiol groups on the receptor in the lacrimal cells does not favor the formation of disulfides with glutathione disulfide or because these cells express a different subtype of IP\(_3\) receptor from, for example, the liver and HeLa cells. Sudhof et al. (1991) reported that the degree of homogeneity between the type 1 and 2 (1,4,5)IP\(_3\) receptors in the brain was lowest in the coupling domains that contain the phosphorylation sites for cAMP. As the lacrimal cells also lack the mechanism by which cAMP potentiates the effects of low concentrations of IP\(_3\), this suggests that the mechanisms by which cAMP and the sulfhydryl reagents induce sensitization are linked at the level of a common modulatory site on the (1,4,5)IP\(_3\) receptor.

**REFERENCES**


